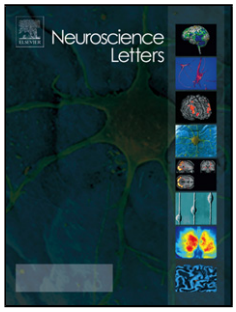
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Title	Romidepsin induces caspase-dependent cell death in human neuroblastoma cells
Authors	Hegarty, Shane V.;Togher, Katie L.;O'Leary, Eimear;Solger, Franziska;Sullivan, Aideen M.;O'Keeffe, Gerard W.
Publication date	2017-05-12
Original Citation	Hegarty, S. V., Togher, K. L., O'Leary, E., Solger, F., Sullivan, A. M. and O'Keeffe, G. W. (2017) 'Romidepsin induces caspase-dependent cell death in human neuroblastoma cells', Neuroscience Letters, 653, pp. 12-18. doi:10.1016/j.neulet.2017.05.025
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.neulet.2017.05.025
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PII: S0304-3940(17)30413-5
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2017.05.025
Reference: NSL 32832

To appear in: *Neuroscience Letters*

Received date: 27-1-2017
Revised date: 2-5-2017
Accepted date: 12-5-2017

Please cite this article as: Shane V.Hegarty, Katie L.Togher, Eimear O'Leary, Franziska Solger, Aideen M.Sullivan, Gerard W.O'Keeffe, Romidepsin induces caspase-dependent cell death in human neuroblastoma cells, Neuroscience Lettershttp://dx.doi.org/10.1016/j.neulet.2017.05.025

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Romidepsin induces caspase-dependent cell death in human neuroblastoma cells.

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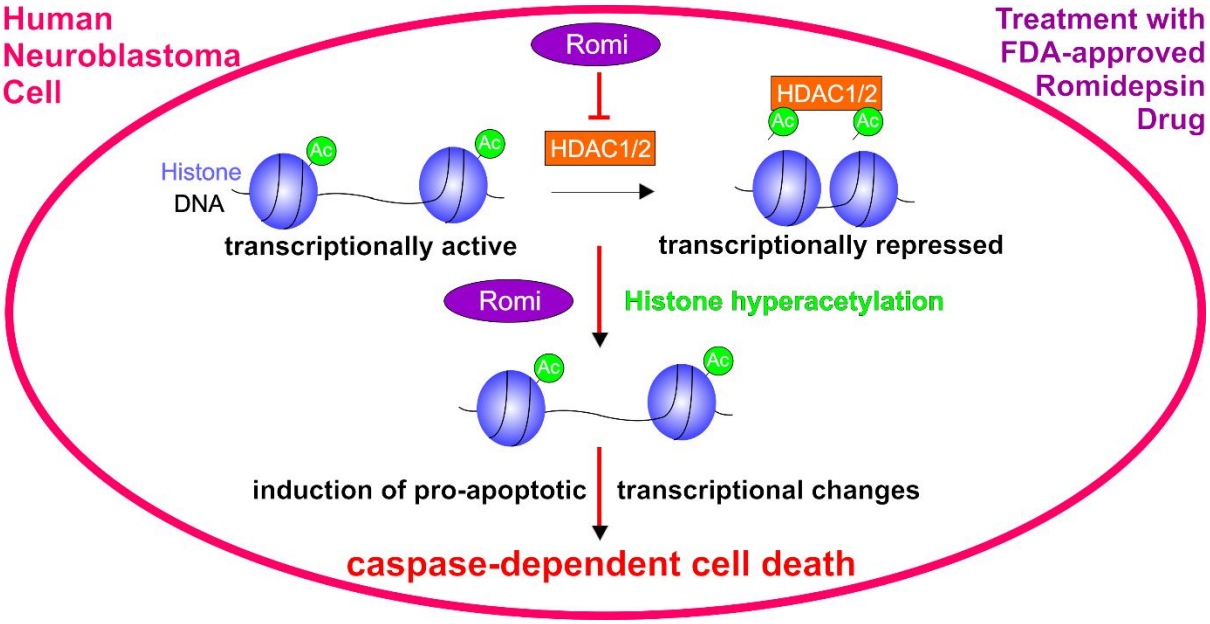
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[1] **Abbreviations:** 6-OHDA - 6-hydroxydopamine; pAcH3 - p-acetylated-histone H3; DIV – day(s) *in vitro;* HDAC - histone deacetylase; LDH - lactate dehydrogenase; MTT - Thiazolyl Blue Tetrazolium Bromide; N - number of repetitions

