**Supplemental Figures Legends**

**Supplemental Fig 1. BRD4 inhibitor MK-8628 and PLK1 inhibitor Volasertib have strong anti-tumor effects against neuroblastoma and rhabdomyosarcoma cell lines in vitro. A** Dose response of rhabdomyosarcoma cell lines treated with the BRD4 inhibitor MK-8628 or the PLK1 inhibitor Volasertib for 72h. **B** Dose response curves for neuroblastoma cell lines treated with BRD4 inhibitor MK-8628 or PLK1 inhibitor Volasertib for 72h. **C** Inhibitory concentration 50% (IC50) values for 18 pediatric tumor cell lines. **D** Maximum effect on cell viability (Emax) for 18 pediatric tumor cell lines **E** Area under the curve values for 18 pediatric tumor cell lines. (Student’s t-test: \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001, \*\*\*\* = p< 0.0001)

**Supplemental Fig. 2. MK-8628 and Volasertib exhibit synergistic anti-tumor effects against neuroblastoma and rhabdomyosarcoma cells. A** Excess over Bliss analysis of neuroblastoma cell line CHP212 (MYC/N high expressing) and NBL (MYC/N low expressing) treated with combinations of MK-8628 and Volasertib. **B** Excess over Bliss analysis of rhabdomyosarcoma cell line RH4 (MYC/N high expressing) and TE441 (MYC/N high expressing) treated with combinations of MK-8628 and Volasertib.

**Supplemental Fig. 3. Simultaneous inhibition of BRD4 and PLK1 synergistically disrupts cell cycle in neuroblastoma and rhabdomyosarcoma cell lines A** FACS-based measurement of the cell cycle distribution of Rh4 cells after treatment with DMSO control, MK-8628 (150nM), Volasertib (3nM), or combination of both (150nM MK-8628 + 3nM Volasertib) for 72h. **B** Fraction of cells in G1 phase as measured in (A). **C** Fraction of cells in S phase as measured in (A). **D** Fraction of cells in G2/M phase as measured in (A). **E** Fraction of cells in Sub G1 phase as measured in (A). **F** FACS-based measurement of the cell cycle distribution of IMR5/75 cells after treatment with DMSO control, MK-8628 (150nM), Volasertib (3nM), or combination of both (150nM MK-8628 + 3nM Volasertib) for 72h. Fraction of cells in G1 phase as measured in Fig 2A. **F** Fraction of cells in G2/M phase as measured in Fig. 2A. (Student’s t-test: \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001, \*\*\*\* = p< 0.0001)

**Supplemental Fig. 4. Screening of 49 dual nanomolar PLK1/BRD4 pediatric tumor cell lines and untransformed control cells**. **A-E** Relative number of viable untransformed fibroblast cells (BJ)**(A)**, *MYCN*-amplified neuroblastoma cells (IMR5) **(B)**, high MYCN-expressing rhabdomyosarcoma cells (RH4) **(C)**, high MYCN-expressing rhabdomyosarcoma cells (RH30) **(D)** and MYC-amplified medulloblastoma cells (HD-MB03) **(E)** after treatment with 49 dual nanomolar PLK1/BRD4 inhibitors for 72h at 20nM. **F** Anti-BRD4 and PLK1 activity in cell free assays for the dual nanomolar BRD4/PLK1 inhibitors as plotted in Figure 3B

**Supplemental Fig. 5** **Candidate dual nanomolar BRD4/PLK1 inhibitors exhibit strong anti-tumoral effects against pediatric tumor cell lines at nanomolar concentrations. A-E** Dose response analysis for rhabdomyosarcoma cell lines treated with increasing concentrations of dual nanomolar BRD4/PLK1 inhibitors UMB160 **(A)** or UM103 **(B)**, for medulloblastoma cell lines treated with UMB160 **(C)** or UMB103 **(D)** and neuroblastoma cell lines treated with UMB160 **(E)**.

