Targeted therapy of *TERT*-rearranged neuroblastoma with BET bromodomain inhibitor and proteasome inhibitor combination therapy

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Running title: OTX015 and carfilzomib against *TERT*-rearranged neuroblastoma

Key words: Neuroblastoma, *TERT* gene rearrangement, BET bromodomain inhibitor, proteasome inhibitor, therapy

Disclosure of Potential Conflicts of Interest: The authors declare no potential conflicts of interest

Statement of translational relevance **150** words, Abstract **249** words, Text **4947** words**,** Main Figures **6**, Supplemental Figures **7**, Supplementary Table **1,** References **40**

Translational Relevance

Neuroblastoma is the most common solid tumor in early childhood. *TERT* gene rearrangement with transcriptional super-enhancers occurs in approximately a quarter of high-risk neuroblastomas, and patients with this subtype of neuroblastoma show very poor prognosis. In this study, through unbiased screening of an approved oncology drug library, we identified the proteasome inhibitor carfilzomib as the approved oncology drug exerting the best synergistic anticancer effects with the BET bromodomain inhibitor OTX015. OTX015 and carfilzomib synergistically blocked TERT protein expression, induced *TERT*-rearranged neuroblastoma cell apoptosis, significantly suppressed tumor progression and improved survival in mice xenografted with *TERT*-rearranged neuroblastoma cell lines or patient-derived xenograft tumor cells, which was largely reversed by forced TERT over-expression. As OTX015 is currently in Phase II clinical trials and carfilzomib is an approved oncology drug, OTX015 and carfilzomib combination therapy is likely to be translated into the first clinical trial of a targeted therapy in *TERT*-rearranged neuroblastoma patients.

Abstract

Purpose: *TERT* gene rearrangement with transcriptional super-enhancers leads to *TERT* over-expression and neuroblastoma. No targeted therapy is available for clinical trials in *TERT*-rearranged neuroblastoma patients.

Experimental Design: Anticancer agents exerting the best synergistic anticancer effects with BET bromodomain inhibitors were identified by screening a US Food and Drug Administration-approved oncology drug library. The synergistic effects of the BET bromodomain inhibitor

OTX015 and the proteasome inhibitor carfilzomib were examined by immunoblot and flow cytometry analysis. The anticancer efficacy of OTX015 and carfilzomib combination therapy was investigated in mice xenografted with *TERT*-rearranged neuroblastoma cell lines or patient-derived xenograft (PDX) tumor cells, and the role of TERT reduction in the anticancer efficacy was examined through rescue experiments in mice.

Results: The BET bromodomain protein BRD4 promoted *TERT*-rearranged neuroblastoma cell proliferation through up-regulating TERT expression. Screening of an approved oncology drug library identified the proteasome inhibitor carfilzomib as the agent exerting the best synergistic anticancer effects with BET bromodomain inhibitors including OTX015. OTX015 and carfilzomib synergistically reduced TERT protein expression, induced endoplasmic reticulum stress, and induced *TERT*-rearranged neuroblastoma cell apoptosis which was blocked by TERT over-expression and endoplasmic reticulum stress antagonists. In mice xenografted with *TERT*-rearranged neuroblastoma cell lines or PDX tumor cells, OTX015 and carfilzomib synergistically blocked TERT expression, induced tumor cell apoptosis, suppressed tumor progression and improved mouse survival, which was largely reversed by forced TERT over-expression.

Conclusions: OTX015 and carfilzomib combination therapy is likely to be translated into the first clinical trial of a targeted therapy in *TERT*-rearranged neuroblastoma patients.

Introduction

High-risk neuroblastoma is a lethal pediatric cancer that accounts for 15% of all childhood cancer death (1, 2). *TERT* gene rearrangements with transcriptional super-enhancers results in chromatin remodeling, massive *TERT* gene over-expression and neuroblastoma in approximately 24% of high-risk neuroblastomas. Patients with *TERT*-rearranged neuroblastoma show very poor prognosis (3, 4).

Transcriptional super-enhancers are found at the loci of oncogenes, and are ideal targets for cancer therapy (5, 6). Super-enhancers are occupied by master transcriptional regulators including the BET bromodomain protein BRD4. BRD4 binds super-enhancers, recognizes acetylated lysine residues, activates super-enhancer activity and considerably up-regulates the transcription and expression of super-enhancer-associated critical oncogenes (5-7).

BET bromodomain inhibitors, such as JQ1, I-BET762 and OTX015, competitively bind and occupy the acetyl lysine recognition pocket of BRD4, disrupt BRD4 recruitment to super-enhancers, suppress super-enhancer-associated oncogene expression and exert anticancer effects (6-9). Currently, several BET bromodomain inhibitors including I-BET762 and OTX015 are in multiple clinical trials in patients with solid tumors or hematologic malignancies (10, 11).

TERT is a component of telomerase which is essential for telomeric DNA maintenance. In addition, TERT regulates gene expression and protein synthesis in a telomerase-independent manner, resulting in cancer stemness and cell proliferation (12, 13). TERT overexpression thus induces tumorigenesis by telomerase activity-dependent and -independent mechanisms (14).

While telomerase inhibitors show anticancer effects in preclinical studies (15), treatment with Imetelstat (GRN163L), the only telomerase inhibitor which has been developed to clinical trials, does not improve the survival of lung, breast and brain cancer patients, and causes life-threatening side effects in childhood brain cancer patients in clinical trials (16-18).

In this study, we examined the role of BRD4 in regulating *TERT* over-expression, cell cycle progression and proliferation in *TERT*-rearranged neuroblastoma cells. Carfilzomib was identified as the approved oncology drug exerting the best synergistic anticancer effects with OTX015 in *TERT*-rearranged neuroblastoma cells. OTX015 and carfilzomib synergistically blocked TERT protein expression, induced endoplasmic reticulum stress, induced *TERT*-rearranged neuroblastoma cell apoptosis, considerably suppressed *TERT*-rearranged neuroblastoma tumor progression and improved mouse survival.

