The polyamine analog verlindamycin promotes differentiation and cell death in neuroblastoma

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Abstract

Neuroblastoma (NB) is the second most common solid tumor in childhood, accounting for 8-10% of the total number of pediatric cancers and 15% of deaths. Deregulated polyamine biosynthesis is a common feature of NB and drugs targeting this metabolic pathway, such as eflornithine (difluoromethylornithine, DFMO), are in preclinical and clinical development. The polyamine analogue verlindamycin (also known as 2d) inhibits the histone demethylase LSD1, as well as homologous enzymes involved in polyamine biosynthesis such as spermine oxidase and N1-acetylpolyamine oxidase. We previously demonstrated that verlindamycin cooperated with all-*trans*-retinoic acid (ATRA) to promote differentiation of acute myeloid leukemia and reasoned that this drug might be effective in NB. Consistent with this notion, we found that treatment of a panel of NB cell lines with verlindamycin and ATRA strongly induced differentiation that was associated with reduced growth and colony formation, as well as induction of G0/G1 arrest and apoptosis. Verlindamycin/ATRA treatment was effective in all NB cell lines tested but effects were more pronounced in those harbouring amplification of the *MYCN* oncogene which is associated with high-risk, poor outcome disease. Transcriptomic analysis revealed that treatment of *MYCN*-amplified SK-N-BE(2)-C cells with verlindamycin/ATRA led to downregulation of MYCN and E2F target genes, as well as genes involved in the G2/M checkpoint, DNA repair, PI3K/AKT/mTOR and unfolded protein response pathways. Conversly, we found upregulation of genes involved in the inflammatory response, apoptotisis, reactive oxygen species, TNFα and TGFβ pathways. These results suggested that the verlindamycin/ATRA combination targeted MYCN and we found that while *MYCN* mRNA was not significantly downregulated, MYCN protein was strongly diminished. This was due to increased turnover of MYCN protein, at least in part via proteasome-dependent destruction. The effects of verlindamycin were not due to it's LSD1 inhibitory activities but appear to be caused, at least in part, by induction of functional Antizyme 1 and 2 via ribosomal frame-shifting. Consistent with previous results describing the function of Antizyme tumor suppressor, we found that verlindamycin treatment led to the selective targeting of ornithine decarboxylase (the rate-limiting enzyme for polyamine biosynthesis) as well as key oncoproteins such as cyclin D and Aurora A kinase. The finding that verlindamycin treatment diminished Aurora A levels is notable in the context of MYCN expressing NB as we have previously shown that Aurora A binds MYCN, preventing its degradation. Retinoid-based multimodal differentiation therapy is one of few interventions that extends relapse-free survival in MYCN-associated high-risk NB and the results presented here strongly argue that the potential inclusion of verlindamycin in this regimen merits further investigation.