**Supplemental Fig. 6. Simultaneous inhibition of BRD4 and PLK1 with dual BRD4/PLK1 inhibitor disrupts cell cycle in neuroblastoma and rhabdomyosarcoma cell lines A** FACS-based measurement of the cell cycle distribution of IMR5/75 cells after treatment with DMSO control or UMB103 (5nM) or UMB160 (5nM) for 72h. Fraction of cells in G1 phase as measured in Fig. 4A. **B** Fraction of cells in G2/M phase as measured in Fig. 4A. **C** neuroblastoma cell lines (LAN1, IMR5/75, SK-N-FI, NBL-S) were treated with two concentrations of dual nanomolar BRD4/PLK1 inhibitor UMB160 for 24h. Changes in proliferation after treatment were analyzed with relative BrdU incorporation. **D** FACS-based measurement of the cell cycle distribution of Rh4 cells after treatment with DMSO control or UMB103 (6.5nM) or UMB160 (4nM) for 72h. Fraction of cells in G1 phase in Rh4. **E** Fraction of cells in S phase in Rh4. **F** Fraction of cells in G2/M phase in Rh4. **G** Fraction of cells in Sub G1phase in Rh4. **H** FACS-based measurement of the cell cycle distribution of HD-MB03 cells after treatment with DMSO control or UMB103 (5 or 10nM) or UMB160 (5 or 10nM) for 72h. Fraction of cells in G1 phase in HD-MB03. **I** Fraction of cells in S phase in HD-MB03. **J** Fraction of cells in G2/M phase in HD-MB03. **K** Fraction of cells in Sub G1 phase in HD-MB03. (Student’s t-test: \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001, \*\*\*\* = p < 0.0001)

**Supplemental Fig. 7. Treatment with dual nanomolar BRD4/PLK1 inhibitors leads to repression of known BRD4 and PLK1 functions** **A** Western immunoblotting of cleaved caspase 3 after treatment of rhabdomyosarcoma cell lines with DMSO control UMB160 (20nM) or UMB103 (20nM) for 24h. **B** Heatmaps of differentially expressed genes as well as Venn diagram showing overlap of differentially expressed genes in MYCN-expressing rhabdomyosarcoma cell line RH4 treated with 500nM MK-8628, 100nM UMB103 or 100nM UMB160 for 6h. **C** Unsupervised clustering of differentially expressed genes in RH4 cells treated with 500nM MK-8628, 100nM UMB103 or 100nM UMB160 for 6h. **D** Gene set enrichment analysis (GSEA) plot for PAX3-FOXO1 target genes in rhabdomyosarcoma cell line RH4 after treatment with 500nM MK-8628, 100nM UMB103 or 100nM UMB160 for 6h. **E** Venn diagram of differentially expressed genes in IMR5 cells after treatment with UMB160, UMB103, Volasertib or MK-8628. **F** Gene set enrichment analysis (GSEA) plot of MYCN target genes in neuroblastoma cell line IMR5 and rhabdomyosarcoma cell line RH4 after treatment with Volasertib or MK-8628. **G**  Top differentially expressed genes in IMR5 cells treated with dual inhibitors compared to treatment with Volasertib, MK-8628 or combination of Volasertib and MK-8628.

**Supplemental table 1:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Target protein** | **Dilution** | **Catalog number** | **Company** |
| MYC | 1:500 | #9402 | Cell Signaling Technology |
| MYCN | 1:1,000 | sc-53993 | Santa Cruz |
| WEE1 | 1:1,000 | Sc-5285 | Santa Cruz |
| CCND1 | 1:500 | #2922 | Cell Signaling Technology |
| BRD4 | 1:200 | sc-48772 | Santa Cruz |
| PLK1 | 1:500 | Sc-5585 | Santa Cruz |
| p-PLK1 | 1:200 | Ab39068 | Abcam, Cambridge, UK |
| Beta-Actin | 1:20,000 | 3700s | Cell Signaling Technology |
| GAPDH | 1:10,000 | Sc-25778 | Santa Cruz |
| Horseradish peroxidase (HPR) -conjugated anti-rabbit IgG | 1:5,000 | 111-035-003 | Dianova, Hamburg, Germany |
| HPR-conjugated anti-mouse IgG | 1:5,000 | 115-035-003 | Dianova, Hamburg, Germany |

**Supplemental tables 2:**

MK-8628

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell line | MYC/N | IC50 | AUC | Emax |
| RH4 | + | 339 | 184402 | 10.3 |
| RH30 | + | 251.1 | 105943 | 5.2 |
| Kym1 | - | 113.9 | 63992 | 2.6 |
| RH18 | - | 301.5 | 167205 | 10.6 |
| TE381.T | - | 334.6 | 15061 | 11.1 |
| RH36 | + | 2971 | 474433 | 24.2 |
| RH41 | + | 4.8 | 13619 | 0.5 |
| T174 | - | 3376 | 484606 | 34.6 |
| RD | - | 153.7 | 194876 | 16.6 |
|  |  |  |  |  |
| UW228 | - | 5316 | 25377487264 | 49.5 |
| ONS76 | + |  | 24166978634 | 97.6 |
| DAOY | + | 124.4 | 22515652253 | 17.1 |
| HD-MB03 | ++ | 261.3 | 498760871 | 0.6 |
|  |  |  |  |  |
| SK-N-FI | - | 5308 | 562527 | 41.1 |
| NBL-S | - | 577.6 | 238938 | 14.5 |
| IMR5/75 | ++ | 91.5 | 69015 | 3.4 |
| Kelly | ++ | 553.1 | 140647 | 5.4 |
| CHP-212 | ++ | 114.4 | 95019 | 7.1 |

