Targeting NRAS via miR-1304-5p or farnesyltransferase inhibition confers collateral sensitivity to ALK inhibitors in high-risk neuroblastoma

Perla Pucci
University of Cambridge  https://orcid.org/0000-0003-1264-3487

Liam Lee
University of Cambridge

Miaojun Han
University of Cambridge

Jamie Matthews
University of Cambridge

Leila Jahangiri
University of Cambridge

Eleanor Manners
University of Cambridge

Annabel Sorby-Adams
MRC Mitochondrial Biology Unit, University of Cambridge, The Keith Peters Building, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0XY, UK

Michaela Schlederer

Joshua Kaggie
Department of Radiology, University of Cambridge, Cambridge Biomedical Campus, Cambridge, CB2 0QQ, UK  https://orcid.org/0000-0001-6706-3442

Ricky Trigg
University of Cambridge

Nina Prokoph
University of Cambridge  https://orcid.org/0000-0002-6429-9895

Stephen Ducray
University of Cambridge

Olaf Merkel
Medical University Vienna  https://orcid.org/0000-0001-5089-344X

Firkret Rifatbegovic
St. Anna Children's Cancer Research Institute, CCRI, Zimmermannplatz 10, 1090 Vienna, Austria  https://orcid.org/0000-0002-9956-2382
Article

Keywords:

Posted Date: November 7th, 2022

DOI: https://doi.org/10.21203/rs.rs-2167328/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: Yes there is potential Competing Interest. Author GAAB has received institutional consultancy fees from Roche, Takeda, Novartis and Janssen. All other authors have no conflicts of interest to declare.

Version of Record: A version of this preprint was published at Nature Communications on April 23rd, 2024. See the published version at https://doi.org/10.1038/s41467-024-47771-x.
Abstract

Targeting Anaplastic lymphoma kinase (ALK) is a promising therapeutic strategy for aberrant ALK-expressing malignancies including neuroblastoma (NB), but resistance to ALK inhibitors (ALK TKI) is a distinct possibility necessitating drug combination therapeutic approaches. Using high-throughput, genome-wide CRISPR-Cas9 knockout screens, we identify miR-1304-5p loss as a desensitizer to ALK TKIs in aberrant ALK-expressing NB; inhibition of miR-1304-5p decreased, while mimics increased the sensitivity of NB cells to ALK TKIs. We show that miR-1304-5p targets NRAS, decreasing cell viability via induction of apoptosis. It follows that the farnesyltransferase inhibitor (FTI) lonafarnib in addition to ALK TKIs act synergistically in NB, inducing apoptosis both in vitro and in vivo. In particular, on combined treatment of NB patient derived xenografts with an FTI and an ALK TKI complete regression of tumour growth was observed although tumours rapidly regrew on cessation of therapy. Overall, our data suggests that combined use of ALK TKIs and FTIs, constitutes a therapeutic approach to treat high risk NB although prolonged therapy is likely required to prevent relapse, rendering high-risk NB a chronic rather than a lethal disease.

Introduction

Resistance to targeted agents such as tyrosine kinase inhibitors (TKIs) is a major cause of relapse and cancer related deaths. Indeed, clinical use of inhibitors of the tyrosine kinase, Anaplastic Lymphoma Kinase (ALK) can lead to rapid relapse. The mechanisms underlying this have yet to be fully elucidated. For example, in the case of aberrant ALK-expressing Non-Small Cell Lung Cancer (NSCLC), resistance has been reported in the context of mutations of the target protein that render it insensitive to ALK TKIs and/or amplification leading to an excess of target. However, resistance mechanisms can also be accountable to so-called bypass tracks which have yet to be fully elucidated. It is therefore clear that the use of ALK TKIs as single agents is not a viable approach and combinations of inhibitors targeting multiple pathways might better prevent relapsed/refractory (r/r) disease and lead to a cure.

One of the cancers that expresses aberrant ALK and is driven by this protein is Neuroblastoma (NB), a solid tumour of the sympathetic nervous system, originating from neural crest cells that largely develops in the adrenal medulla and in the sympathetic ganglion chain running from the neck to the pelvis. NB is a complex and heterogeneous malignancy due to its varying molecular and clinical features which make it the most common and deadly extracranial solid malignancy in children, accounting for 7–8% of all childhood cancers and 15% of all paediatric malignancy-related deaths. NB is classified as being of a low-, intermediate-, or high-risk with low and intermediate risk disease effectively responding to surgery or mild chemotherapy, with some rare patients experiencing spontaneous regression in less aggressive cases. Nevertheless, most NB patients are diagnosed with high-risk NB (60%), for which there are a lack of effective treatments, resulting in a poor prognosis with a 5-year survival rate of less than 50%. These patients sometimes present with aberrant molecular profiles often associated with aggressive phenotypes, such as MYCN amplification (20% overall and 50% of high-risk cases), ALK amplification
and/or ALK kinase-domain point mutations (8–10% of cases)\(^9,10,15,16\). For these children, novel therapeutic approaches are urgently needed and ALK TKIs are slowly making their way into the paediatric oncology setting\(^17\). However, r/r disease is a distinct possibility as discussed above, and has been reported for ALK-expressing NSCLC and Anaplastic Large Cell Lymphoma\(^4,18–20\). Hence, additional drug targets for combination therapy of NB and other ALK-aberrant cancers to prevent r/r disease are urgently needed\(^1–3\).

Herein, we describe a genome-wide CRISPR-Cas9 knockout (GecKO) screen\(^21\) to identify additional pathways modulating ALK TKI response in aberrant ALK-expressing NB cells. Interestingly, 9 miRNA genes were identified, including 4 miRNAs that were consistently detected in at least 3 of the 4 screens conducted. Due to the ability of miRNAs to control several signalling pathways\(^22–26\), we focussed on these hits for further investigation. Notably, following *in vitro* validation, *miR-1304-5p* knockdown decreased sensitivity to the ALK TKIs, ceritinib and brigatinib. Moreover, we show that *miR-1304-5p* acts as a tumour suppressor in NB cells via induction of apoptosis, leading to a decrease in cell viability. Furthermore, we show that *miR-1304-5p* targets NRAS, which is of prognostic relevance to NB patients. These data suggest that combined inhibition of ALK and NRAS, via upregulation of *miR-1304-5p*, might constitute a therapeutic approach to treat TKI-naïve disease. Indeed, a combination of *miR-1304-5p* mimics and ALK TKIs increased the response to treatment of NB cells. Knockdown of *NRAS* phenocopied the effect of *miR-1304-5p* mimics in response to ALK TKI. Furthermore, the farnesyl transferase inhibitor (FTI) lonafarnib showed synergistic activity when administered to aberrant ALK-expressing NB cells in combination with ALK TKIs both *in vitro* and *in vivo*. Notably, all NB patient derived xenograft (PDX) mice in the ALK TKI-lonafarnib combination treatment group were alive at the end of the study without any evidence of a growing tumour mass.

Overall, this study shows that *miR-1304-5p* is a novel NB tumour suppressor and regulator of ALK TKI response, targeting oncogenic NRAS and paving the way for the use of miRNA- or FTI- based ALK TKI combination treatments for aberrant ALK-expressing NB.