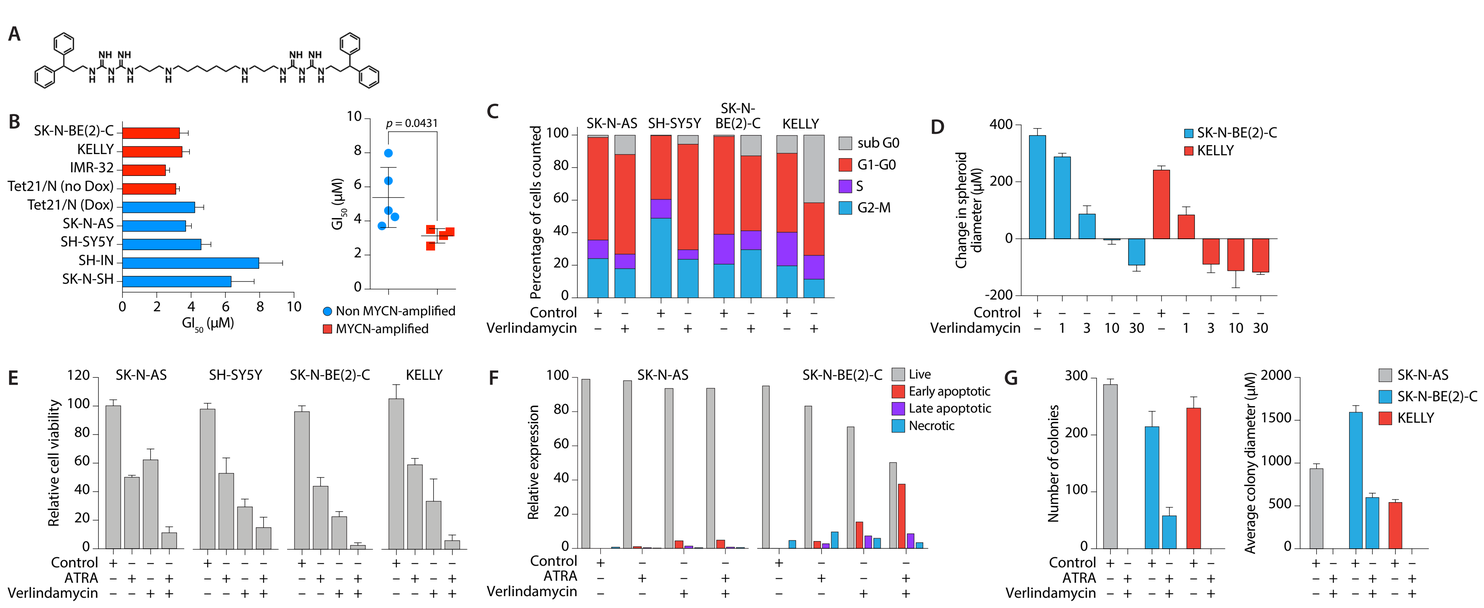

Figure 1. Verlindamycin and its combination with AtRA inhibits neuroblastoma cell growth and induces apoptosis.
(A) Structure of verlindamycin. **(B)** Neuroblastoma cells were treated with a range of verlindamycin concentrations to establish Gi50 with the use of SRB assay; graph on the right shows differences in Gi_{50} values between *MYCN*-amplified and non-amplified cells. **(C)** Cells treated with verlindamycin (1.75 μM = ½ Gi_{50}) for 6 days were stained with propidium iodide (PI) to assess cell cycle distribution. **(D)** Cells grown as spheroids were exposed to a range of verlindamycin concentrations, spheroid diameter was measured every 24h for 96 hours with Celigo Imaging Cytometer. **(E)** Metabolic activity of cells treated with 1.75 μM verlindamycin combined with 1 μM ATRA was measured by CellTiter Glo after 6 days of treatment. **(F)** Cell death was assessed by FITC-annexin V and PI staining after 6 days of combined 1.75 μM verlindamycin and 1 μM ATRA treatment. **(G)** Cells treated with 1.75 μM verlindamycin and 1 μM ATRA for 10 days were tested for their ability to form colonies from single cells for within 14 days.