Materials and Methods

Cell culture

Human neuroblastoma GI-MEN, CHP134 and SHEP cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. CLB-GA cells were grown in Roswell Park Memorial Institute medium with 10% fetal calf serum. WI38 fetal lung fibroblasts were cultured in minimum essential medium with 10% fetal calf serum. GI-MEN, CLB-GA and SHEP cells were provided by Dr Jo Vandesompele (Center for Medical Genetics Ghent, Belgium), Dr Valérie Combaret (Centre Léon Bérard, Lyon, France) and Dr Barbara Spengler (Fordham University, New York, NY) respectively. CHP134 cells were obtained from the European Collection of Cell Cultures in 2010, and WI38 cells from the American Type Culture Collection 20 years ago. Umbilical cord blood for research was provided by the Sydney Cord Blood Bank and experiments were approved by the Sydney Children's Hospital Human Research Ethics Committee. Mononuclear cells were purified from umbilical cord blood using Lymphoprep (Axis-Shield, Oslo, Norway). CD34+ cells were isolated to greater than 95% purity and cultured in Stemline II serum-free medium (Sigma, St Louis, MO) with a cytokine cocktail of Flt3 ligand, stem-cell factor and trombopoietin (R&D Systems, Minneapolis, MN). All cell lines were verified by small tandem repeat profiling by Garvan Institute of Medical Research (Darlinghurst, NSW, Australia) or Cellbank Australia. PDX CCI-NB07-RMT cells were extracted from mouse tumors and cultured in Iscove's Modified Dulbecco's medium with 20% fetal calf serum and insulin-transferrin-sodium (Thermo Fisher Scientific, Waltham, MA).

Drug screening

CLB-GA neuroblastoma cells were treated with vehicle control, I-BET762 at inhibition of cell viability by 20% (IC_{20}, 0.5μM), the Approved Oncology Drugs (AODs) Set IV (the US

National Cancer Institute) compounds at 1µM, or combination for 72 hours. Cell viability was determined by Alamar blue assays. Synergistic interaction between I-BET762 and the AODs was examined by R value using fractional product method (19).

Compounds which reduced the number of viable CLB-GA cells by ≥ 90% on their own and compounds which reduced the number of viable CLB-GA cells by ≥ 80% when combined with I-BET762 with a R value of less than 0.7 were shortlisted for secondary drug screening. CLB-GA and GI-MEN neuroblastoma cells were treated with a range of doses of I-BET762 or OTX015 (0, 125, 250, 500 and 1,000nM), the shortlisted AODs (0, 3.90625, 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, 1000nM), or combination. To determine if the effects of I-BET762/OTX015 and the AOD were additive, bliss-additivity was calculated with Bliss-additive formula (20). Synergy was further validated by the Chou-Talalay method (21) to calculate combination indexes (CIs) for effective doses for 75% (ED75) and 90% (ED90) cell number reduction with CompuSyn software (Combosyn Inc, http://www.combosyn.com/).

ChIP sequencing and data analysis

ChIP sequencing was performed as we described (22, 23). PDX CCI-NB07-RMT neuroblastoma cells were treated with vehicle control or 2uM OTX015 for 48 hours, followed by ChIP with the ChIP Assay kit (17-295, Millipore) and a rabbit anti-H3K27ac antibody (ab4726, Abcam), rabbit anti-BRD4 antibody (A301-985A, Bethyl Laboratories, Montgomery, TX) or control rabbit IgG (10500C, Invitrogen). After immunoprecipitation, DNA was purified and subjected to sequencing with Illumina HiSeq 2000 at Ramaciotti Centre for Genomics, University of New South Wales, Australia. The ChIP sequencing data has been deposited at the Gene Expression Omnibus website (GSE147181). In addition, published ChIP sequencing data from BE(2)-C and CHP134 cells with H3K27ac, H3K4me3 and BRD4 antibodies were downloaded from GEO websites (GSM2113517, GSM2113518, GSM2113520 and Series

GSE113139) and also analysed. For the CHP134 cell line, H3K4me3 data was available which was used to filter out potential promoter peaks. In all other cell lines only H3K27ac was used to perform super-enhancer detection. Peaks identified were used for super enhancer detection with *ROSE* (5) using parameters -s 12500 -t 1000. HOMER (24) and R Bioconductor packages (org.Hs.eg.db and TxDb.Hsapiens.UCSC.hg19.knownGene) were used for peak annotation. Genes whose transcription start site fell within 600kbp flanking of detected super enhancers were considered candidate associated genes.

Experimental therapy in mice

Animal experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales (Sydney, New South Wales, Australia). Female Balb/c nude mice aged 5 to 6 weeks were injected subcutaneously into the right flank under anaesthesia with GI-MEN, wild type CLB-GA cells or CLB-GA cells stably transfected with an empty vector or TERT ORF expression construct per mouse (25). In addition, non-obese diabetic severe combined immune deficiency gamma (NSG) mice aged 5-6 weeks old were injected with 2 x 10^6 PDX CCI-NB07-RMT cells. When the engrafted tumors reached 200 or 100 mm^3, the mice were randomly divided into 4 groups and treated with vehicle control, OTX015 at 50 mg/kg body weight/day via oral gavage, carfilzomib at 6 mg/kg body weight/once every other day via intraperitoneal injection, or OTX015 plus carfilzomib. The treatments were continued until the mice were humanely culled when the tumors reached 1,000 mm^3, and survival curves were plotted. Tumor tissues were collected, snap-frozen or formalin-fixed and paraffin-embedded for immunoblot, telomere length assays, telomere dysfunction-induced foci assay or immunohistochemistry analysis respectively.

Statistical analysis

For statistical analysis, experiments were conducted three times. Data were analysed with Prism 6 software (GraphPad) and presented as mean ± standard error. Differences were analysed for significance using ANOVA among groups or unpaired Student's *t*-test for two groups. All statistical tests were two-sided. A p value of less than 0.05 was considered statistically significant. Synergy or additivity was calculated by combination index (CI) method for combinations of multiple doses of drugs, or by the fractional product (R) method for combinations of a single dose of drugs.