## Results

**Genome wide CRISPR-Cas9 Knockout (GeCKO) screens identify miRNA targets as potential ALK TKI synergistic therapeutic vulnerabilities in NB**

A GeCKO CRISPR screen was conducted, and quality control performed as detailed in Fig. 1A and Extended Data Fig. 1. The NB cell line SH-SY5Y that expresses the ALK\(^{F1174L}\) mutant (expressing Cas9) was exposed to the ALK TKIs ceritinib or brigatinib, at their respective effective dose (ED\(_{50}\) and ED\(_{75}\) concentrations for 14 days, following lentiviral transduction of a sgRNA library containing 3 sgRNA/gene, targeting 19,050 genes and 1,864 microRNAs (miRNAs)\(^27\). Surviving clones were subject to next-generation sequencing (NGS) to identify which genes, following their ablation, allowed the aberrant ALK-expressing NB cells to survive in the presence of an ALK inhibitor, therefore representing potential
therapeutic targets. Analysis of the sequencing data by k-means clustering showed common but also diverging genes/microRNAs that on KO conferred a survival advantage across different concentrations (300 and 750nM) of ALK TKIs (brigatinib and ceritinib) (Extended Data Fig. 1C). For each treatment condition, an arbitrary threshold (>1.8) of fold difference (FD) in gene expression between DMSO and ALK TKI treatment was set, and the genes with multiple sgRNAs exceeding the FD threshold were considered to be significantly enriched (Fig. 1B and C and Extended Data Fig. 1D, Supplementary File 1). The stringency of these data was further increased, as only those genes which on knockout enabled cell survival following exposure to both brigatinib and ceritinib at either concentration (i.e., 300 nM or 750 nM) were identified as ‘hits’ (Fig. 1B and C and Extended Data Fig. 1D). Within these data, 9 miRNAs met these criteria, of which only one (hsa-mir-136) was common to the collective higher and lower doses of both ALK TKIs, suggesting that different sensitivities may arise at variable TKI concentrations (Fig. 1B). The top 4 miRNAs (hsa-mir-136, hsa-mir-1304, hsa-mir-7975 and hsa-mir-4746) were identified in at least 3 of the 4 screens (Fig. 1C-G). As miRNAs are negative regulators of several signalling pathways, these were further investigated.

Inhibition of miRNA miR-1304-5p decreases sensitivity to ALK TKIs in NB

To validate the GeCKO screen hits individually, inhibitors of hsa-mir-136-5p, hsa-mir-1304-5p, hsa-mir-4746-5p or hsa-mir-7975-5p, along with a scrambled negative control, were individually transfected into the SH-SY5Y cell line. At 48 h following transfection, the cells were treated with increasing concentrations of brigatinib (Fig. 2A and B) or ceritinib (Fig. 2C and D) for 72 h. Of all the miRNAs analysed, only inhibition of miR-1304-5p significantly decreased sensitivity to both of the ALK TKIs, brigatinib (Fig. 2A and B) and ceritinib (Fig. 2C and D).

miR-1304-5p inhibits NB cell viability and induces apoptosis

In order to determine whether miR-1304-5p acts as a novel tumour suppressor for NB, an inhibitor of miR-1304-5p was transfected into a panel of 17 NB cell lines of differing ALK, p53 and MYCN status (Extended Data Table 1) (Fig. 2E). Among the cell lines tested, 16 demonstrated a significant increase in viability when miR-1304-5p was inhibited. In keeping with these observations, mimics of mature miR-1304-5p transfected into the same cell line panel led to a significant reduction in cell viability for all of the NB cell lines (Fig. 2F).

To identify the cellular mechanism(s) underlying the observed decrease in cell viability of NB cells transfected with mature miR-1304-5p mimics, cell cycle and apoptosis were assessed. Transfection of a miR-1304-5p mimic into cells led to a 17000-fold increase in miR-1304-5p expression in SH-SY5Y (ALK F1174L, MYCN non-amplified; Extended Data Fig. 2A) and a 3000-fold increase in KELLY cells (ALK F1174L, MYCN amplified; Extended Data Fig. 2B). Correspondingly, a significant (p < 0.05) increase in caspase 3/7 activity representative of apoptosis was observed 72 h after transfection of the miRNA in both SH-SY5Y and KELLY cell lines (Extended Data Fig. 2C and D, respectively). However, no significant effects on the cell cycle were observed (Extended Data Fig. 2E and F).
miR-1304-5p targets NRAS

To identify the molecular mechanisms driving the cellular phenotype induced by *miR-1304-5p*, genome-wide expression microarray (HT-12 v4) analysis was performed on SH-SY5Y and KELLY cells transfected with a *miR-1304-5p* mimic or a scrambled negative control, detecting 1267 and 1103 altered genes in SH-SY5Y and KELLY cell lines, respectively (Fig. 3A, Supplementary File 2 and 3). Gene set enrichment analysis (GSEA) was then performed with MSigDB’s ‘hallmark gene sets’ (Extended Data Fig. 3A)29. The top-10 enriched gene sets were ranked by the normalized enrichment score (NES) index; for both KELLY and SH-SY5Y cell lines, 4 gene sets were identified that were significantly altered following *miR-1304-5p* transfection with a negative correlation: xenobiotic metabolism, apoptosis, KRAS signalling and IL-6-JAK-STAT3 signalling (Fig. 3B and Extended Data Fig. 3A).

To investigate *miR-1304-5p* target genes, we next compared the genome-wide expression microarray data with miRNA-target *in silico* predictions. The 3′-UTRs of 3,336 coding genes were predicted to bind *miR-1304-5p* by Target Scan (cumulative weighted context ++ score ≤ -0.01; Fig. 3A and Extended Data Fig. 3B). Among these predicted target genes, the mRNA levels of 393 and 391 genes were downregulated in SH-SY5Y and KELLY cell lines respectively according to our microarray results, and 276 in both cell lines (Fig. 3A). KEGG pathway analysis of these 276 genes, showed that 28 were involved in pathways associated with cancer regulation (Fig. 3A, Extended Data Fig. 4A). Expression of these 28 genes was validated via RT-qPCR after transfection with *miR-1304-5p* mimics into SH-SY5Y cells (Extended Data Fig. 3C). Among them, 22 genes were downregulated by more than 20% of their original expression levels due to the *miR-1304-5p* mimic (Extended Data Fig. 3C). Based on the GSEA analysis and evidence gleaned from the literature30–32, 4 genes associated with the RAS/MAPK pathway were singled out as *bona fide* *miR-1304-5p* targets and therefore selected for further investigation: NRAS (neuroblastoma RAS viral oncogene homolog), RRAS (related RAS viral oncogene homolog), PTPN11 (Protein Tyrosine Phosphatase, Non-Receptor Type 11; also referred to as *SHP2*) and IQGAP1 (IQ motif containing GTPase activating protein 1).