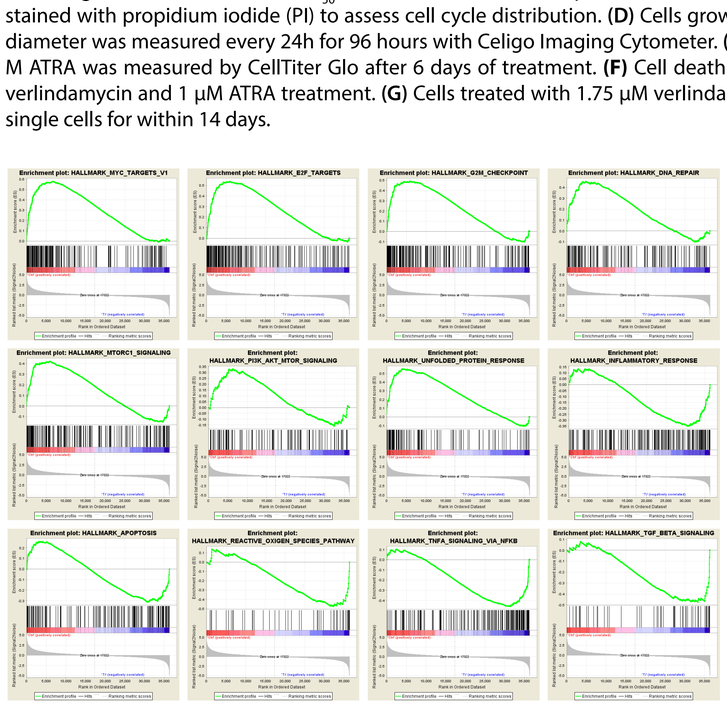

Figure 3. Treatment with verlindamycin and AtRA changes expression patterns in neuroblastoma cells.
Expression microarray was performed on SK-N-BE(2)-C and SK-N-AS neuroblastoma cell lines treated with 1.75 μM verlindamycin and 1 μM AtRA for 6 days. Selected Gene Set Enrichment Analysis (GSEA) plots for SK-N-BE(2)-C are shown.

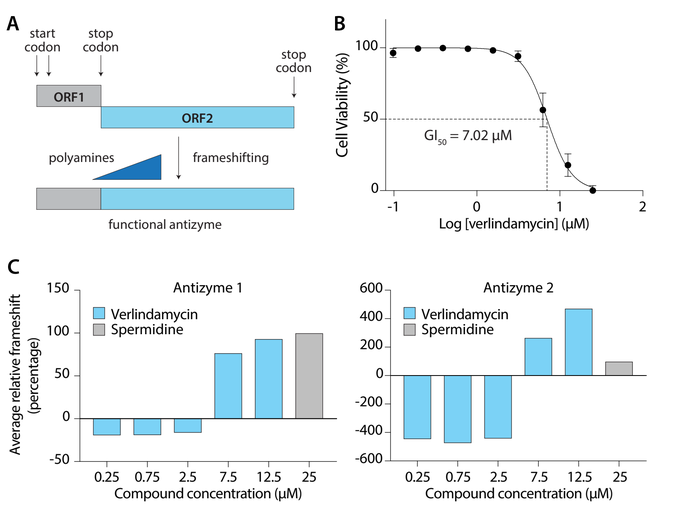

Figure 6: Verlindamycin treatment induces frameshifting of Antizyme 1 and 2.
(A) Schematic showing polyamine-induced frameshifting of Antizyme. **(B)** GI_{50} of Verlindamycin in 293T cells. **(C)** Verlindamycin-induced frameshifting of antizyme 1 and 2 relative to 25μM spermadine. 293T cells grown in medium containing 2.5 mM Eflornithine (DFMO) were transfected with Antizyme 1 or 2 frameshifting reporters, or in-frame controls. After 6 hours, medium was replaced with medium containing 25μM Spermidine, or Verlindamycin as indicated. After 48hrs reporter activity was assayed using Dual-Glo (Promega). Percentage frameshifting (%FS) activity was determined by obtaining firefly:renilla luciferase ratios, then dividing reporter values by in-frame control values. Relative FS was calculated by the following method: background %FS activity determined from the 2.5mM DFMO control was subtracted from the %FS activity for treated samples. The background-corrected %FS activity of each compound was then divided by the background-corrected %FS activity induced by 25mM spermidine and multiplied by 100.

Figure 2. Verlindamycin enhances AtRA-induced differentiation in neuroblastoma.
The neuroblastoma cell lines SK-N-BE(2)-C (*MYCN*-amplified) and SK-N-AS (*MYCN*-non-amplified) were treated with 1.75 μM verlindamycin and 1 μM ATRA for 6 days. **(A)** Cells were fixed and stained with neurofilament-light (NF-L, red) and TO-PRO-3 (blue). **(B)** mRNA expression of ATRA-target gene (*CRABP2*) and neural markers (*RARB*, *RET*) was measured by RT-qPCR and normalized to *GAPDH*.