Results

BRD4 is required for TERT expression and cell proliferation in *TERT*-rearranged neuroblastoma cells

We first examined the effect of TERT siRNAs on *TERT*-rearranged neuroblastoma cell proliferation and cell cycle progression. RT-PCR and immunoblot analyses confirmed that two independent TERT siRNAs, TERT siRNA-1 and TERT siRNA-2, effectively knocked down TERT mRNA and protein expression in *TERT*-rearranged GI-MEN and CLB-GA neuroblastoma cells (3) (Supplementary Fig. S1A and S1B). Telomerase activity and Alamar blue assays showed that transfection with TERT siRNAs significantly reduced telomerase activity (Supplementary Fig. S1C) and the numbers of GI-MEN and CLB-GA cells (Supplementary Fig. S1D). Cell cycle analysis revealed that TERT knockdown for 72 hours significantly increased the percentage of GI-MEN and CLB-GA cells at the G1 phase and decreased the percentage of the cells at the S phase (Supplementary Fig. S1E and S1F).

Next, immunoblot analysis confirmed that transfection with wild type or D712A mutant telomerase activity-deficient TERT open reading frame expression construct (26) led to considerable TERT protein over-expression in GI-MEN and CLB-GA cells (Supplementary Fig. S1G). Cell cycle analysis showed that both the wild type and the D712A mutant TERT expression construct rescued the *TERT*-rearranged neuroblastoma cells from G1 cell cycle arrest and growth inhibition due to TERT siRNA-2 which targeted the 3'-untranslated region of TERT mRNA (Supplementary Fig. S1H and Fig. S1I). As TERT is known to induce cancer cell proliferation by enhancing global protein synthesis (13), our puromycin incorporation assays showed that TERT knockdown with TERT siRNAs significantly reduced global protein synthesis (Supplementary Fig. S1J). The data suggest that TERT promotes global protein

synthesis and thus induces *TERT*-rearranged neuroblastoma cell proliferation and cell cycle progression.

We examined whether BRD4 regulated *TERT* oncogene expression in *TERT*-rearranged neuroblastoma cells. RT-PCR and immunoblot analyses showed that transfection with two independent BRD4 siRNAs effectively knocked down BRD4 mRNA and protein expression and reduced TERT mRNA and protein expression 48 hours after siRNA transfection (Supplementary Fig. S2A and S2B). In comparison, transfection with BRD4 siRNAs for 48 hours reduced N-Myc but not TERT mRNA and protein expression in *MYCN*-amplified CHP134 neuroblastoma cells (Supplementary Fig. S2C and S2D).

GI-MEN and CLB-GA cells stably expressing doxycycline (DOX)-inducible control shRNA or one of two independent BRD4 shRNAs (shRNA-1 and shRNA-2) were established using the FH1tUTG construct (27, 28). The BRD4 siRNAs and shRNAs targeted different BRD4 mRNA sequences. RT-PCR and immunoblot analyses showed that treatment with DOX considerably reduced BRD4 and TERT mRNA and protein expression in DOX-inducible BRD4 shRNA, but not control shRNA, GI-MEN and CLB-GA cells (Fig. 1A, Fig. 1B and Supplementary Fig. S2E). Furthermore, BRD4 knockdown by DOX significantly reduced telomerase activity in DOX-inducible BRD4 shRNA-1 and BRD4 shRNA-2 GI-MEN and CLB-GA cells (Supplementary Fig. S2F). The data demonstrate that BRD4 is required for *TERT* expression and telomerase activity in *TERT*-rearranged neuroblastoma cells.

We next examined whether BRD4 is required for *TERT*-rearranged neuroblastoma cell proliferation and cell cycle progression. Alamar blue assays showed that knocking down BRD4 with siRNAs reduced the number of *TERT*-rearranged neuroblastoma cells (Supplementary Fig. S2G). We then established GI-MEN and CLB-GA cells stably transfected with an empty vector or a TERT open reading frame (ORF) expression construct. The exogenous TERT ORF expression was controlled by a viral promoter and was therefore not expected to respond to

BRD4. Immunoblot analysis confirmed that BRD4 siRNAs down-regulated TERT protein expression in empty vector expressing cells, but did not have an effect on TERT protein expression in TERT ORF construct expressing cells (Fig. 1C). Alamar blue assays demonstrated that exogenous TERT ORF expression induced neuroblastoma cell proliferation in both GI-MEN and CLB-GA cells, and largely reversed cell growth inhibition mediated by BRD4 siRNAs (Fig. 1D).

In addition, Alamar blue assays showed that treatment with DOX significantly reduced the number of DOX-inducible BRD4 shRNA, but not control shRNA, GI-MEN and CLB-GA cells (Supplementary Fig. S2H). Cell cycle analysis revealed that BRD4 knockdown increased the proportion of DOX-inducible BRD4 shRNA GI-MEN and CLB-GA cells at the G1 phase and decreased the proportion of the cells at the S phase (Fig. 1E), and clonogenic assays showed that BRD4 knockdown considerably reduced the numbers of colonies formed by GI-MEN and CLB-GA cells (Fig. 1F and Fig. 1G).

Taken together, these data demonstrate that TERT promotes *TERT*-rearranged neuroblastoma cell proliferation and cell cycle progression through telomerase-independent mechanism including enhancing protein synthesis, and that BRD4 promotes *TERT*-rearranged neuroblastoma cell proliferation and cell cycle progression through modulating TERT expression.

Approved Oncology Drug screening identifies carfilzomib as the anticancer agent exerting considerable synergistic anticancer effects with BET bromodomain inhibitors

As BET bromodomain inhibitors I-BET762 and OTX015 block BRD4 function and are promising anticancer agents in clinical trials (10, 11), we examined whether BET bromodomain inhibitors showed anticancer effects against *TERT*-rearranged neuroblastoma cells. Alamar blue assays showed that treatment with the BET bromodomain inhibitors JQ1, I-BET762

(GSK525762) or OTX015 reduced the number of viable GI-MEN and CLB-GA cells, however, the anticancer effect plateaued at higher doses (Supplementary Fig. S3A).