The expression levels of NRAS, RRAS, PTPN11 and IQGAP1 were assessed by RT-qPCR and Western blot upon *miR-1304-5p* mimic transfection into SH-SY5Y cells (Extended Data Fig. 3C, Fig. 3C). NRAS, RRAS PTPN11 and IQGAP1 levels were significantly reduced following transfection of the *miR-1304-5p* mimic (Fig. 3C), with phospho- and total-ERK protein levels also lowered (Fig. 3C). In order to confirm *miR-1304-5p* regulation of NRAS, RRAS, PTPN11 and IQGAP1 expression, the 3′-UTRs of these 4 genes were cloned into the psiCHECK2 dual-luciferase plasmid vector and then individually transfected into SH-SY5Y cells. Luciferase activity was significantly decreased for all the target genes upon *miR-1304-5p* mimic transfection, strongly suggesting that NRAS, RRAS, IQGAP1 and PTPN11 are direct targets of *miR-1304-5p* (Fig. 3D). Notably, rescue experiments by transduction of the cDNAs of the 4 genes showed a significant increase in SH-SY5Y cell viability from 20–50% for NRAS and PTPN11, and to 30% for IQGAP1; no significant increase was observed upon RRAS cDNA transfection (Fig. 3E). Furthermore, analyzing previously published expression data derived from NB primary patient tumours33, we show that NRAS transcript levels positively correlate with PTPN11 but not with RRAS or IQGAP1 (Extended Data
Fig. 4B and D). Additionally, NRAS is of prognostic significance in the same cohort of NB patients whereby those with higher levels of NRAS have a worse prognosis ($p < 0.05$; Fig. 3F). No significant prognostic value could be associated with RRAS, IQGAP1 nor PTPN11 expression. Additionally, NRAS is the only gene amongst our target dataset that is significantly upregulated in ceritinib resistant (n = 2) versus sensitive (n = 2) orthotopic NB xenograft models $^{34}$ (Extended Data Fig. 3D). Therefore, we focused on the potential of NRAS as a miR-1304-5p target for further investigation as a therapeutic target.

miR-1304-5p and NRAS are therapeutic targets in combination with ALK TKIs in aberrant ALK-expressing NB cell lines

In order to investigate the therapeutic utility of ALK TKIs and miR-1304-5p in NB, cells were transfected with miR-1304-5p mimics prior to ALK TKI treatment, which significantly decreased the $ED_{50}$ for both brigatinib and ceritinib in MYCN non-amplified SH-SY5Y (Fig. 4A and B) and MYCN amplified KELLY cells (Fig. 4C and D). These data suggest that miRNA-based therapies could be promising strategies to sensitize cells to ALK inhibition. Indeed, transfection of NB cells with miR-1304-5p mimics prior to treatment with ALK TKIs significantly increased apoptosis as determined by caspase 3/7 activity compared to ceritinib alone for SH-SY5Y and KELLY (Fig. 4E and F) as well as for brigatinib alone in SH-SY5Y cell lines (Fig. 4E and F). However, the latter did not reach significance for the KELLY cell line perhaps due to a dosage effect.

As miRNAs are yet to be efficaciously implemented as therapeutics in the clinic, we chose to study the effects of the validated miR-1304-5p target, NRAS whose expression also has prognostic significance in NB and (Fig. 3). NB cells were transfected with NRAS Dicer-Substrate Short Interfering RNAs (DsiRNA) prior to ALK TKI treatment. NRAS knockdown was confirmed via RT-qPCR and WB (Extended Data Fig. 5A, B) and significantly decreased the $ED_{50}$ values for both brigatinib and ceritinib in ALK-mutant NB cells (SH-SY5Y) (Extended Data Fig. 5C, D). As expected, transfection of NB cells with NRAS siRNA prior to treatment with ALK TKIs significantly increased apoptosis as determined by caspase 3/7 activity compared to either ceritinib or brigatinib treatment alone (Extended Data Fig. 5E). Overall, these data suggest that the miR-1304-5p target, NRAS, is a valid therapeutic target.

NRAS is of prognostic significance for NB (Fig. 3F) and has previously been shown to be a potential druggable target$^{35,36}$. However, there are no specific NRAS inhibitors and those drugs that target downstream pathway proteins such as MEK have previously been shown to lack efficacy in NB $^{37}$. Therefore, we treated cells with lonafarnib (Zokinvy), an oral FTI that was recently (November 2020) approved for the treatment of Hutchinson-Gilford Progeria Syndrome (HGPS or Progeria) and processing-deficient Progeroid Laminopathies (PL) $^{36,38}$. Whilst FTIs have multiple targets, we reasoned that their activity upstream in the MAPK and other RAS-regulated pathways would provide broader inhibition of downstream pathways and in doing so, prevent pro-survival feedback signaling from occurring as has previously been reported in NB with the use of MEK inhibitors$^{37}$. Effective concentrations of single agents were determined for use in drug combination experiments by treating cells with increasing doses and
measuring cell viability (Fig. 5A-C). Treatment of SH-SY5Y cells with lonafarnib in combination with the ALK TKIs decreased cell viability compared to administration of ALK TKIs alone, with additive effects at lower concentrations (synergy score: >-10 and < 10) and synergistic effects at higher concentrations (synergy score: >10) for both brigatinib (Fig. 5D, Extended Data Fig. 6A) and ceritinib (Fig. 5E, Extended Data Fig. 6B). All concentrations are clinically achievable as they are below the lower C_{max} per dose used in the clinic 39. Notably, this combination treatment was additive, rather than synergistic at higher concentrations in MYCN amplified KELLY cells, suggesting that MYCN status could affect the cellular response to lonafarnib when given in combination with brigatinib (Fig. 5F, Extended Data Fig. 6C) or ceritinib (Fig. 5G; Extended Data Fig. 6D). Similar to the effects observed on treatment of cell lines with miR-1304-5p mimics in combination with ALK TKIs, the combination of brigatinib or ceritinib with lonafarnib increased apoptosis as determined by caspase 3/7 activity in SH-SY5Y (Fig. 5H) and KELLY cells (Fig. 5I), although this increase did not reach significance for brigatinib and lonafarnib treated KELLY cells.

**ALK TKIs and lonafarnib show synergistic activity with tumour regression in the treatment of NB patient derived xenografts**

Due to the noted effect of ALK TKI combination treatments with both miR-1304-5p mimics or lonafarnib in NB cells, we further investigated this regulatory axis in 3 ALK mutant, high-risk NB PDXs: PDX FELIX – COG-N-426x (ALK_{F1245C}; MYCN WT), COG-N-415 (ALK_{F1174L}; MYCN amplified) and COG-N-557 (ALK_{F1245L}; MYCN amplified)) (Fig. 6A-F, Extended Data Fig. 7). In all cases, this combination resulted in decreased cell viability compared to administration of either agent alone, with highly synergistic effects observed at higher concentrations (synergy score: >10) (Fig. 6A, C, E, Extended Data Fig. 7A-C) accountable to increased apoptosis (Fig. 6B, D, F). Of note, COG-N-415 PDX cells grow more aggressively than the other PDX cells and despite the sub-optimal effect observed on monotherapy (exposure to either lonafarnib or ceritinib alone), significant synergy was observed between ALK TKIs and lonafarnib (Fig. 6E-F).