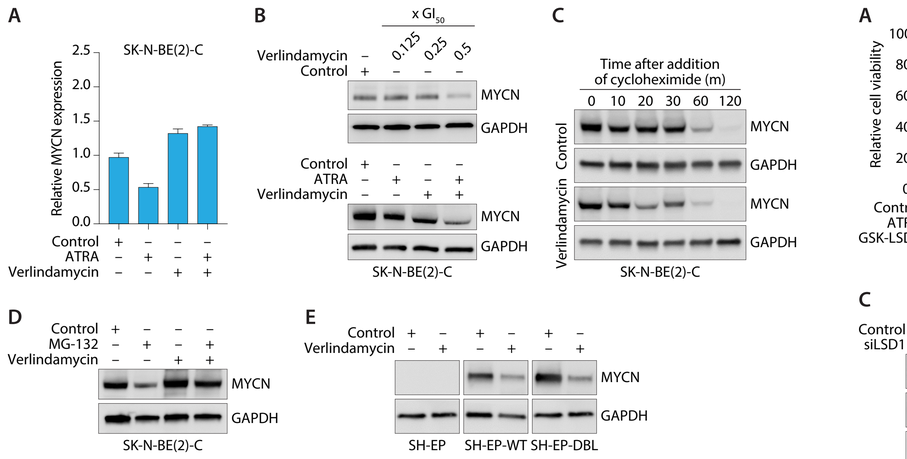

Figure 4. Verlindamycin down-regulates expression of MYCN protein in MYCN-amplified neuroblastoma.
(A) *MYCN* mRNA expression was measured by RT-qPCR (relative to *GAPDH*) in SK-N-BE(2)-C cells treated for 6 days with 1.75 μM verlindamycin alone or combined with 1μM ATRA. **(B)** MYCN protein levels were assessed in SK-N-BE(2)-C cells treated for 6 days: with different concentrations of verlindamycin (upper panel; Gi_{50} = 3.5 μM) and with 1.75 μM verlindamycin combined with 1 μM ATRA (lower panel). **(C)** SK-N-BE(2)-C cells pre-treated with verlindamycin for 4 days were exposed to 25 μg/ml cycloheximide for up to 2h. **(D)** SK-N-BE(2)-C cells pre-treated with verlindamycin for 4 days were exposed to 10 μM MG-132 for 16h. **(E)** Following 3 days treatment with verlindamycin, MYCN expression was assessed in parental SH-EP cells lacking MYCN, SH-EP cells expressing exogenous wild-type MYCN (SH-EP-WT) or SH-EP cells expressing exogenous MYCN mutated at T58 and S62 (SH-EP-DBL).