To identify the anticancer agents that synergize with I-BET762, we undertook a chemical library screen using the Approved Oncology Drugs (AODs) Set IV from the US National Cancer Institute. CLB-GA cells were treated with 101 AODs at 1μM either alone or in combination with 0.5μM I-BET762 which reduced the number of CLB-GA cells by 20% on its own. As single agents, 11 AODs at 1μM reduced CLB-GA cell viability by ≥ 90% (Supplementary Fig. S3B and Table S1) and were therefore subjected to multiple-dosage secondary drug screening. When combined with I-BET762, 5 out of the remaining 90 AODs showed synergistic anti-cancer effects with $R < 0.7$ (fractional product method), and the combination therapies reduced the number of CLB-GA cells by more than 80% (Supplementary Fig. S3C and Table S1).

The 11 compounds which reduced cell viability by ≥ 90% on their own and the 5 compounds which synergized with I-BET762, were subjected to a secondary drug screen with multiple dosages of I-BET762, AODs or combination in GI-MEN and CLB-GA cells. Three classes of AODs were found to exert strong synergy with I-BET762 with combination indexes (CIs) of < 0.7: proteasome inhibitors including carfilzomib and bortezomib; DNA topoisomerase II inhibiting and DNA intercalating chemotherapy agents; and tubulin inhibitor ixabepilone which was less effective (Fig. 2A). However, Alamar blue assays showed that combination therapy with I-BET762 and the chemotherapy agents at the same dosages as used in neuroblastoma cells, induced cytotoxicity in embryonic fibroblast WI38 cells (Supplementary Fig. S4A). In contrast, combination therapy with I-BET762 and the proteasome inhibitors carfilzomib or bortezomib did not show toxicity to the normal cells (Supplementary Fig. S4B).

The synergistic anticancer effects between the proteasome inhibitors carfilzomib and bortezomib and the BET bromodomain inhibitors I-BET762 and OTX015 were further

examined. GI-MEN and CLB-GA cells were treated with vehicle control, various dosages of carfilzomib or bortezomib, various dosages of I-BET762 or OTX015, or combinations, followed by Alamar blue assays. Synergy/additivity analysis using the Bliss-additivity model and the combination index (CI) method showed the strongest synergy by carfilzomib and OTX015 combination therapy over other combinations (Fig. 2B, Fig. 2C and Supplementary Fig. S5).

GI-MEN and CLB-GA cells were treated with vehicle, OTX015, carfilzomib or combination, followed by staining with Annexin V and flow cytometry analysis of apoptosis. While treatment with OTX015 or carfilzomib alone slightly increased the percentage of cells positively stained with Annexin V, OTX015 and carfilzomib significantly and synergistically induced apoptosis (R = 0.28 for GI-MEN cells, and R = 0.56 for CLB-GA cells, fractional product method) (Fig. 2D). Importantly, treatment of the normal embryonic fibroblast WI38 and CD34+ cells with vehicle control, OTX015, carfilzomib or combination did not induce apoptosis (Fig. 2D).

We next examined whether BRD4 siRNA, like the BET bromodomain BRD4 inhibitors, also synergized with carfilzomib. Flow cytometry analysis showed that BRD4 siRNA and carfilzomib also synergistically induced GI-MEN and CLB-GA cell apoptosis (Fig. 2E).

Taken together, the data confirm that the proteasome inhibitor carfilzomib is the AOD exerting considerable synergistic anticancer effects with BET bromodomain inhibitors against *TERT*-rearranged neuroblastoma cells, and that OTX015 and carfilzomib synergistically induce *TERT*-rearranged neuroblastoma cell apoptosis with little toxicity against normal cells.

OTX015 and carfilzomib exert synergistic anticancer effects partly by synergistically repressing TERT protein expression

BET bromodomain inhibitors are well-known to suppress oncogene expression (5, 6, 8), and proteasome inhibitors regulate protein expression (29). We next examined whether OTX015

and carfilzomib synergistically regulated TERT expression, telomerase activity and telomere length in *TERT*-rearranged neuroblastoma cells. RT-PCR and immunoblot analyses showed that OTX015, but not carfilzomib, reduced TERT mRNA and protein expression in both cell lines, that OTX015 and carfilzomib did not co-operatively reduced TERT mRNA expression in CLB-GA cells, and that OTX015 and carfilzomib co-operatively reduced TERT protein expression in both of the cell lines (Fig. 3A and Fig. 3B). Telomerase activity and telomere length assays showed that OTX015 or carfilzomib alone reduced telomerase activity, and that OTX015 and carfilzomib significantly and synergistically decreased telomerase activity ($R = 0.78$ for GI-MEN cells and $R = 0.49$ for CLB-GA cells, fractional product method), but showed no effect on telomere length (Fig. 3C and Fig. 3D).

TERT protein is targeted for ubiquitination and degradation by DYRK2-mediated EDD-DDB1-VprBP E3 ligase complex activation (30). Our immunoblot data showed that OTX015 and carfilzomib synergistically up-regulated DYRK2 protein expression in GI-MEN and CLB-GA cells (Fig. 3E). Importantly, immunoblot analysis showed that DYRK2 knockdown blocked OTX015 and carfilzomib combination therapy-mediated TERT protein reduction (Fig. 3F).

To examine whether OTX015 and carfilzomib exerted synergistic anticancer effects through reducing TERT protein expression, we treated GI-MEN and CLB-GA cells stably transfected with an empty vector or a TERT ORF expression construct with vehicle control, OTX015, carfilzomib, or combination. The exogenous TERT ORF expression was controlled by a viral promoter without super-enhancers. Treatment with OTX015 and carfilzomib showed no effect on TERT protein expression in GI-MEN and CLB-GA cells transfected with the TERT ORF expression construct, but synergistically reduced TERT protein expression in empty vector cells (Fig. 3G). Flow cytometry analysis showed that OTX015 and carfilzomib significantly increased the proportion of empty vector cells undergoing apoptosis, and that forced over-expression of TERT ORF significantly reduced this effect (Fig. 3H).

Taken together, the data demonstrate that OTX015 and carfilzomib synergistically induce *TERT*-rearranged neuroblastoma cell apoptosis partly through synergistically reducing TERT protein expression.

