Kinome expression profiling of human neuroblastoma tumors identifies potential drug targets for ultra high-risk patients

Roberta Russo1,2,†, Flora Cimmino1,2,†, Lucia Pezone2,3, Francesco Manna1,2, Marianna Avitabile1,2, Concetta Langella1,2, Jan Koster4, Fiorina Casale5, Maddalena Raia2, Giampietro Viola6, Matthias Fischer7,8, Achille Iolascon1,2 and Mario Capasso1,2,9,*

1Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Napoli, Italy, 2CEINGE Biotecnologie Avanzate, Napoli, Italy, 3Department of Medicine, University of Verona, 4Department of Oncogenomics, Academic Medical Center, University of Amsterdam, Meibergdreef, Amsterdam, The Netherlands, 5Servizio di Oncologia Pediatrica, Dipartimento della Donna, del Bambino e di Chirurgia Generale e Specialistica—Seconda Università degli Studi di Napoli, Italy, 6Dipartimento di Salute della Donna e del Bambino, Università degli Studi di Padova, Italy, 7Department of Pediatric Oncology and Hematology, University of Cologne Children’s Hospital, Cologne, Germany, 8Center for Molecular Medicine Cologne (CMMC), Cologne, Germany and 9IRCCS SDN, Istituto di Ricerca Diagnostica e Nucleare, Napoli, Italy

*To whom correspondence should be addressed. Tel: +39 081 3737 889; Fax: +39 081 3737 804; Email: mario.capasso@unina.it
†These authors equally contribute to the manuscript.

Abstract

Neuroblastoma (NBL) accounts for >7% of malignancies in patients younger than 15 years. Low- and intermediate-risk patients exhibit excellent or good prognosis after treatment, whereas for high-risk (HR) patients, the estimated 5-year survival rates is still <40%. The ability to stratify HR patients that will not respond to standard treatment strategies is critical for informed treatment decisions. In this study, we have generated a specific kinome gene signature, named Kinome-27, which is able to identify a subset of HR-NBL tumors, named ultra-HR NBL, with highly aggressive clinical behavior that not adequately respond to standard treatments. We have demonstrated that NBL cell lines expressing the same kinome signature of ultra-HR tumors (ultra-HR-like cell lines) may be selectively targeted by the use of two drugs [suberoylanilide hydroxamic acid (SAHA) and Radicicol], and that the synergic combination of these drugs is able to block the ultra-HR-like cells in G2/M phase of cell cycle. The use of our signature in clinical practice will allow identifying patients with negative outcome, which would benefit from new and more personalized treatments. Preclinical in vivo studies are needed to consolidate the SAHA and Radicicol treatment in ultra-HR NBL patients.

Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy. It accounts for >7% of malignancies in patients younger than 15 years and around 15% of all pediatric oncology deaths (1). NBL exhibits a broad range of clinical behaviors. Particularly, NBL patients are assigned to three different risk categories according to clinicopathological variables such as age at diagnosis, MYCN oncogene amplification, tumor histology and DNA ploidy. Thus, tumor-specific genetic markers and histopathological assessment are crucial determinants.
INSS  International Neuroblastoma Staging System
HR  high-risk
HDAC  histone deacetylase
ALK  anaplastic lymphoma kinase
MYCN  R-spondin
ΔCt  ∆Ct
CI  combination index
G1\text{\textsubscript{0}}  50% growth inhibition
Hsp90  heat shock protein 90
INSS  International Neuroblastoma Staging System
HR-NBL  high-risk neuroblastoma
NBL  neuroblastoma
SAHA  suberylanilide hydroxamic acid

of treatment planning, especially for children younger than 18 months. During the past few decades, tailored treatment approaches based on the presence or absence of specific clinical and biological factors have been used, and risk-based clinical trials have led to substantial improvement in outcome for patients classified as low or intermediate risk. Indeed, low- and intermediate-risk patients generally have good prognosis after treatment with surgery and standard chemotherapy. Current standard-of-care treatment strategy for high-risk (HR) NBL includes consolidation therapy with autologous hematopoietic stem cell transplantation and irradiation, and post-consolidation therapy with pro-differentiation agents and immunotherapy (2). New therapies that include anaplastic lymphoma kinase (ALK), MYCN and angiogenesis inhibitors are in development (3,4). Despite substantial improvement in the treatment of these well-defined subsets of patients, their outcome has improved only modestly, with estimated 5-year survival rates <40% (1,2,5).