Volasertib

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell line | MYC/N | IC50 | AUC | Emax |
| RH4 | + | 4.7 | 78093470 | 9.9 |
| RH30 | + | 9.5 | 92916025 | 12.3 |
| Kym1 | - | 9.2 | 162790272 | 3.9 |
| RH18 | - | 65.3 | 266862804 | 44.7 |
| TE381.T | - | 8.8 | 225106484 | 14.3 |
| RH36 | + | 14.1 | 15249496 | 23.4 |
| RH41 | + | 4.1 | 16650732 | 26.2 |
| T174 | - | 5.1 | 127881158 | 11.4 |
| RD | - | 6.2 | 142256885 | 6.1 |
|  |  |  |  |  |
| UW228 | - | 106.6 | 320943084 | 59.7 |
| ONS76 | + | 5.8 | 24825066 | 7.5 |
| DAOY | + | 14.4 | 362461802 | 29.8 |
| HD-MB03 | ++ | 3.0 | 5387811 | 0.3 |
|  |  |  |  |  |
| SK-N-FI | - | 31.6 | 4588 | 27 |
| NBL-S | - | 18.6 | 3179 | 20.4 |
| IMR5/75 | ++ | 10.5 | 1395 | 1.8 |
| Kelly | ++ | 13.8 | 1674 | 0.2 |
| CHP-212 | ++ | 22.7 | 3630 | 19.8 |

GSK461364

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | MYC/N | IC50 | AUC | Emax |
| RH4 | ++ | 122.6 | 12618 | 12.1 |
| IMR5/75 | + | 183.9 | 17419 | 36.4 |

++ MYC/N amplification, + MYC/N high expression, - low MYC/N expression

**Supplemental tables 3:**

UMB103

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell line | MYC/N | IC50 | AUC | Emax |
| TE381.T | - | 19.9 | 153235273 | 23.6 |
| T174 | - | 9.3 | 147147082 | 15.2 |
| RH30 | + | 9.7 | 89491522 | 5.7 |
| RH4 | + | 6.5 | 42751396 | 6.6 |
| Kym1 | - | 58.9 | 740583870 | 4.9 |
| RD | - | 43.4 | 641553271 | 9.6 |
| Rh41 | + | 25.6 | 67551788 | 14.4 |
|  |  |  |  |  |
| DAOY | - | 59.2 | 1013746466 | 25.3 |
| HD-MB03 | + | 60.9 | 541778244 | 10.9 |
| ONS76 | + | 27.0 | 855676660 | 2.5 |
| UW228 | ++ | 178.3 | 617459632 | 45.6 |
|  |  |  |  |  |
| CHP-212 | ++ | 133.1 | 492743486 | 11.5 |
| GI-MEN | - | 139.4 | 1412486083 | 32.7 |
| IMR5/75 | ++ | 27.3 | 276927038 | 0.2 |
| Kelly | ++ | 62.5 | 575143297 | 0.2 |
| NBL-S | - | 106.6 | 554019269 | 3.4 |

UMB160

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell line | MYC/N | IC50 | AUC | Emax |
| TE381.T | - | 4.8 | 159119920 | 23.6 |
| T174 | - | 3.7 | 175729522 | 15.2 |
| RH30 | + | 5.3 | 123790777 | 5.7 |
| RH4 | + | 2.9 | 34496680 | 6.6 |
| Kym1 | - | 41.4 | 559566326 | 4.9 |
| RD | - | 63.5 | 777899383 | 9.6 |
| Rh41 | + | 41.9 | 95197564 | 14.4 |
|  |  |  |  |  |
| DAOY | - | 25.2 | 770511938 | 23.9 |
| HD-MB03 | + | 14.9 | 207701999 | 0.3 |
| ONS76 | + | 13.6 | 448108713 | 4.9 |
| UW228 | ++ | 87.0 | 511047965 | 35.1 |
|  |  |  |  |  |
| CHP-212 | ++ | 225.3 | 656767783 | 8.8 |
| GI-MEN | - | 361.4 | 2257663523 | 38.5 |
| IMR5/75 | ++ | 45.3 | 716942927 | 0.3 |
| Kelly | ++ | 144.8 | 1018945730 | 0.3 |
| NBL-S | - | 116.4 | 726888626 | 2.2 |

++ MYC/N amplification, + MYC/N high expression, - low MYC/N expression