OTX015 and carfilzomib exert synergistic anticancer effects partly by inducing endoplasmic reticulum stress

Treatment with the proteasome inhibitor bortezomib results in up-regulation of Nrf2 protein which suppresses endoplasmic reticulum stress, leading to cancer cell resistance (31, 32). We have reported that treatment with the BET bromodomain inhibitor JQ1 blocks Nrf2 pathway activation and enhances cancer cell death (33). We therefore examined whether OTX015 and carfilzomib synergistically induced *TERT*-rearranged neuroblastoma cell death partly by blocking Nrf2 expression and synergistically inducing endoplasmic reticulum stress.

Immunoblot analysis showed that carfilzomib considerably up-regulated the expression of Nrf2 and its canonical targets HMOX1 and NQO1, and that OTX015 blocked carfilzomib-induced Nrf2, HMOX1 and NQO1 up-regulation in GI-MEN and CLB-GA cells (Fig. 4A). Additionally, OTX015 alone or carfilzomib alone did not have an effect, but OTX015 and carfilzomib combination therapy considerably activated the expression of CHOP, ATF4 and phosphorylated eIF2a proteins (Fig. 4A), the markers for endoplasmic reticulum stress (34), and activated oxidative stress (Supplementary Fig. S6A), demonstrating that OTX015 and carfilzomib synergistically induce endoplasmic reticulum stress and oxidative stress.

Next, our immunoblot analysis confirmed that Nrf2 siRNAs blocked carfilzomib-induced up-regulation of Nrf2, its targets HMOX1 and NQO1, and similar to OTX015, considerably activated the expression of the endoplasmic reticulum stress marker proteins CHOP, ATF4 and phosphorylated eIF2a (Fig. 4B). Alamar blue assays showed that combination of carfilzomib and Nrf2 siRNAs, similar to OTX015, synergistically reduced the number of

viable GI-MEN and CLB-GA cells (Fig. 4C), and that combination of CHOP siRNAs and ATF4 siRNAs partly rescued cells from OTX015 and carfilzomib combination-induced cytotoxicity (Supplementary Fig. S6B).

GI-MEN and CLB-GA cells were then transfected with a construct expressing empty vector or Nrf2 (33), followed by treatment with vehicle control, OTX015, carfilzomib or combination. Alamar blue assays showed that Nrf2 over-expression significantly suppressed the anticancer effect of OTX015 and carfilzomib combination therapy (Fig. 4D). In addition, Alamar blue assays showed that treatment with the endoplasmic reticulum stress inhibitor salubrinal or the oxidative stress inhibitor *N*-acetyl-l-cysteine (NAC) (35, 36) significantly suppressed the anticancer effect of OTX015 and carfilzomib combination therapy (Fig. 4E and Supplementary Fig. S6C).

Taken together, the data suggest that treatment with carfilzomib leads to Nrf2 protein over-expression, Nrf2 pathway activation and resistance to endoplasmic reticulum stress, that OTX015 blocks the effects, and that OTX015 and carfilzomib exert synergistic anticancer effects partly by synergistically inducing endoplasmic reticulum stress.

OTX015 and carfilzomib synergistically block TERT expression, suppress tumor progression and improve survival in neuroblastoma-bearing mice

No targeted therapy against *TERT*-rearranged neuroblastoma has been tested in clinical trials in patients. We investigated the anticancer efficacy of OTX015 and carfilzomib combination therapy in two xenograft models of *TERT*-rearranged neuroblastoma. GI-MEN and CLB-GA cells were each engrafted into 48 mice, which were randomized into four treatment groups (vehicle, OTX015, carfilzomib, or combination of OTX015 and carfilzomib) when tumors reached 0.2 cm^3. While 44 out of 48 mice in the CLB-GA group developed tumors three weeks after xenografting, only 15 out of 48 mice in the GI-MEN group developed tumors 6

months after xenografting. Treatment with OTX015 or carfilzomib alone inhibited neuroblastoma growth, compared with vehicle control, in mice xenografted with CLB-GA or GI-MEN cells. In combination, OTX015 and carfilzomib caused considerable tumor growth inhibition and initial tumor regression (Fig. 5A and Fig. 5B). Immunoblot analysis of mouse tumor tissues revealed that carfilzomib did not alter TERT protein expression, while OTX015 modestly reduced TERT protein expression. In combination, OTX015 and carfilzomib abolished TERT protein expression in both GI-MEN and CLB-GA cell xenograft tumors (Fig. 5C). In addition, OTX015 and carfilzomib combination therapy synergistically and considerably induced apoptosis in tumors, as characterized by significantly increased proportion of tumor cells positively-stained by TUNEL ($R = 0.42$ for CLB-GA xenografts and $R = 0.33$ for GI-MEN xenografts, fractional product method) (Fig. 5D and Fig. 5E).

Taken together, the data demonstrate that OTX015 and carfilzomib synergistically block TERT protein expression, induce tumor cell apoptosis, suppress tumor progression and improve survival in mice with established *TERT*-rearranged neuroblastoma tumors.

OTX015 and carfilzomib exert synergistic anticancer effects in neuroblastoma-bearing mice through a TERT-dependent mechanism

To examine whether OTX015 and carfilzomib exert synergistic anticancer effects against *TERT*-rearranged neuroblastoma *in vivo* through blocking TERT protein expression, we xenografted CLB-GA cells stably transfected with an empty vector or TERT ORF expression construct into nude mice. In mice xenografted with CLB-GA cells stably transfected with an empty vector, OTX015 and carfilzomib considerably and synergistically reduced tumor progression and improved mouse survival (Fig. 6A). By contrast, in mice xenografted with CLB-GA cells stably transfected with a TERT ORF expression construct, the anticancer effect of OTX015 and carfilzomib combination therapy was substantially reversed (Fig. 6B).

Immunoblot analysis showed that OTX015 and carfilzomib synergistically blocked TERT protein expression in mice xenografted with empty vector CLB-GA cells, but not TERT-ORF CLB-GA cells (Fig. 6C). TUNEL assays demonstrated that OTX015 and carfilzomib synergistically and considerably induced apoptosis in tumor tissues from the mice xenografted with empty vector CLB-GA cells, and that the effect was significantly reduced in tumor tissues from the mice xenografted with TERT-ORF CLB-GA cells (Fig. 6D). In addition, in tumors from the mice xenografted with empty vector CLB-GA cells, OTX015 and carfilzomib did not show co-operative effect on telomere length (Fig. 6E), but synergistically induced DNA damage response at telomere, as revealed by positive γ-H2AX staining (Fig. 6F).