Subsequently, NSG mice were injected sub-cutaneously with early passage NB PDX cells (FELIX-PDX COG-N-426x) suspended in Matrigel, and when the tumours reached approximately 75 mm\(^3\) the mice were treated daily with either vehicle (20% hydroxypropyl-beta-cyclodextrin), single-agent ceritinib (30 mg/kg), single-agent lonafarnib (40 mg/kg) or both agents in combination (Extended Data Fig. 8). Single-agent treatment with ceritinib or lonafarnib significantly delayed tumour growth compared to vehicle alone (median EFS = 21.5 days, \(p<0.001\), and median EFS = 18.5 days, \(p<0.001\) versus median EFS = 10.5 days respectively; an endpoint event was defined as a tumour reaching 15 mm in any direction; Fig. 7A, B). Combination treatment with ceritinib and lonafarnib led to an exceptional response with all the animals in this treatment group showing no obvious signs of tumour expansion in the following 30 days of continuous treatment (Fig. 7A and B). All compounds were well-tolerated, with no significant decrease in body weight nor lethal toxicity observed (Fig. 7C). MRI analysis was performed after 30 days of daily treatment and showed that one of the mice had no detectable tumour although the others had residual tumours of 1-6 mm diameter (Fig. 7D). However, 2 mice with small palpable tumours from the combination treatment group analysed at day 9 and 10 following cessation of treatment showed tumour
progression (Fig. 7D, Extended Data Fig. 9A). Indeed, by 24 days following cessation of treatment, all of
the mice in the combination treatment group (including the mouse with no tumour observable by MRI)
had relapsed, suggesting that continual and/or further optimisation of the dosing regimen is required to
sustain control of tumour growth (Extended Data Fig. 9B).

Tumours were harvested from the vehicle, lonafarnib and ceritinib-only treated mice at the experimental
endpoint showing typical NB morphology of small, round, monomorphic cells with nuclear
hyperchromasia and scant cytoplasm (Extended Data Fig. 9C). As expected, treatment with lornafarnib or
ceritinib led to a decrease in pERK expression levels although this was more pronounced in the
lornafarnib-treated mice (Fig. 7E and Extended Data Fig. 9C). Given that MAPK inhibitors have previously
been shown to lack efficacy in ALK-aberrant NB due to activation of a negative feedback loop through PI
3-Kinase, we also examined expression of pAkt in the tumour cells on inhibition of NRAS via a specific
siRNA (Fig S10A), and in the PDX tumours shown in Fig. 7E (Fig S10B). No change in pAkt levels were
observed, suggesting that targeting proteins upstream of MAPK (and other RAS-regulated pathways)
prevents this feedback loop from occurring.

Overall, these studies confirm the high efficacy and tolerability of combined ALK TKI and FTI treatment in
xenograft models of high-risk, ALK-aberrant, NB.

Discussion

Resistance to chemotherapy is a major contributor to poor event-free and overall survival outcomes in
cancer\(^1,40\). The introduction of targeted therapies into clinical practice paved the way for personalized
medicine and improvements in the efficacy of cancer treatment but resistance remains a problem and
has been reported in many studies, including those conducted with ALK TKIs\(^2-4,41\). Clinical trials for ALK
TKIs in the treatment of paediatric malignancies including NB are in their relative infancy\(^42-46\).
Nevertheless, \textit{de novo} and acquired resistance can occur for all first, second and third generation ALK
inhibitors, thereby making ALK TKI efficacy challenging\(^2,3,41,47,48\). Hence, additional targets for
combination treatments of NB and ALK-aberrant cancers are urgently needed\(^1-3\).

In this study we performed a CRISPR-Cas9 GeCKO screen of NB cells in order to investigate the genes that
when downregulated render cells less sensitive to ALK TKIs. These genes and the pathways in which they
reside could play a role as tumour suppressors in signalling pathways that sensitize NB to ALK inhibitors,
thereby representing potential therapeutic targets for drug combinations. Via our GeCKO screen, NB cells
for which gene-specific knockout permitted cell survival in the presence of ALK TKIs (i.e., decreased
sensitivity to ALK TKIs) were identified. Most of the genes identified have either previously not been
associated with cancer, or act as known tumour suppressors in a variety of cancers, including NB\(^49-51\). In
the latter regard, \textit{sMEK1} is a gene that encodes a Serine/threonine-protein phosphatase that stimulates
apoptosis in response to paclitaxel treatment in ovarian cancer via inhibition of mTOR signalling\(^52\), and
\textit{NPRL2} promotes sensitivity to irinotecan in colon cancer by activating the DNA damage checkpoint
pathway\(^50\). Notably, one IncRNA was also identified in our “top” hit list, \textit{MRPL39}, which acts as a tumour
suppressor in gastric cancer. Most of the miRNAs identified by the screens have previously been reported to act as tumour suppressors in NB and other cancers, including \textit{miR-1304} and \textit{miR-141}, the latter via stimulation of drug sensitivity in NB. None of the hits identified has been studied in the context of an ALK TKI response before.

We highlighted \textit{miR-1304-5p} inhibition as a potential desensitizer of NB cells to ALK TKIs. Increasing evidence implicates non-coding RNAs (ncRNAs) in response to cancer treatment with miRNAs often downregulated in resistant tumour cells with subsequent upregulation of the oncogenes they regulate. As such, novel therapeutic approaches have been studied including ncRNA-based treatments such as antisense-oligonucleotides (ASOs) and miRNA mimic-based formulations. Hence, the potential of targeting \textit{miR-1304-5p} in ALK-expressing NB was explored further. Indeed, \textit{miR-1304-5p} inhibition enhanced cell viability, in multiple genetically distinct NB cell lines regardless of ALK or other genetic status; \textit{miR-1304-5p} upregulation had the opposite effect, suggesting this as a potential therapeutic vulnerability across multiple genetic sub-types of NB. The decrease in cell viability on \textit{miR-1304-5p} upregulation was attributed to apoptosis rather than cell cycle arrest suggesting that this might represent an efficacious therapeutic approach in keeping with data previously reported by Li et al., which identified \textit{miR-1304} as a tumour suppressor in NSCLC.

Targeted delivery of miRNAs can simultaneously affect a range of proteins, therefore having great potential as therapeutic agents, as shown by pre-clinical studies of pancreatic and liver cancer. Furthermore, miRNA mimics have also been investigated in clinical trials, suggesting that miRNA induction is a promising cancer treatment although not yet clinically actionable. Despite this, clinical miRNA-based therapeutics are still in their infancy and further studies are needed to establish well-tolerated doses and delivery methods to increase the overall treatment benefits and to avoid high toxicity. Additionally, efficient miRNA delivery requires chemical modifications due to their instability and rapid degradation in biological fluids. This might affect the specificity of miRNAs and lead to off-target and saturation effects, thus leading to unexpected gene regulation and toxicities.