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Graphical abstract



Highlights

- • Romidepsin is an FDA-approved small molecule drug and a selective HDAC1/2 inhibitor
- • Romidepsin potently induces caspase-dependent cell death in human neuroblastoma cells
- • MYCN-amplified neuroblastoma cells are more sensitive to Romidepsin-induced death
- • The cytotoxic dose of Romidepsin can be lowered by combination with other cytotoxins
- • Romidepsin may be a promising mono-/combination- chemotherapeutic for neuroblastoma

Abstract

Neuroblastoma is the most common extracranial pediatric solid tumor, arising from the embryonic sympathoadrenal lineage of the neural crest, and is responsible for 15% of childhood cancer deaths. Although survival rates are good for some patients, those children diagnosed with high-risk neuroblastoma have survival rates as low as 35%. Thus, neuroblastoma remains a significant clinical challenge and the development of novel

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therapeutic strategies is essential. Given that there is widespread epigenetic dysregulation in neuroblastoma, epigenetic pharmacotherapy holds promise as a therapeutic approach. In recent years, histone deacetylase (HDAC) inhibitors, which cause selective activation of gene expression, have been shown to be potent chemotherapeutics for the treatment of a wide range of cancers. Here we examined the ability of the FDA-approved drug Romidepsin, a selective HDAC1/2 inhibitor, to act as a cytotoxic agent in neuroblastoma cells. Treatment with Romidepsin at concentrations in the low nanomolar range induced neuroblastoma cell death through caspase-dependent apoptosis. Romidepsin significantly increased histone acetylation, and significantly enhanced the cytotoxic effects of the cytotoxic agent 6-hydroxydopamine, which has been shown to induce cell death in neuroblastoma cells through increasing reactive oxygen species. Romidepsin was also more potent in MYCN-amplified neuroblastoma cells, which is an important prognostic marker of poor survival. This study has thus demonstrated that the FDA-approved chemotherapeutic drug Romidepsin has a potent caspase-dependent cytotoxic effect on neuroblastoma cells, whose effects enhance cell death induced by other cytotoxins, and suggests that Romidepsin may be a promising chemotherapeutic candidate for the treatment of neuroblastoma.

Key words:
Romidepsin; Neuroblastoma; Cell Death; Epigenetic Regulation; Chemotherapeutic Drug.

Introduction

Neuroblastoma is the most common extracranial pediatric solid tumor, and arises from the sympathoadrenal lineage of the neural crest. The incidence rate has been reported to be 10.9 cases per million children [1,2]. Neuroblastoma accounts for 8 to 10% of pediatric cancers and is responsible for 15% of childhood deaths from cancer [3]. Although survival rates for patients with loco-regional tumours are greater than 90%, the prognosis for those less than 18

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months of age at diagnosis with metastatic disease remains dismal, and can be as low as 35% [2]. Therefore, neuroblastoma remains a significant clinical challenge, especially for those children with poorer prognoses, and the development of novel therapeutic strategies is essential.

Cancer is now understood to be a disease of widespread epigenetic dysregulation, which contributes to almost every step of tumour progression and interacts extensively with underlying genetic mutations [4-6]. Therefore, epigenetic modulators may offer an effective means to therapeutically alter the regulation of proto-oncogenes and tumor suppressor genes in cancer cells. One key epigenetic process is histone acetylation which regulates the accessibility of genes for transcription factor binding, thereby controlling the levels of gene expression [7,8]. Histone acetylation involves a dynamic interplay between histone acetyltransferase and histone deacetylase (HDAC) enzymes, which are responsible for histone acetylation and deacetylation respectively [9]. In recent years, small molecule HDAC inhibitors have been shown to be potent anti-cancer agents, several of which are now FDA-approved cancer therapies. Indeed, HDAC inhibitors are being developed as drugs for the treatment of a wide range of cancers including: glioblastoma; leukemia; lymphoma; myeloma; and breast, colorectal, gastrointestinal, lung, ovarian, pancreatic, and prostate cancer [4,10-20]. In neuroblastoma, HDAC inhibitors have been shown to induce apoptosis in a number of neuroblastoma cell lines, and thus have promise for treating high-risk neuroblastoma [21-25].