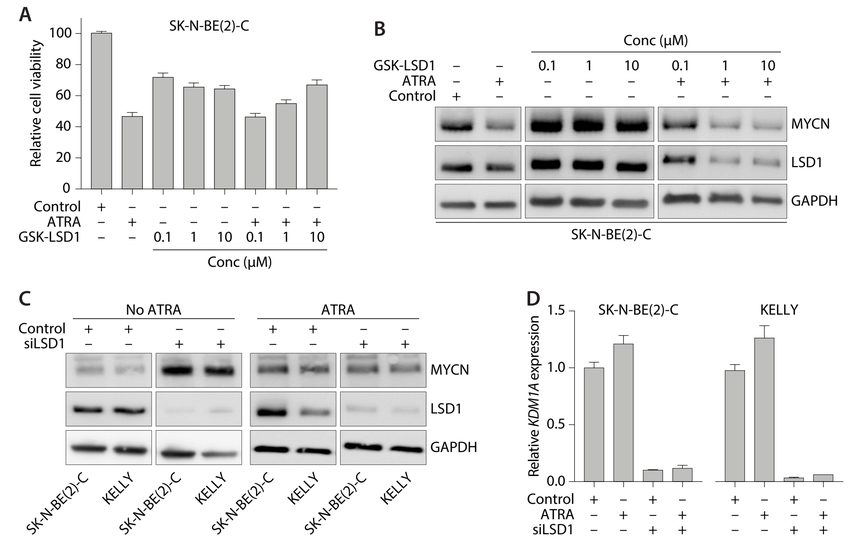

Figure 5 Inhibition of KDM1A/LSD1 is not the mechanism of action of verlindamycin.
(A) SK-N-BE(2)-C cells were treated with a range of GSK-LSD1 concentrations combined with 1μM ATRA for 6 days after which cell viability was assessed by CellTiter Glo. **(B)** MYCN expression level was tested in cells treated with GSK-LSD1 with or without addition of 1μM ATRA for 6 days. **(C)** SK-N-BE(2)-C and Kelly cells with siRNA-mediated *KDM1A* knock-down and treated with ATRA (for 6 days) were harvested to assess the expression of MYCN and LSD1. **(D)** mRNA expression of *KDM1A* was also tested in SK-N-BE(2)-C and KELLY cells treated with ATRA and *KDM1A* -targeting siRNA; expression measured by RT-qPCR relative to *GAPDH*.

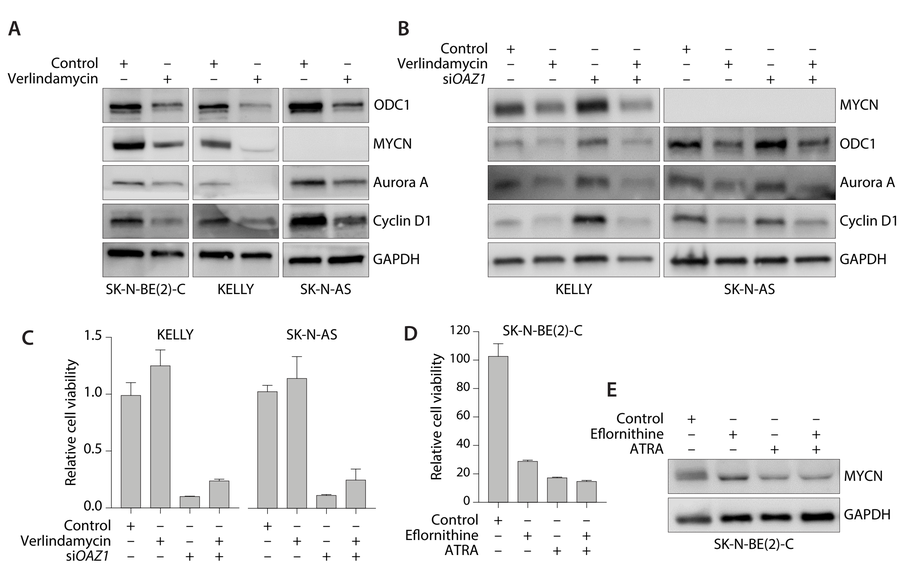

Figure 7. Functional analysis of verlindamycin-induced frameshifting of antizyme.
(A) SK-N-BE(2)-C, Kelly and SKNAS cells were treated with 1.75 μM verlindamycin for 4 days, after which the expression of Cyclin D1, Aurora A, and MYCN was measured. **(B)** After 96h of siRNA knock-down of *OAZ1* combined with verlindamycin treatment expression of Antizyme 1 targets was measured by Western blotting. **(C)** Expression of *OAZ1* mRNA was assessed by RT-qPCR (relative to GAPDH) in cells transfected with siRNA. **(D)** SK-N-BE(2)-C cells were treated with Eflornithine (DFMO), a well-studied ODC1 inhibitor, combined with 1μM AtRA; after 6 days relative viability was measured by CellTiter Glo. **(E)** In those cells MYCN protein level was also assessed.

Conclusions

- Verlindamycin induces expression of Antizyme 1 and 2
- Verlindamycin combines with ATRA to strongly diminish levels of MYCN
- Verlindamycin/ATRA combination treatment targets mutiple pathways involved in the pathogenesis of NB
- Inhibition of LSD1 may not be an effective treatment for MYCN-amplified NB
- Addition of verlindamycin to mutimodal therapy of high-risk MYCN-amplified NB merits further investigation

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