Taken together, the data demonstrate that OTX015 and carfilzomib exert considerable anticancer effects against *TERT*-rearranged neuroblastoma *in vivo* through TERT-dependent but telomerase activity-independent mechanisms including DNA damage.

OTX015 and carfilzomib synergistically improve mouse survival in a PDX model of *TERT*-rearranged neuroblastoma

We have established patient-derived xenograft (PDX) *TERT*-rearranged neuroblastoma cells, CCI-NB07-RMT cells, with the massive deletion of chromosome 5:50791668 - 5:1295647 (20). RT-PCR confirmed that TERT mRNA expression was considerably higher in PDX CCI-NB07-RMT cells than *TERT*-rearranged or *MYCN*-amplified neuroblastoma cell lines, while N-Myc was hardly detectable (Supplementary Fig. S7A). ChIP sequencing showed an enhancer region rearranged to the *TERT* gene due to chromosome 5:50791668 - 5:1295647 deletion, and BRD4 protein binding to the enhancer in PDX CCI-NB07-RMT cells (Supplementary Fig. S7B). In comparison, the enhancer existed at the same locus in *TERT*-non-rearranged/*MYCN*-amplified BE(2)-C but not CHP134 cells, and the enhancer was > 49 Mb away from the *TERT* gene in BE(2)-C cells (Supplementary Fig. S7B). In addition, after PDX CCI-NB07-RMT cells

were treated with OTX015, ChIP PCR showed that BRD4 protein binding at the enhancer region was dramatically reduced (Supplementary Fig. S7C).

PDX CCI-NB07-RMT cells extracted from tumors from non-obese diabetic severe combined immune deficiency gamma (NSG) mice were xenografted into new groups of NSG mice, and treated with vehicle control, OTX015, carfilzomib, or combination. As shown in Supplementary Fig. S7D, OTX015 and carfilzomib synergistically suppressed tumor progression and improved mouse overall survival. Immunoblot analysis of tumor tissues from the mice showed that OTX015 and carfilzomib synergistically and significantly diminished TERT protein expression (Supplementary Fig. S7E). The data further demonstrate OTX015 and carfilzomib combination as an effective therapeutic strategy for *TERT*-rearranged neuroblastoma.

Discussion

BET bromodomain inhibitors exert anticancer effects by displacing BRD4 from super-enhancers, leading to transcriptional silencing of target oncogenes, such as *MYCN*, *MYC* and *BCL2* (5, 6, 8). In this study, we have found that BRD4 knockdown or BET bromodomain inhibitor treatment significantly reduces TERT mRNA and protein expression as well as telomerase activity in *TERT*-rearranged neuroblastoma cells, leading to *TERT*-rearranged neuroblastoma cell cycle arrest at the G1 phase and growth inhibition.

BET bromodomain inhibitor monotherapy does not cause tumor regression in mice or patients (9-11). We have previously identified anti-microtubule drugs as the AODs exerting the best synergistic anticancer effects with BET bromodomain inhibitors against *MYCN*-amplified neuroblastoma cells (33). In the current study, we have identified the proteasome inhibitors bortezomib and carfilzomib as the AODs exerting the best synergistic anticancer effects with BET bromodomain inhibitors against *TERT*-rearranged neuroblastoma cells with little toxicity to normal cells, and demonstrated that OTX015 and carfilzomib exert more effective anticancer effects than I-BET762 and bortezomib combinations. This is consistent with our observation that OTX015 is slightly more effective than I-BET762 as monotherapy, and consistent with the literature that the second generation proteasome inhibitor carfilzomib shows better anticancer efficacy and safety than the first generation proteasome inhibitor bortezomib (37, 38). While BET bromodomain inhibitors mainly suppress BRD4 but also show weak effect on other BRD proteins (39, 40), we have confirmed that BRD4 siRNAs, similar to OTX015, exert synergistic and significant anticancer effects with carfilzomib against *TERT*-rearranged neuroblastoma cells. The data suggest that BET bromodomain inhibitors exert synergistic anticancer effects with carfilzomib mainly through blocking BRD4 function.

Cancer cells develop resistance due to proteasome inhibitor-mediated Nrf2 up-regulation (32). In this study, we have found that carfilzomib activates the Nrf2 pathway and suppresses endoplasmic reticulum stress, that OTX015 blocks carfilzomib-mediated Nrf2 pathway activation, and that combination therapy with OTX015 and carfilzomib synergistically induces endoplasmic reticulum stress and cell death in *TERT*-rearranged neuroblastoma cells. In addition, cell death due to OTX015 and carfilzomib combination therapy is partly blocked by Nrf2 over-expression or treatment with an endoplasmic reticulum stress inhibitor. These data suggest that OTX015 and carfilzomib induce *TERT*-rearranged neuroblastoma cell death partly by regulating Nrf2 pathway and causing endoplasmic reticulum stress.

TERT is targeted for ubiquitination and degradation through DYRK2-mediated EDD-DDB1-VprBP E3 ligase activation (30). In this study, we have found that OTX015 and carfilzomib synergistically block TERT protein but not mRNA expression and up-regulate DRYK2 expression, and that DYRK2 knockdown blocks OTX015 and carfilzomib-modulated TERT protein reduction. The data suggest that OTX015 and carfilzomib synergistically reduce TERT protein expression through up-regulating DYRK2 expression, and that DYRK2 is a therapeutic target for *TERT*-rearranged neuroblastoma. We have also demonstrated that forced TERT over-expression largely blocks OTX015 and carfilzomib-induced *TERT*-rearranged neuroblastoma cell apoptosis *in vitro*. Importantly, OTX015 and carfilzomib combination therapy considerably blocks TERT protein expression, induces tumor cell apoptosis, suppresses *TERT*-rearranged neuroblastoma tumor progression and improves survival in mice xenografted with *TERT*-rearranged neuroblastoma GI-MEN, CLB-GA cell lines or PDX cells, and forced TERT over-expression in neuroblastoma cells largely blocks the anticancer effects of OTX015 and carfilzomib combination therapy in mice. In addition, GI-MEN cell tumors progress much slower than CLB-GA and PDX cell tumors, and OTX015 and carfilzomib combination therapy is more effective in mice xenografted with GI-MEN cells. Taken together, the data demonstrate

that OTX015 and carfilzomib exert synergistic anticancer effects against *TERT*-rearranged neuroblastoma partly by reducing TERT protein expression.