Romidepsin (FK228; FR901228 or Istodax®) is a depsipeptide small molecule (MW=540.7) that belongs to bicyclic peptide selective inhibitors of HDAC1 and HDAC2. It was approved by FDA for the treatment of refractory cutaneous T-cell lymphoma based on evidence from phase II clinical trials [20,26-28]. Romidepsin is being used/developed as a monotherapy or combination therapy for a number of cancers, in particular T-cell lymphoma [10,12,13,18,20,26,27,29,30-32]. Romidepsin is well tolerated in pediatric patients with refractory solid tumors, as well as patients with advanced cancers [33,34], meaning that it may be promising for clinical use in neuroblastoma patients. In the present study, we investigated the ability of Romidepsin to induce cytotoxic effects in the human neuroblastoma cells.

Materials and Methods

Cell Culture

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SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12, supplemented with 10% foetal calf serum (FCS), 100 nM L-Glutamine, 100 U/ml Penicillin/Streptomycin. IMR-32 cells were cultured in Eagle's Minimum Essential Medium with 2 mM L-Glutamine, 1% Non-Essential Amino Acids (NEAA) with 10% FCS. SK-N-BE cells were cultured in Eagle's Minimum Essential Medium with 2mM L-Glutamine, 1% NEAA: Hams F12 (1:1) with 15% FCS (all from Sigma). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. For cell viability assays, cells were plated at a density of 1×10^5 cells/well in a 24-well plate. For western blotting, 2×10^6 cells were plated per well of a 6-well plate. For RNA extraction 1.5×10^6 cells were plated per well in a 12-well plate. Where indicated, cells were treated with 5–5,000 nM of Romidepsin (MedChem Express) and/or 1 µg/ml caspase-3 inhibitor (Calbiochem) or with 15 µM 6-hydroxydopamine (6-OHDA; Sigma) for 24 h or daily for 4 days *in vitro* (DIV).

Cell Viability and Cell Death Assessment

Thiazolyl Blue Tetrazolium Bromide (MTT) assays were performed to assess cell viability [35]. MTT was added to cells at a concentration of 0.5 mg/ml. Lactate dehydrogenase (LDH) activity was measured in 100 µl of the cell culture medium of each treatment group using an LDH Activity Assay Kit (Sigma), according to the manufacturer's instructions. Fresh cell culture medium, which had no contact with cultured cells, was used as the negative control.

Immunocytochemistry

Cultures were immunocytochemically stained for p-acetylated-histone H3 (Ser 11/Lys 15) (pAcH3; 1:200; rabbit polyclonal IgG; Santa Cruz) and β-actin (1:200; mouse monoclonal IgG; Sigma) [36]. Cells were imaged with an Olympus IX70 inverted microscope fitted with an Olympus DP70 camera and AnalysisD^{TM} software. The fluorescence intensity of individual cells stained for pAcH3 was measured using Image J and the relative fluorescence intensity was calculated as the intensity of each individual cell after subtraction of the background noise.

Measurement of Cellular Morphology

Neurite growth analysis as a proxy measure differentiation of SH-SY5Y cells was performed as previously described [36], using the using the formula: neurite length = α x T x (π/2), where α is the number of times the neurite intersects the grid lines, and T is the distance between the gridlines on the magnified image.

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Western Blotting

Western blotting was carried out as described [37]. Cells were lysed in RIPA buffer for 1h on ice, and insoluble debris was removed by centrifugation. 15 µg of protein was run by SDS-PAGE and transferred to nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, CA, USA). The membranes were incubated with primary antibodies against pAcH3 (1:1000; rabbit polyclonal IgG; Santa Cruz) or β-actin (1:1000; mouse monoclonal IgG; Sigma) overnight at 4°C, washed, incubated with the appropriate horseradish peroxidase-labelled secondary antibodies (1:2000; Promega), washed and developed with enhanced chemiluminescence (GE Healthcare). Protein expression was normalised to β-actin by densitometry using Image J.