As over-expression of miRNAs is yet to be successfully applied in the clinic, we identified targets of \textit{miR-1304-5p} as being potential alternative therapeutic options. Genome-wide expression arrays identified \textit{miR-1304-5p} to significantly impact four gene sets, including those associated with the RAS/MAPK pathway. In particular, a cross-comparison analysis, between \textit{in silico} predictions of \textit{miR-1304-5p} target genes and genes significantly downregulated (> 20%) by a \textit{miR-1304-5p} mimic was conducted to narrow down the list of potential direct targets of \textit{miR-1304-5p}. Among these targets, 4 are effectors of the RAS/MAPK pathway (\textit{NRAS}, \textit{RRAS}, \textit{PTPN11}, \textit{IQGAP1}) and hence were validated as potential direct targets of \textit{miR-1304-5p} in NB. PTPN11 preferentially dephosphorylates RAS proteins increasing their association with RAF ultimately activating the downstream MAPK cascade; \textit{NRAS} and \textit{RRAS} interact with RAF proteins to activate the MAPK pathway with a preference over other kinase pathways such as PI3K; \textit{IQGAP1} is a scaffold protein that enhances the interaction between MEKs (MAPK/ ERK kinase) and ERKs (extracellular signal–regulated kinases) and is indispensable for RAS-driven neoplastic cell survival.
Downregulation of these genes by miR-1304-5p was confirmed by Western blot and dual-luciferase assays, the latter of which identified a direct interaction between the 3'-UTRs of these genes and miR-1304-5p.

NRAS was selected for further study since it was the only miR-1304-5p target identified in our study to negatively associate with NB patient survival (albeit across all NB genotypes rather than specific to ALK-expressing NB) and ALK TKI resistance in in vivo models. Additionally, PTPN11 expression positively correlates with that of NRAS in NB patients. Notably, recent evidence regarding the use of a PTPN11 inhibitor in NRAS mutated NB, showed increased survival and a decreased tumour volume in preclinical models; this evidence suggests that the RAS/MAPK pathway may be an effective therapeutic target for this malignancy. In addition, NRAS activating mutations, amplification or upregulation have been implicated in resistance to targeted therapies such as TKIs used in NB and other cancers, such as hepatocellular carcinoma (HCC), metastatic colorectal cancer and NSCLC.

As MAPK pathway inhibitors, in particular the MEK inhibitor Trametinib has previously been shown to have limited efficacy in ALK-expressing NB due to activation of pro-survival feedback signaling through Akt/mTORc, we decided to investigate an inhibitor of NRAS activity, a protein upstream of both PI 3-Kinase and MEK. In particular, we investigated the therapeutic efficacy of the FTI lonafarnib. Lonafarnib already has an orphan drug designation and has been approved for use in children with Hutchinson-Gilford progeria syndrome and for the treatment of certain processing-deficient progeroid laminopathies in patients one year of age and older. Indeed, we showed that targeting NRAS via either molecular knock-down or pharmacological inhibition with lonafarnib did not affect the pAKT signalling both in vitro and in vivo (Extended Data Fig. 10A and B), suggesting that lonafarnib could overcome this feedback activation and result in a stronger anti-tumour effect.

In particular, our results show that lonafarnib acts synergistically with ALK TKIs in the treatment of NB both in vitro and in vivo particularly when MYCN is non-amplified. Since RAS inhibition could have a downstream effect on MYCN inhibition, it is reasonable to suggest that when MYCN is amplified, this effect could be partially reduced. MYCN-amplified NB could synergistically benefit from MYCN inhibition in addition to combination therapy targeting the RAS/MAPK pathway, as suggested in the literature. Another study has shown an inverse correlation between MAPK signalling and MYCN status in response to targeted therapy (MEK and CDK4/6 inhibitors) in preclinical models of NB, suggesting that therapy targeting the MAPK pathway could have improved efficacy when MYCN is not amplified.

Overall, our study shows synergistic activity of an ALK TKI and lonafarnib. Notably this evidence was observed in 3 different PDX models, of differing ALK and MYCN status. Additionally, none of the animals treated with a combination of ALK TKI and lonafarnib showed any signs of toxicity and all the animals in the combination treatment group were alive at the end of the study without any evidence of a growing tumour mass, showing an exceptional response. This is a significant finding and whilst later
relapse was observed on cessation of therapy, the remission observed on treatment, rendering this a chronic disease rather than a lethal one, is of great clinical significance.

Online Methods

Cell lines and cell culture

The neuroblastoma cell lines CHLA-15, CHLA-20, CHLA-90, CHLA-95, CHLA-171, COG-N-426 (Felix), COG-N-415, COG-N-557, LA-N-5, LA-N-6, NB-1643, NB-EBC1, SK-N-FI, and SMS-LHN were obtained from the Children’s Oncology Group Childhood Cancer Repository. CHP-134, KELLY, LA-N-1 and SH-SY5Y were obtained from the European Collection of Authenticated Cell Cultures. GI-ME-N, NBL-S and NGP were obtained from German Collection of Microorganisms and Cell Cultures and 293FT was obtained from Thermo Fisher Scientific.

CHLA-15, CHLA-20, CHLA-90, CHLA-95, CHLA-171, NB-1643, NB-EBC1, COG-N-426 (Felix), COG-N-415, COG-N-557, and NBL-S cells were cultured in IMDM (Gibco, #21980032) supplemented with 20% FBS, 1% insulin-transferrin-selenium (ITS; Gibco, Cat#41400045) and 1% penicillin and streptomycin (PS). CHP-134, GI-ME-N, KELLY, LA-N-1, LA-N-6, NGP, SK-N-FI and SMS-LHN cells were cultured in RPMI 1640 medium (Gibco, Cat#21875091) supplemented with 10% FBS, 1% ITS and 1% PS. SH-SY5Y and 293FT cells were cultured in DMEM (Gibco, Cat#41966029) supplemented with 10% FBS and 1% PS. Cells were grown at 37°C in a humidified incubator with 5% CO₂. All cells were mycoplasma-free and subjected to quarterly in-house testing.

CRISPR GeCKO screens

The GeCKO screen library was prepared as detailed in the supplementary methods. Cells (10⁶ per well) were plated in a 6-well plate (Corning) in 10% FBS/DMEM. After 24 h, virus libraries (in volumes ranging from 0 to 500 ul) were added to the cells for 24 h and cells were selected in 1 µg/mL of puromycin (ThermoFisher). The effective MOI was calculated after 3 days of selection, as the average cell counts from the duplicate wells following puromycin selection divided by the average cell counts from the duplicates with no selection reagent. The virus volume yielding a MOI nearest to the 0.3–0.4 range was selected leading to the use of 8 x 10⁶ cells per 10 cm plate with a total of 40x 10 cm plates (320 x 10⁶ cells) being transduced. Cells were selected in puromycin, and the effective MOI of the cells transduced for the screens was verified once again. SH-SY5Y cells (320 x 10⁶) were transduced as described above and puromycin (1 µg/mL) was added to the cells 24 h after transduction and maintained for 7 days. On day 7, cells were split into 5 different conditions before drugs were added (4 TKI conditions plus DMSO vehicle control) in duplicates with a minimum of 35 x 10⁶ cells per replicate (plated in 12 x 15 cm plates) with a separate 35 x 10⁶ cells frozen down to be used as the Day 0 sample for genomic DNA analysis. The 4 ALK TKI conditions were: 300 nM AP26113 (brigatinib), 750 nM AP26113 (brigatinib), 300 nM LDK-378 (ceritinib) and 750 nM LDK-378 (ceritinib). Cell pellets with a minimum of 35 x 10⁶ cells were taken
13 days after the addition of ALK TKIs, at which point the screens were terminated and the cell pellets were frozen at -80°C until library preparation as detailed in the supplementary methods.