In addition to telomere maintenance, TERT promotes tumorigenesis by telomere-independent mechanisms, including enhancing global protein synthesis (13). In this study, we have found that TERT knockdown leads to a reduction in global protein synthesis in *TERT*-rearranged neuroblastoma cells, and that forced over-expression of wild type or telomerase-deficient TERT both blocks TERT or BRD4 knockdown-induced neuroblastoma cell growth inhibition and cell cycle arrest. In addition, while OTX015 and carfilzomib synergistically blocks TERT protein expression and suppress telomerase activity, forced over-expression of wild type or telomerase-deficient TERT both blocks the anticancer effects of the combination therapy. These data indicate a non-canonical function of TERT in *TERT*-rearranged neuroblastoma.

In summary, BRD4 is required for *TERT* oncogene over-expression, cell cycle progression and proliferation in *TERT*-rearranged neuroblastoma cells. The proteasome inhibitor carfilzomib is the agent exerting the best synergistic anticancer effects with the BET bromodomain inhibitor OTX015 against *TERT*-rearranged neuroblastoma cells. OTX015 and carfilzomib synergistically induce *TERT*-rearranged neuroblastoma cell apoptosis by blocking TERT protein expression and inducing endoplasmic reticulum stress, and substantially suppress tumor progression and improve survival in mice xenografted with *TERT*-rearranged neuroblastoma cell lines or PDX cells (Supplementary Fig. S7F). Although OTX015 itself has not been tested in children, OTX015 is currently in Phase II clinical trials in adult cancer patients and the BET bromodomain inhibitor BMS-986158 is now in a Phase I clinical trial in pediatric cancer patients (ClinicalTrials.gov Identifier: NCT03936465). Carfilzomib is an approved oncology drug and is currently in Phase I clinical trials in pediatric cancer patients (ClinicalTrials.gov Identifiers: NCT02303821 and NCT02512926). OTX015 and carfilzomib

combination treatment is likely to be translated into the first clinical trial of targeted therapy against *TERT*-rearranged neuroblastoma in patients.

Acknowledgments

We thank Dr Marco Herold at Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, for providing the FH1tUTG construct. Children's Cancer Institute Australia is affiliated with UNSW Australia and Sydney Children’s Hospitals Network.

Grant Support

The authors were supported by Cancer Council NSW and National Health & Medical Research Council Australia. P.Y.L. is a research fellow of Cancer Institute NSW. V. B. was supported by the ATIP-Avenir Program, the ARC Foundation (RAC16002KSA - R15093KS), the ‘Who Am I?’ Laboratory of Excellence (ANR-11-LABX-0071) and the French Government [ANR-11-IDEX-0005-02].

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Figure Legends

Figure 1. BRD4 is required for TERT expression and cell proliferation in *TERT*-rearranged neuroblastoma cells. **A-B,** DOX-inducible control shRNA, BRD4 shRNA-1 or BRD4 shRNA-2 GI-MEN and CLB-GA cells were treated with vehicle control or 2µg/ml DOX for 48 hours, followed by RT-PCR (**A**) and immunoblot (**B**) analyses of BRD4 and TERT mRNA and protein expression. **C,** GI-MEN and CLB-GA cells stably transfected with a TERT open reading frame (ORF) expression construct (TERT ORF) or empty vector were transfected with control siRNA, BRD4 siRNA-1 or BRD4 siRNA-2. Immunoblot analysis of BRD4 and TERT protein expression was performed 48 hours after siRNA transfection (**C**), and Alamar blue assays were performed 96 hours later (**D**). **E**, DOX-inducible control shRNA, BRD4 shRNA-1 or BRD4 shRNA-2 GI-MEN and CLB-GA cells were treated with vehicle control or 2µg/ml DOX, followed by staining with propidium iodide and flow cytometry analysis of the cell cycle 72 hours later. The percentages of cells at the G1 phase and the S phase were shown. **F-G,** DOX-inducible control shRNA, BRD4 shRNA-1 or BRD4 shRNA-2 GI-MEN and CLB-GA cells were treated with vehicle control or 2µg/ml DOX for 14 days, followed by clonogenic assays (**F**) and quantification of colonies (**G**). Data were shown as the mean ± standard error of three independent experiments. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively.

Figure 2. Approved Oncology Drug screening identifies carfilzomib as the anticancer agent exerting considerable synergistic anticancer effects with BET bromodomain inhibitors. **A,** For secondary screening, GI-MEN and CLB-GA cells were treated with vehicle control, various dosages of I-BET762, 16 Approved Oncology Drugs, or combination, followed by Alamar blue assays. Combination Indexes (CIs) for effective doses for 75% (ED75) and 90% (ED90) cell number reduction were calculated with CalcuSyn. **B-C,** GI-MEN and CLB-GA cells were

treated with vehicle control, various dosages of the BET bromodomain inhibitor I-BET762 or OTX015, the proteasome inhibitor bortezomib or carfilzomib, 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 the BET bromodomain inhibitor alone or the proteasome inhibitor 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 (**B**). Combination effects were further summarized by combination indexes (CIs), and CIs for effective doses for 75% (ED75) and 90% (ED90) cell number reduction were calculated with CalcuSyn (**C**). **D,** GI-MEN neuroblastoma, WI-38 embryonic fibroblast and CD34+ cells were treated with vehicle control, 2μM OTX015, 4nM carfilzomib or combination of OTX015 and carfilzomib for 72 hours, and CLB-GA cells were treated with vehicle control, 1μM OTX015, 2nM carfilzomib or combination of OTX015 and carfilzomib for 72 hours. Cells were then stained with Annexin-V and 7-AAD, followed by flow cytometry analyses. The percentage of cells positively stained by Annexin-V was quantified. **E,** GI-MEN and CLB-GA cells were transfected with control siRNA or BRD4 siRNA-1 and treated with vehicle control or carfilzomib for 72 hours. Cells were then stained with Annexin-V and 7-AAD, followed by flow cytometry analyses. The percentage of cells positively stained by Annexin-V was quantified. Data were shown as the mean ± standard error of three independent experiments. *** indicates $p < 0.001$.