RNA extraction, cDNA synthesis and real-time PCR

RNA was extracted from the cells using Trizol reagent as per the manufactures instructions (Invitrogen). RNA concentration was quantified using a Spectrophotometer (NanoDrop 1000). Reverse transcription was performed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems) under the following parameters: 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; 4 °C for 10 min. The reaction mix for real-time PCR consisted of 5 µl TaqMan® Gene Expression Master Mix (Applied Biosystems), 4 µl of RNase-free H_2O (Applied Biosystems), 0.5 µl 20× TaqMan® Gene Expression Assays (*ACTB*, *Bax*, *Bcl2*, *caspase-8*, *caspase-3*, *PPAR-γ*; Applied Biosystems) and 0.5 µl of cDNA (10 ng/ml). Each sample was run in duplicate under the following cycling parameters: 50 °C for 2 min; 95 °C for 10 min; 40 repetitions of 95 °C for 15 s and 60 °C for 1 min. Expression levels were calculated using the 2-δCT method (Schmittgen and Livak, 2008).

Statistical Analysis

Unpaired Student's t-test or one-way ANOVA with a *post hoc* Tukey's or Bonferroni's test was performed, as appropriate, to determine significant differences between groups. For non-parametric data, Kruskal-Wallis ANOVA with Dunn's *post hoc* test was used. Outliers were removed prior to analysis if Grubb's Test $p < 0.05$. Results were expressed as means with SEM and deemed significant when $p < 0.05$.

Results

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Romidepsin induces cell death of human SH-SY5Y neuroblastoma cells.

We first examined the effects of Romidepsin using human SH-SY5Y neuroblastoma cells, which were treated with 5 – 500 nM of Romidepsin daily for 4 DIV. These concentrations were selected based on IC_{50} values of Romidepsin being in the nanomolar range (36 and 47 nM for HDAC1 and HDAC2, respectively), and that Romidepsin has been shown to reduce viability at 10 nM in the HL-60 cell line and at 500 nM in the HP100 cell line [38]. An MTT assay revealed that Romidepsin had no effect on cell viability at concentrations $\leq$ 40 nM when compared to control (Fig. 1A), but significantly reduced cell viability at concentrations $\geq$ 100 nM (Fig. 1A). Phase contrast microscopy confirmed that Romidepsin treatment led to a decrease in cell number and an increase in dead, non-adherent cells (Fig. 1B). We further examined the effects of the non-cytotoxic doses of Romidepsin on cellular morphology by immunostaining for β-actin. Treatment with 40 nM of Romidepsin significantly reduced the length of SH-SY5Y cell neurites (Fig. 1C), indicating a neuritotoxic effect. These data demonstrate that Romidepsin exerts potent cytotoxic effects in SH-SY5Y neuroblastoma cells in the low nano-molar range.

Romidepsin increases histone acetylation in human SH-SY5Y neuroblastoma cells.

As Romidepsin is a selective HDAC1 and HDAC2 inhibitor, we next assessed the ability of Romidepsin to increase histone acetylation. Given that 40 nM Romidepsin had a neuritoxic effect, and to eliminate the confounding effects of low cell numbers at the higher doses, cells were treated with 20 nM and 40 nM of Romidepsin and the levels of acetylated histones (pAcH3) were first measured by western blotting. 20 nM and 40 nM Romidepsin significantly increased the levels of pAcH3 relative to control, which was measured by densitometric quantification of pAcH3 protein levels relative to β-actin loading control (Fig. 2A). Immunocytochemistry was used to confirm these findings and Romidepsin treatment resulted in a significant increase in the levels of pAcH3 relative to control, as quantified by densitometry (Fig. 2B). Taken together, these results suggest that Romidepsin leads to increases in histones in acetylation in SH-SY5Y neuroblastoma cells.

The effects of Romidepsin on SH-SY5Y cells are caspase-dependent.

To determine whether Romidepsin induces apoptosis in SH-SY5Y cells, cultures were co-treated with a broad-spectrum caspase-3 inhibitor, which inhibits to the pro-apoptotic caspase-3 that functions upstream of other pro-apoptotic caspases; caspase-6 and caspase-7 [39]. An MTT assay revealed that caspase-3 inhibition significantly inhibited Romidepsin-

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induced cell death in the SH-SY5Y neuroblastoma cells at 4 DIV (Fig. 3A). We next aimed to investigate whether the cytotoxic dose of Romidepsin could be reduced by combination therapy, in which neuroblastoma cells are exposed to another cytotoxic agent. To provide proof of principle for this, SH-SY5Y cells were co-treated with 6-OHDA, which is a potent cytotoxin that has been shown to induce SH-SY5Y cell death through increasing reactive oxygen species [40,41]. SH-SY5Y cells were treated with 15 μM of 6-OHDA [36] with or without 20 nM of Romidepsin (a concentration which does not adversely affect cell viability or morphology, but induces hyperacetylation) for 24 h at which time LDH assays were performed to assess cell death. Interestingly, 20 nM Romidepsin significantly enhanced the cytotoxic effects of 6-OHDA (Fig. 3B). These data suggest that it may be possible for the therapeutic dose of Romidepsin to be lowered when used as an add-on pharmacotherapy in combination therapy.