**Transfection of miRNA mimics, hairpin inhibitors and DsiRNAs**

Cells were seeded in 96-well (10^4) or 6-well plates (2 x 10^5) with a lipid:miRNA mixture prepared using the Lipofectamine RNAiMAX reagent (Invitrogen, Loughborough, UK), according to the manufacturer’s protocol. The final miRNA mimic, inhibitor and DsiRNA concentration used was 40nM. All mimics and hairpin inhibitors were purchased from Dharmacon miRIDIAN™ /Horizon (Extended Data Table 2). DsiRNAs were purchased from Integrated DNA Technologies, Inc. (IDT).

**Cell viability assays**

Cells (10^4) were seeded into 96-well White Polystyrene Microplates (Corning, Cat# CLS3610) and after 24 h, the media was aspirated before one of the following procedures was carried out:

- Transfection with miRNA mimics, inhibitors or DsiRNAs (see corresponding methods section) for 48/72 h.
- Transfection with miRNA mimics, inhibitors or DsiRNAs for 48 h and subsequent addition of 100 µL of fresh media containing drugs in log-scale concentrations (0, 1, 10, 30, 100, 300, 1000 nM) for 72h.
- Addition of 100 µL of fresh media containing drugs in log-scale concentrations (0, 1, 10, 30, 100, 300, 1000 nM) for 72 h.

The DMSO concentration was maintained at 0.02% for all conditions. For analysis of cell viability, 100 µL CellTiter-Glo reagent (Promega, Cat#C7571) was added to the cells, according to the manufacturer’s protocol. Luminescence was read on a SpectraMax i3 microplate reader (Molecular Devices).

**Cell cycle analysis**

Approximately 500,000 SH-SY5Y or KELLY cells were collected by centrifugation following trypsinization, washed with cold PBS, resuspended in 300 µL of cold PBS and fixed by the dropwise addition of 700 µL of 70% ice-cold ethanol (Sigma-Aldrich). Cells were fixed for 30 minutes on ice, then washed twice with ice-cold PBS before resuspending in 100µg/mL RNase (Sigma-Aldrich) for 30 minutes at 37°C. Propidium iodide (PI; 50 µg/mL, Sigma-Aldrich) was added to the cells which were analyzed using a FACS Accuri™ C6 Plus Flow Cytometer (BD Biosciences). Single cells were gated using the FL2-area and –width parameters and a minimum of 10,000 events were collected per sample. Data analysis was conducted with FlowJo software (Treestar).

**Apoptosis assays**

SH-SY5Y or KELLY cells (10^4) were seeded in 96-Well White Polystyrene Microplates (Corning, Cat# CLS3610) and after 24 h, the media was aspirated and cells were transfected with 40nM of a miRNA mimic, inhibitors or DsiRNAs (see corresponding methods section) for 72 h before 100 µL of Caspase
3/7-Glo apoptosis reagent (Promega, Cat#C7571) was added to each well and cells were incubated at room temperature for 1.5 h. Luminescence was read on a SpectraMax i3 microplate reader (Molecular Devices). Data were normalized to the scrambled non-targeting control (NC) and cell viability was assessed using GraphPad Prism 8 software (GraphPad Software Inc).

**RNA and miRNA extraction and RT-qPCR analysis**

Total RNA was isolated from cell lines and processed as described previously except for analysis of NRAS expression when the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific cat# 4368814) was used followed by TaqMan RT-qPCR assay (NRAS, Hs00180035_m1, cat# 4331182, ThermoFisher). For isolation of miRNA, miRNeasy mini (Qiagen, cat# 217004) and PureLink miRNA Isolation kits were according to the manufacturer’s instructions. *miR-1304-5p* expression was analyzed using TaqMan RT-qPCR with the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, cat# 4366596) and the *hsa-miR-1304-5p* - TaqMan® MicroRNA Assay (cat# 4427975, ThermoFisher). Relative quantification (ΔΔCₜ method) analysis was conducted with normalization to GAPDH (Thermo Fisher Scientific, Hs02786624_g1, 4331182), HPRT1 (Thermo Fisher Scientific, Hs02800695_m1, 4331182), U6 snRNA (TaqMan® MicroRNA Assays, cat# 4427975) and RNU24 (Thermo Fisher Scientific, cat# 001001) as indicated.

**HT-12 v4 genome-wide expression microarray**

A *Hsa-miR-1304-5p* mimic (Qiagen) or the AllStars Negative Control siRNA was transfected at 40 nM with Lipofectamine RNAiMAX (ThermoFisher) into SH-SY5Y and KELLY cells. Total RNA was extracted and applied to Illumina Human HT-12v4 expression arrays (Eurofins Genomics, Germany). The microarray readouts were re-formatted to Gene Cluster Text (GCT) file format and a Categorical class (CLS) file was generated. Both files were used as input for Gene Set Enrichment Analysis (GSEA) on quantile normalized mRNA read counts from each cell line with the MSigDB’s ‘hallmark gene sets’ on the javaGSEA Desktop Application to compare *hsa-miR-1304-5p* mimic (Qiagen) versus Negative Control gene expression profiles of SH-SY5Y and KELLY cell lines.

**Immunoblot analysis**

Immunoblot analysis was conducted as previously described. Primary antibodies used were as follows: anti-IQGAP1 (1:200; Santa Cruz Biotechnology (SCBT), cat# sc-376021), PTPN11 (1:200; SH-PTP2 B-1, SCBT, cat# sc-7384), anti-p42/44 MAP Kinase (1:1000; Cell Signalling Technology (CST), cat# 9102), anti-pan-Ras (1:200; SCBT, cat# sc-166691), anti-phospho-p42/44 MAP Kinase (Thr202/Tyr204) (1:1000; CST, cat# 9101S), anti-phospho-AKT (1:1000; CST, cat# 9271) and vinculin (1:200; SCBT, Cat# sc-73614).

**3’-UTR dual-luciferase assays**

The 3’-UTR of the genes of interest were amplified by PCR from SH-SY5Y cDNA, using the ProtoScript II First Strand cDNA Synthesis Kit (NEB). To generate the positive control plasmid, an oligonucleotide that is complementary to the *hsa-miR-1304-5p* sequence (5’- UUUGAGGCUACAGUGAGAUGUG – 3’) was annealed and cloned into the psiCHECK-2 vector (Promega) vector:
psiUTR-pos_F 5’- TCGACACATCTCAGCTTAGCCTCAAAGT – 3’

psiUTR-pos_R 5’- GGCCACTTTGAGGCTACAGTGAGATGTG − 3’.

Similarly, to generate the negative (scrambled; scr) control plasmid, the positive control oligonucleotide sequence was scrambled (http://www.invivogen.com/sirnawizard/scrambled.php) to achieve least base pairing to the miRNA while maintaining the overall base composition:

psiUTR-scr_F 5’- TCGAACAACTATCCGTCCATAATCCGGT − 3’

psiUTR-scr_R 5’ - GGCCACCGGATTATGGACGGATAGTTGT − 3’.