Figure 3. OTX015 and carfilzomib exert synergistic anticancer effects partly by synergistically repressing TERT protein expression. **A-D,** GI-MEN cells were treated with control solvent, 2μM OTX015, 4nM carfilzomib or combination, and CLB-GA cells were treated with control solvent, 1μM OTX015, 2nM carfilzomib or combination. RNA and protein were extracted for RT-PCR (**A**) and immunoblot (**B**) analyses of BRD4 and TERT mRNA and protein expression

48 hours post-treatment. Telomerase activity (**C**) and telomere length assays (**D**) were performed 72 hours post-treatment. HT1080 and VA13 cells were used as controls without treatment. **E,** GI-MEN and CLB-GA cells were treated with control solvent, OTX015, carfilzomib, or combination for 48 hours, followed by immunoblot analysis of TERT and DYRK2. **F,** GI-MEN and CLB-GA cells were transfected with control siRNA, DYRK2 siRNA-1 or siRNA-2 and treated with control solvent or combination of OTX015 and carfilzomib for 48 hours, followed by immunoblot analysis of TERT and DYRK2. **G,** GI-MEN and CLB-GA cells stably transfected with an empty vector or TERT ORF expression construct were treated with control solvent, OTX015, carfilzomib or combination. Immunoblot analyses of TERT protein expression was performed 48 hours after treatments. **H,** Empty vector or TERT ORF expression GI-MEN and CLB-GA cells were treated with control solvent, OTX015, carfilzomib or combination for 72 hours, followed by staining with 7-AAD and Annexin-V, flow cytometry analysis and quantification of Annexin-V positively stained apoptotic cells. Data were shown as the mean ± standard error of three independent experiments. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively, and ns indicates no significant difference.

Figure 4. OTX015 and carfilzomib exert synergistic anticancer effects partly by inducing endoplasmic reticulum stress. **A,** GI-MEN cells were treated with control solvent, 2µM OTX015, 4nM carfilzomib or combination, and CLB-GA cells were treated with control solvent, 1µM OTX015, 2nM carfilzomib or combination, followed by immunoblot analysis of Nrf2, HMOX1, NQO1, CHOP, ATF4, total and phosphorylated eIF2a proteins. **B-C,** GI-MEN and CLB-GA cells were transfected with control siRNA, Nrf2 siRNA-1 or Nrf2 siRNA-2, and treated with control solvent or carfilzomib, followed by immunoblot analysis 48 hours later (**B**) or Alamar blue assays 72 hours later (**C**). **D,** GI-MEN and CLB-GA cells were transfected with a construct expressing empty vector or Nrf2, and treated with control solvent, OTX015,

carfilzomib or combination, followed by Alamar blue assays 72 hours later. **E,** GI-MEN and CLB-GA cells were co-treated with control solvent, OTX015, carfilzomib or combination, together with control solvent or 200nM salubrinal, followed by Alamar blue assays 72 hours later. Data were shown as the mean ± standard error of three independent experiments. *** indicates $p < 0.001$.

Figure 5. OTX015 and carfilzomib synergistically block TERT expression, suppress tumor progression and improve survival in neuroblastoma-bearing mice. **A-B,** BALB/c nude mice were xenografted with CLB-GA or GI-MEN neuroblastoma cells. When tumors reached 0.2 cm^3, mice were divided into four sub-groups, and treated with control solvent, OTX015 at 50 mg/kg body weight/day (oral gavage), carfilzomib at 6 mg/kg body weight/2 days (i.p.), or OTX015 plus carfilzomib. Tumor size was monitored and mice were culled when tumor reached 1 cm^3 (**A**). Survival curves showed the probability of mouse overall survival. Long-rank test was used to determine statistically significant difference between combination therapy group and the other groups (**B**). **C,** Protein was extracted from mouse tumor tissues and subjected to immunoblot analysis of BRD4 and TERT protein expression. Actin was used as a loading control. **D-E,** Paraffin-embedded tumor tissues from the mice were subjected to TUNEL labeling and Scale bars represent 100μM (**D**). Tumor cells positively stained by TUNEL were quantified (**E**). Data were shown as the mean ± standard error and evaluated by one-way ANOVA. *** indicates $p < 0.001$.

Figure 6. OTX015 and carfilzomib exert synergistic anticancer effects in neuroblastoma-bearing mice through a TERT-dependent mechanism. **A-B,** BALB/c nude mice were xenografted with CLB-GA cells stably transfected with an empty vector (**A**) or TERT ORF expression construct (**B**). When tumors reached 0.1 cm^3, the mice were divided into four sub-

groups, and treated with control solvent, OTX015 at 50 mg/kg body weight/day (oral gavage), carfilzomib at 6 mg/kg body weight/2 days (i.p.), or OTX015 plus carfilzomib. Mice were culled when the tumor reached 1 cm^3. Survival curves showed the probability of mouse overall survival. Long-rank test was used to determine statistically significant differences between the combination therapy group and the other groups. **C,** Protein was extracted from mouse tumor tissues and subjected to immunoblot analysis of TERT protein expression. Actin was used as a loading control. **D,** Paraffin-embedded tumor tissues from the mice were subjected to TUNEL labeling. Tumor cells positively stained by TUNEL were quantified. Scale bars represent 100μM. Data were shown as the mean ± standard error. *** indicates $p < 0.001$. **E,** Tumor tissues from mice xenografted with empty vector CLB-GA cells were subjected to telomere length assays. **F,** Tumor sections from mice xenografted with empty vector CLB-GA cells were double-stained with a telomere marker and an antibody against γ-H2AX for telomere dysfunction-induced foci assays and quantified. Data were shown as the mean ± standard error and evaluated by one-way ANOVA. * indicates $p < 0.05$.