Romidempsin exerts cytotoxic effects in MYCN amplified cells.

As SH-SY5Y cells express wild-type p53 and are not MYCN amplified, we carried out additional experiments in IMR-32 (MYCN amplified, p53 wild-type) and SK-N-BE(2) (MYCN amplified, p53 mutant) human neuroblastoma cell lines. MYCN amplification is the primary and most important prognostic marker of poor survival in neuroblastoma [54]. Based on the dose-response experiments carried out in SH-SY5Y cells, these cells were treated with 40 nM or 100 nM of Romidempsin for 4 DIV. An MTT assay revealed that both MYCN amplified neuroblastoma cell lines were more sensitive to the cytotoxic effects of Romidempsin than SH-SY5Y cells (Fig. 1A and 4A). Indeed, Romidempsin doses of 40 nM and 100 nM significantly reduced IMR-32 and SK-N-BE cell viability (Fig. 4A). Real-time PCR revealed that 40 nM Romidempsin treatment significantly increased the expression levels of *caspase-3* mRNA (Fig. 4B), and increased the ratio of *Bax* mRNA to *Bcl2* mRNA in all the neuroblastoma cell lines tested (Fig. 4C). These data show that Romidemspin is a potent cytotoxin that leads to pro-apoptotic transcriptional changes in MYCN amplified and non-amplified human neuroblastoma cells.

Discussion

The selective inhibitor of HDAC1 and HDAC2, Romidepsin, is an FDA-approved chemotherapeutic drug that is being used/developed as a monotherapy or combination therapy for a number of cancers [10,12,13,18,20,26-29,30-32]. To investigate the potential of

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Romidepsin for the treatment of neuroblastoma, we examined the cytotoxic effects of Romidepsin in human neuroblastoma cell lines. In the present study, we first demonstrated that Romidepsin induces cell death of SH-SY5Y cells at concentrations in the low nanomolar range through caspase-dependent apoptosis. We next found that Romidepsin significantly increases histone acetylation in these neuroblastoma cells, most likely through inhibition of HDAC1 and HDAC2 activity, and that the cytotoxic dose of Romidepsin can be lowered when combined with other cytotoxic agents. Finally, we demonstrated that Romidepsin is a potent cytotoxin that leads to pro-apoptotic transcriptional changes in MYCN amplified and non-amplified human neuroblastoma cell lines. As a result, we believe that Romidepsin may be useful in monotherapy or combination therapy approaches for neuroblastoma.

In the present study, following a dose-response experiment, we initially found that concentrations >100 nM of Romidepsin induce a significant reduction in cell viability of SH-SY5Y cells. We observed extensive cell death by phase contrast microscopy, and then showed that a non-cytotoxic dose of Romidepsin (40 nM) significantly reduced the length of SH-SY5Y cell neurites, while lower doses had no effect on neurite length. These data indicate that Romidepsin has a neuritotoxic effect on SH-SY5Y cells. Moreover, Romidepsin did not increase the length of SH-SY5Y cell neurites, and thus did not induce their neuronal differentiation. Taken together, we concluded that Romidepsin has potent cytotoxic effects on SH-SY5Y neuroblastoma cells. In support of our findings, HDAC inhibitors have been shown to induce cell death in a number of neuroblastoma cell lines [21-25].

We subsequently confirmed the ability of Romidepsin to induce hyperacetylation of histone H3 in the SH-SY5Y cells, which was most likely due to selective inhibition of HDAC1 and HDAC2. Romidepsin induced significant increases in the levels of acetylated histone H3 at 20 nM and 40 nM. In support of this, the IC_{50} values of Romidepsin are 36 nM and 47 nM for HDAC1 and HDAC2, respectively. Furthermore, Romidepsin has been consistently demonstrated to inhibit HDAC1 and HDAC2 activity *in vitro*, *in vivo* and in cancer patients, in which histone acetylation levels were assessed [28,38,42]. This suggests that the Romidepsin-induced cytotoxic effects observed in this study may be mediated by hyperacetylation, following inhibition of HDAC1 and HDAC2 activity. Indeed, epigenetic dysregulation has been reported to contribute to a cancer phenotype [4-6].