The isolated plasmids were transfected into 293FT cells with TransIT-293 (MirusBio) reagent in 24-well plates at 50% confluence. The next day, cells were transfected with hsa-miR-1304-5p mimics (Qiagen) or AllStar Negative Control siRNA (Qiagen). Following transfection of the miRNA (at 48 h), firefly and renilla luciferase activities were assayed with the Dual-Glo Luciferase Assay System (Promega) using a SpectraMax i3 (Molecular Devices).

Patient derived xenograft studies

NSG mice were obtained from Charles River and housed in groups of 2–6 mice per cage. All procedures were carried out under UK Home Office licence P4DBEFF63 according to the Animals (Scientific Procedures) Act 1986 and were approved by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB). COG-N-426x patient-derived xenograft (PDX) cells were obtained from the Childhood Cancer Repository maintained by the Children's Oncology Group (COG). Cells were suspended in Matrigel (Corning) diluted 1:2 with PBS and 5 × 10^5 cells (300 µL) were injected into the left flank of NSG mice at 8 weeks of age. Tumours were measured daily with manual calipers and tumour volumes estimated using the modified ellipsoid formula: \( V = \frac{ab^2}{2} \), where a and b (a > b) are length and width measurements respectively. Once tumours reached approximately 75 mm^3, mice were randomly allocated into four treatment groups (n = 6 per group) and treated daily with the following agents by oral gavage at 10 µL/g body weight: vehicle (20% hydroxypropyl-beta cyclodextrin), ceritinib (30 mg/kg), lonafarnib (40 mg/kg) or a combination of ceritinib and lonafarnib at the same doses. Mice were euthanized once tumours reached 15 mm in any direction (defined as an event for event-free survival analysis).

Immunohistochemistry (IHC)

IHC was performed as reported previously. Briefly, tumours were fixed in 10% neutral-buffered formalin for 48 h before paraffin-embedding. Tissue sections were stained with hematoxylin and eosin or with antibodies against ALK (CST, Cat#3633), pERK (CST, Cat#9101s) or pAKT (CST, Cat#3787). The heat antigen retrieval was conducted with a citrate buffer pH6. The signal was developed under visual control with 3-Amino-9-ethylcarbazole.

Patient sample analysis
CBioPortal (www.cbioportal.org) was queried using a publicly available TARGET study (TARGET, 2018, phs000218 (https://ocg.cancer.gov/programs/target) available at https://portal.gdc.cancer.gov/projects.” Study ID phs000467). This dataset consists of 1,089 samples from 1,076 patients. Among them, 143 samples with bulk RNA seq RPKM data, 249 with Agilent microarray and 59 with copy number alterations are available. We analyzed the association of miR-1304-5p and NRAS expression with clinical features such as Risk group, INSS stage, MYCN status, tumour histology and overall survival.

**Magnetic resonance imaging (MRI)**

Magnetic resonance imaging (MRI) data was acquired using a 3T BioSpec Bruker system (Ettlingen, Germany) with a 40 mm quadrature volume coil. Animals were anaesthetised with isoflurane (induction 3%, maintenance 2%) in 100% oxygen, adjusted thereafter to normalise respiration rate, which was maintained at 40–60 breaths per minute using a pneumatic pillow (ERT Control Gating Module, SA Instruments, New York, United States). Animals were positioned in an MRI compatible cradle fitted with a heated air supply (Thermo Fisher Scientific, Massachusetts, United States), allowing the temperature to be maintained at 35–36°C using a rectal probe. Anatomical 3D FISP sagittal images were acquired with a scan time of 4 m 28 s, with the following parameters: field-of-view (FOV) = 30 × 30 × 30 mm³, matrix = 128 × 128 × 64, TR = 6.5 ms, TE = 3 ms, bandwidth = 37 kHz, averages = 3, with a flip angle of 15 degrees and eight segments. For tumoural contrast, sagittal 3D T2-weighted images were acquired with turboRARE and the following parameters: TE = 84 ms, TR = 1200 ms, averages = 1, scan time = 7 m 40 s, echo spacing 12 ms, RARE factor = 16, FOV = 30 × 30 × 30 mm³, matrix size = 100 × 100 × 64, bandwidth = 20.8 kHz, with fat suppression. Following image acquisition animals were placed in a recovery box on a heated pad set to 37°C. Images were viewed in ParaVision 360 V2.0 with the T2-weighted image (green) overlaid onto the anatomical 3D FISP image (grey), which then allowed for 3D visualisation and high contrast.

**Statistical analysis**

All Student’s t-tests, One- and Two-way ANOVA models, correlation analyses and Kaplan-Meier survival analyses were conducted with GraphPad Prism 8/9 software.

**References**


84. Synergy Finder.
Figure 1

A GeCKO screen conducted in SH-SY5Y NB cells identifies genes that following CRISPR cas9-induced excision, alter sensitivity to ALK TKIs.
(A) Experimental schema of the CRISPR GeCKO screen conducted on SH-SY5Y cells stably expressing Cas9 and transduced with the GeCKO Version 2 sgRNA library before incubation with the indicated ALK inhibitors, followed by high throughput sequencing. (B) Venn diagram of miRNA genes identified by the CRISPR GeCKO screens, whose loss of expression desensitizes SH-SY5Y cells to brigatinib and ceritinib (at 300nM and/or 750nM concentrations of each drug). (C) Heatmap of miRNA hits identified in both brigatinib and ceritinib screens (at 300nM and/or 750nM of each drug) showing the top 4 miRNAs that were identified in ≥3 screens (in the red square). Colored squares represent the mean values of fold changes (ALK TKI versus DMSO treated) of the selected sgRNAs, from lower (blue) to higher (yellow) fold changes. (D-G) gRNAs ranked by fold-change vs difference in log$_2$ normalized read counts between DMSO and ALK TKI-treated SH-SY5Y cells. Only miRNA sgRNAs common to brigatinib and ceritinib treatments in at least 3 GeCKO screens are shown (has-miR-1304, has-miR-136, hsa-miR-7975, has-miR-4746).
Inhibition of miR-1304 decreases the sensitivity of a range of NB cell lines to brigatinib and ceritinib