To determine the cellular mechanisms through which Romidepsin induced SH-SY5Y cell death, we next showed that Romidepsin-induced cell death in SH-SY5Y neuroblastoma cells was caspase-dependent. In support of these findings, Romidepsin has previously been reported to induce apoptosis in a number of cancer cell lines [24,38,43]. SH-SY5Y cell death

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induced by treatment with 100 nM of Romidepsin was prevented by co-treatment with a caspase-3 inhibitor. Similarly, Romidepsin has been shown to induce apoptosis at 10 nM in the HL-60 cell line and at 500 nM in the HP100 cell line [38]. Taken together, these results suggest that Romidepsin induces caspase-dependent apoptosis in neuroblastoma cells.

We next sought to determine if the cytotoxic dose of Romidepsin in neuroblastoma cells could be reduced by combination therapy with another cytotoxic agent. Using a concentration of Romidepsin which does not adversely affect cell viability or morphology but induces hyperacetylation (20 nM), we showed that Romidepsin significantly increases the SH-SY5Y cell death induced by the cytotoxin 6-OHDA, which has been shown to induce SH-SY5Y cell death through increasing reactive oxygen species [40,41]. These data suggest that it may be possible for the therapeutic dose of Romidepsin to be lowered when used as an add-on pharmacotherapy in combination therapies for neuroblastoma.

Finally, we examined the effects of Romidepsin in MYCN amplified human neuroblastoma cell lines, IMR-32 and SK-N-BE, as MYCN amplification is the primary and most important prognostic marker of poor survival in neuroblastoma [54]. MYCN amplified neuroblastoma cell lines were significantly more sensitive to the cytotoxic effects of Romidempsin than the non-MYCN amplified SH-SY5Y cells. This suggests that Romidepsin may be particularly effective in neuroblastoma cases with MYCN amplification, which accounts for ~25% of cases and correlates with high-risk disease and poor prognosis [54]. Lastly, Romidempsin was shown to cause significant pro-apoptotic transcriptional changes in both MYCN amplified and non-amplified human neuroblastoma cells.

Collectively these *in vitro* data demonstrate the potential of Romidepsin to be used as chemotherapy for neuroblastoma patients. Indeed, the FDA-approved Romidepsin is being used/developed as a monotherapy or combination therapy for various other cancer subtypes [10,12,13,18,20,26,27,29,30-32]. Furthermore, Romidepsin has been demonstrated to be well tolerated in pediatric patients with refractory solid tumors, as well as patients with advanced cancers [33,34], which gives it promise for clinical use in neuroblastoma patients. Before Romidepsin is considered for clinical use, it should be examined in animal models of neuroblastoma (for examples see [47]) as the next stage in rationalizing its use as a potential therapy for this cancer. Despite the use, effectiveness and promise of Romidepsin as a chemotherapy, Romidepsin has been ineffective in its treatment of some cancers in clinical trials, including glioblastomas and colorectal cancer, with some patients experiencing adverse effects [42,48-52]. Thus, preclinical efficacy with Romidepsin does not necessarily predict effectiveness in clinical trials. In the event of Romidepsin-related clinical complications,

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Romidepsin has been shown to be metabolized by cytochrome P450 enzymes [53], which provides a potential avenue to counteract any adverse effects of Romidepsin. However, clinical trials aimed at harnessing these enzymes to metabolise Romidepsin were unsuccessful [52]. Given the dismal survival rates in children with high-risk neuroblastoma [2], it is important that novel therapeutic approaches are developed for these patients. Romidepsin has demonstrated promise in this regard, and warrants further investigation in this context.

Acknowledgements

Studies in the authors' laboratories are supported by grants from the Irish Research Council (R15897; SVH/AS/G'OK) the National University of Ireland (R16189; SVH/AS/G'OK). This publication has also emanated from research supported in part by a research grant from Science Foundation Ireland (SFI) under the Grant Number 15/CDA/3498 (G'OK).

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Figures and Figure Legends

Figure 1: Romidepsin induces cell death of human SH-SY5Y neuroblastoma cells.

(A) MTT assay with (B) representative photomicrographs of Romidepsin-treated SH-SY5Y cells treated daily for 4 DIV with 0 - 500 nM of Romidepsin (*** $p < 0.001$; ANOVA with post-hoc Tukey's test; N = 4). (C) Graph with (D) representative photomicrographs (Green =

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β-actin) showing the average total neurite length of cells in the control or 40 nM Romidepsin group (** $p < 0.01$; 20 images analysed per group per experiment. N = 3). Scale bar = 50 µm.

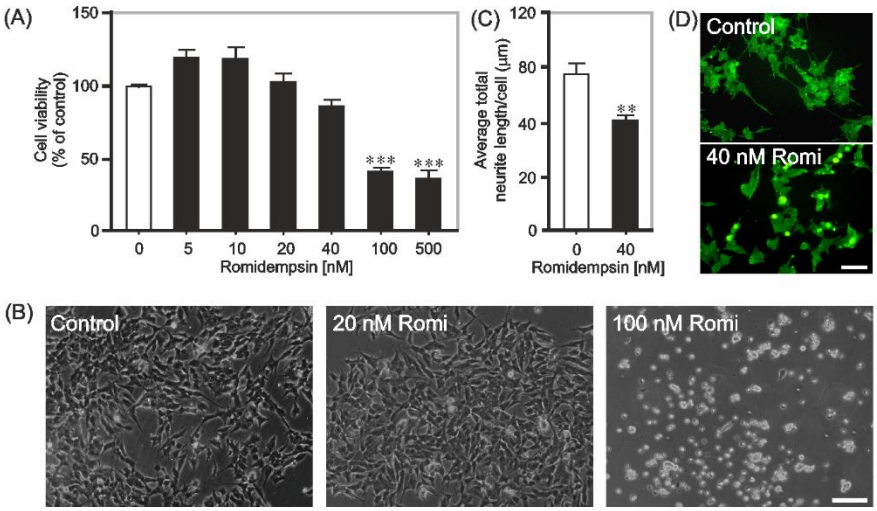


Figure 2: Romidepsin increases histone acetylation in SH-SY5Y neuroblastoma cells. Western blots of (A) pAcH3 and β-actin levels in SH-SY5Y cells cultured for 24 h with 0 to 40 nM of Romidepsin, and (B) normalised to β-actin levels (N = 3). (C) Quantification of the relative fluorescence intensity of (D) pAcH3 staining in SH-SY5Y cultured for 24 h with 0 to 40 nM of Romidepsin (* $p < 0.05$, *** $p < 0.001$ v control; One-way ANOVA with post-hoc Tukey's test; 50 cells analysed per group per experiment. N = 3). Scale bar = 100 µm.

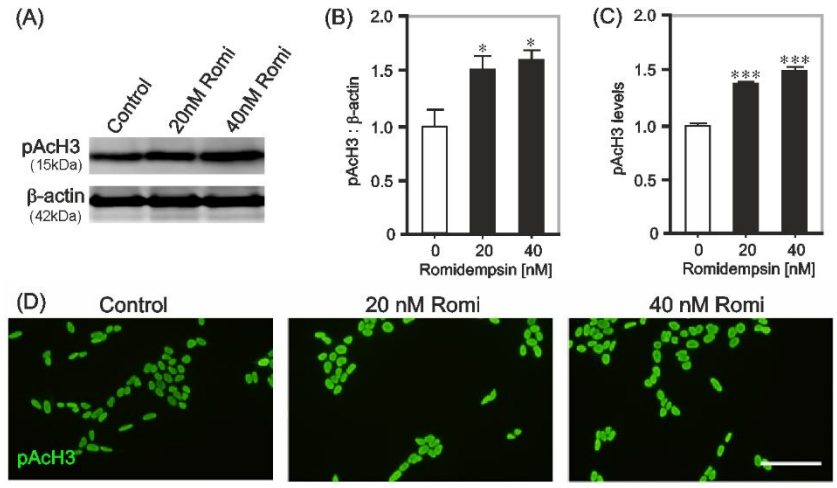


Figure 3: Caspase-dependent cytotoxic effects of Romidepsin in neuroblastoma cells. (A) Representative photomicrographs and (B, C) MTT assay of SH-SY5Y cells treated daily with without or with 1 µg/ml of caspase-3 inhibitor and/or 40 or 100 nM of Romidepsin for 4 DIV (** $p < 0.01$ v control; One-way ANOVA with post-hoc Tukey's test; N = 3). Scale bar = 50 µm. (D) Standardised LDH assay of SH-SY5Y cells treated with 15 µM 6-OHDA in the presence or absence of 20 nM of Romidepsin for 24 h (*** $p < 0.001$ v control; ++ $p < 0.01$ v 6-OHDA alone; One-way ANOVA with post-hoc Tukey's test; N = 6).