(A-D) SH-SY5Y cell viability and ALK TKI ED$_{50}$s, 5 days post transfection of the indicated miRNA inhibitors followed by 72 h exposure to either brigatinib (A, B) or ceritinib (C, D). All results are normalized to the viability of the untreated (DMSO) control for each miRNA inhibitor treatment conducted. ED$_{50}$s
were calculated using a non-linear regression curve. Results are shown as mean ± SEM and are representative of two independent experiments each conducted with technical triplicates. (E-F) Cell viability 72 h following transfection with (E) the \textit{miR-1304-5p} inhibitor or (F) a \textit{miR-1305-5p} mimic in a panel of 17 NB cell lines. Statistical comparison has been conducted using a 2-way ANOVA with Dunnett`s post-test in B and D, and Student`s t-test in E-F. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \).
Figure 3

miR-1304-5p targets NRAS

(A) Experimental schema to identify target genes of miR-1304-5p with selection of the top-target genes (NRAS, PTPN11, IQGAP1, RRAS). (B) GSEA of ‘Hallmark_KRAS_signaling_up’. GSEA was performed to rank the genes based on their differential expression levels between cells transduced with a non-targeting control (NT) compared to a miR-1304-5p mimic (miR1304). A deviation from an enrichment score (ES) of 0 in the profile (green line) reflects the degree of overrepresentation of genes at the top (positive ES score) or bottom (negative ES score) of the ranked gene list. Expression profiles derived from biological triplicate samples were analyzed by GSEA. (C) Western blot of the indicated proteins 72 h post-transfection of SH-SY5Y with a miR-1304-5p mimic. (D) Dual luciferase assay signal driven from the 3’UTR of the 4 target genes transfected into SH-SY5Y, upon miR-1304-5p mimic transfection (ratio of target gene-firefly luciferase (Fluc) to the Renilla luciferase (Rluc) control vector and normalized to the negative control (Scr control)). (E) Cell viability (CTG) of SH-SY5Y transduced to express the 4 target genes. Data represent the means ± SEM of three independent experiments. Statistical comparison: One-way ANOVA with Tukey’s post-test (D, F, G), **p<0.01, ****p<0.0001. (F) Overall survival of 143 NB patients with high or low expression of NRAS (z-score=1), measured by bulk RNA Sequencing (Log-rank test, *p<0.05).
Figure 4

The miR-1304-5p mimic is a therapeutic target when used in combination with ALK TKIs

(A-D) SH-SY5Y (A, B) and KELLY (C, D) cell viability (measured via CTG) and ED$_{50}$ upon a combination of miR-1304-5p mimic transfection and brigatinib (SH-SY5Y (A) and KELLY (C)) or ceritinib (SH-SY5Y (B) and KELLY (D)) treatment for 72 h at the indicated doses. ED$_{50}$ values shown in the graphs on the right.
were calculated from the non-linear fit curves on the left. Data shown are representative of three independent experiments. Statistical comparisons were conducted with a two-way ANOVA with Sidak post-test and Students t-test on means ± SEM. *p<0.05, ***p<0.001. (E, F) Apoptosis determined by caspase 3/7 activity in SH-SY5Y (E) and KELLY (F) cell lines treated with a combination of a miR-1304-5p mimic and brigatinib (1-1000nM) or ceritinib (1-1000nM). Results shown are representative of three independent experiments. Significance was determined using a one-way ANOVA with Tukey’s post-test of the means ± SEM. *p<0.05, ****p<0.0001.
ALK inhibitors and FTI act synergistically in ALK-aberrant MYCN non-amplified NB cell lines

(A-C) SH-SY5Y, KELLY and FELIX cell viability (CTG) upon treatment with brigatinib, ceritinib or lonafarnib for 72 h. (D-G) Dose-response matrices of lonafarnib plus brigatinib or ceritinib in SH-SY5Y (D, E) and KELLY (F, G), treated for 72 h with the combination of agents or agents alone. The synergy scores and viability levels shown result from the average of two independent experiments. Loewe synergy scores were calculated with Synergy Finder and are shown in each square for each dose pair. Color gradients represent cell viability as a % compared to the DMSO vehicle control treated cells (from green 100% viability to red 0%). Synergy scores >10 represent synergism in activity of the inhibitors. (H, I) Apoptosis determined by caspase 3/7 activity per cell population, normalized for the untreated (DMSO) control, in SH-SY5Y (H) and KELLY (I) cells treated with a combination of lonafarnib (1µM) and brigatinib (1µM) or ceritinib (1µM), or each agent alone for 48h. Results shown are representative of three independent experiments. Significance was determined using a one-way ANOVA with Tukey’s post-test on means ± SEM. *p<0.05, ***p<0.001, ****p<0.0001.
Figure 6

A combination of an ALK inhibitor and an FTI act synergistically in PDX cell models via induction of apoptosis

(A) Dose-response matrix of lonafarnib (1-3000nM) and ceritinib (1-1000nM) alone or in combination, following 72 h incubation with COG-N-426 (FELIX) PDX cells. Loewe synergy scores (Synergy Finder) and
cell viability (CTG) result from two biological replicates. Color gradients: % cell viability normalised to DMSO (from green: 100%, to red: 0%). Scores >10 represent synergism. **(B)** Apoptosis (caspase 3/7 activity per cell population normalized to DMSO) of COG-N-426 cells treated with a combination of lonafarnib (1µM) and ceritinib (1µM), or single agents (same doses), for 48 h. Results shown are representative of three biological replicates. **(C, E)** Dose-response matrix of lonafarnib (1-1000nM) and ceritinib (1-1000nM) alone or in combination, following 72 h incubation in COG-N-557 **(C)** and COG-N-415 **(E)** PDX cells. Loewe synergy scores (Synergy Finder) and cell viability (CTG) result from two biological replicates. Color gradients: % cell viability normalised to DMSO (from green: 100%, to red: 0%). Scores >10 represent synergism. **(D, F)** Apoptosis (caspase 3/7 activity per cell population normalized to DMSO) of COG-N-557 **(D)** and COG-N-415 **(F)** cells treated with a combination of lonafarnib (1µM) and ceritinib (1µM), or single agents (same doses), for 72 h. Results shown are representative of three biological replicates. One-way ANOVA with Tukey’s post-test has been used for statistical analysis. ****p<0.0001.
Figure 7

A combination of an ALK inhibitor (ceritinib) with a FTI (lonafarnib) significantly reduces PDX tumour growth in vivo

(A) Tumour volume over time of NSG mice injected sub-cutaneously with COG-N-426x primary NB cells which reached 75\(mm^3\) before daily administration of either vehicle (20% hydroxypropyl beta cyclodextrin), ceritinib (30 mg/kg), lonafarnib (40 mg/kg), or ceritinib and lonafarnib (combo, same doses). Study endpoint is tumours reaching 15 mm diameter or following 30 days of treatment, whichever came first. (B) Kaplan–Meier event-free survival analysis. Data points (n=6) represent means ± SEM, shown until the experimental endpoint (as defined above) of the first animal within each treatment group, ***\(p<0.001\) (Log-rank test). (C) Mouse body weight at the experimental endpoint relative to
baseline weights for each treatment group. Data points (n=6) represent means ± SEM. One-way ANOVA with Tukey’s post-test determined significance at each experimental endpoint in A and C. ****p<0.0001. (D) MRI scan of coronal and sagittal sections of animals treated with a combination of lonafarnib and ceritinib at the end of treatment (left panel) compared to relapses after 9 (central panel) and 10 (right panel) days following the end of treatment. Red arrows = tumour mass; green bar = 1cm. (E) Immunohistochemistry (pERK) in tumours from mice treated with vehicle (20% hydroxypropyl-beta-cyclodextrin), ceritinib (30 mg/kg) or lonafarnib (40 mg/kg) at the study endpoint. Magnification bar= 100µm. Inserts = 200x magnification.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ExtendedData.docx
- SupplementaryinformationSupplementaryfile1normalizedreadcountsmageckcrisprko.xlsx
- SupplementaryinformationSupplementaryFile2GeneAnalysisSHSY5Y.xlsx
- SupplementaryinformationSupplementaryFile3GeneAnalysisKELLY.xlsx