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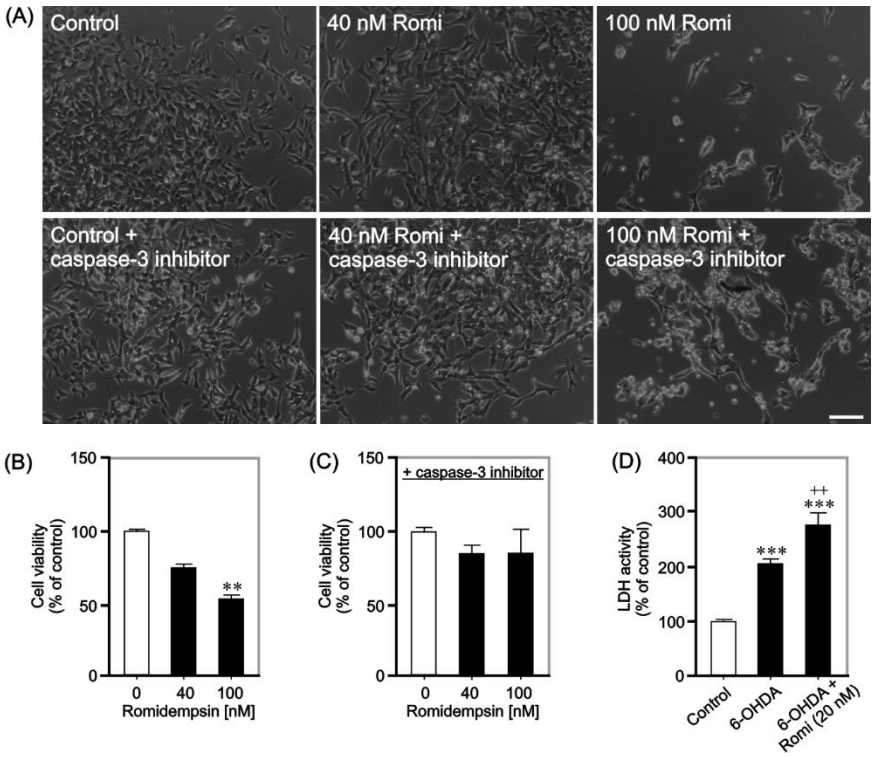
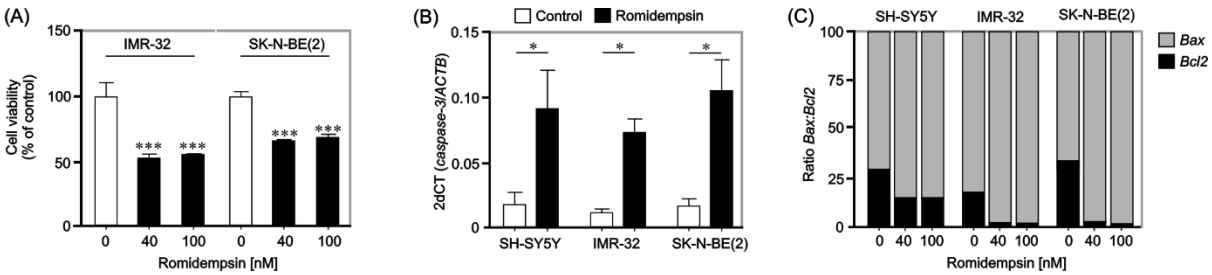


Figure 4: Effects of Romidepsin in MYCN-amplified and non-amplified neuroblastoma cells.

(A) MTT assay of Romidepsin-treated IMR-32 and SK-N-BE(2) cells treated daily for 4 DIV with 0, 40 or 100 nM of Romidepsin (*** $p < 0.001$ vs control; ANOVA with post-hoc Tukey's test. Number of repetitions (N) = 4). (B) Real-time PCR of *caspase-3* mRNA in all three neuroblastoma cells lines treated for 24 h with 40 nM Romidempsin (* $p < 0.05$ vs control; Student's t-test). (C) Graphical representation of real-time PCR showing the ratio of *Bax* to *Bcl2* mRNA in all three cells lines treated for 24 h with 40 nM Romidempsin.



C