The ability to stratify HR patients that will not respond to standard treatment strategies is critical for informed treatment decisions. Indeed a subclassification of HR patients by gene signatures could be a useful tool to develop novel therapeutic strategy and to improve patient survival. Of note, the prognostic value of numerous low-risk and HR NBL signatures have been discovered (6–12). Particularly, some of these signatures have been proven to be able to identify a subset of HR patients with significantly worse survival, named ultra-HR patients (13).

Oncogenic kinases, which constitute to ~1.7% of human genes, are often activated or over-expressed in tumor cells. Indeed, misregulation of kinases’ expression has been described by means of kinome profiling analysis in several human tumors, as thyroid tumor (14) and clear cell renal cell carcinoma (15). Moreover, therapies targeting oncogenic kinases have provided promising results in inhibiting proliferation and invasion of cancer cells, and some kinases have been targeted in preclinical and clinical studies, e.g. in childhood sarcomas (16). An unbiased approach to identify alteration in kinases expression in cancer is to perform kinome profiling screen. In this study, we have built a powerful kinome signature able to subclassify the subset of ultra-HR NBL tumors among those HR. Moreover, we have demonstrated that NBL cell lines expressing the same kinome signature of ultra-HR tumors respond to the combined treatment with suberylanilide hydroxamic acid (SAHA) and Radicicol. Therefore, the specific kinase expression of ultra-HR patients could represent the starting point to select potential target in the treatment of these patients that will not adequately respond to standard therapy of HR ones. Preclinical in vivo studies will be needed to identify the most powerful compounds able to modulate ultra-HR kinome signature.

Materials and methods

Selection of the data sets

We identified HR-NBLs based on Children’s Oncology Group (COG) risk assignment. Among these, we distinguished two subsets: (i) ultra-HR patients, HR-NBLs who died of disease within 18 months ( ultra-HR) and (ii) non-ultra-HR, HR alive without an event for at least 2 years (non-ultra-HR) as control group. Although there is not yet a unique definition of this term (ultra-HR NBL) in the scientific literature, we defined ultra-HR NBL as death of disease within 18 months after diagnosis, referring to an operational definition of the ultra-HR group of the International Neuroblastoma Response (INRC) consortium (P.Depuydt, personal communication at ANR 2016).

DNA microarray-based gene expression and clinical data of 740 NBLs from two independent freely available data sets (accession no. GSE16476 and GSE45547) were collected (Supplementary Figure 1). Overall, 230 HR-NBLs were included in these two data sets. We selected 19 and 49 ultra-HR and 5 and 63 non-ultra-HR from GSE16476 and GSE45547 data sets, respectively.

Kinome analysis

Human genes coding for protein kinases (n = 539) were selected from KinBase (http://kinase.com/kinbase/). We performed a meta-analysis on 68 ultra-HR and 68 non-ultra-HR from the two aforementioned gene expression data sets using the web tool NetworkAnalyst—network-based visual analytics for gene expression profiling, meta-analysis and interpretation (http://www.networkanalyst.ca/), a user-friendly web-based tool designed to support meta-analysis of multiple gene expression data sets (Supplementary Figure 1). NetworkAnalyst uses a horizontal data integration, which involves the combination and multi-faceted analysis of different data sets measuring the same molecular events under similar experimental conditions; e.g. combining the same type of cancer gene expression data sets from different studies (17).

Kinome-27 signature

K-mean clustering was performed using default parameters by methods implemented in program R (http://r2.amc.nl). The probability of overall survival was calculated using Kaplan–Meier method, and the significance of the difference between Kaplan-Meier curves was calculated by the log-rank test. Multivariate Cox proportional regression analysis was performed to evaluate the prognostic significance of Kinome-27 signature and the currently used risk factors such as age at diagnosis (>18 months versus <18 months), International Neuroblastoma Staging System (INSS) stage (stages 4 and 3 versus stages 1, 2 and 4) and MYCN status (amplified versus not amplified). Hazard ratios and 95% confidence interval for survival were calculated. The analyses were performed on the following gene expression datasets: GSE16476, GSE62564, Asgharzadeh (validation set 1) and GSE3446 (validation set 2) deposited in the R2 database.

Gene Ontology

Differentially expressed genes were categorized in Gene Ontology groups using the DAVID Functional Annotation Bioinformatics Microarray Analysis tool (http://david.abcc.ncifcrf.gov/) and WEB-based Gene Set Analysis Toolkit (WebGestalt, http://bioinfo.vanderbilt.edu/webgestalt/).

Quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent (Life Technologies, Carlsbad, CA). Synthesis of cDNA from total RNA (2 μg) was performed using cDNA synthesis kit (Applied Biosystems, Milan, Italy). Quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) was performed to evaluate the expression of marker genes: EPHB2, CAMKV, BAZIA, MAP2K2 as up tags and MAP2K1, PKRACB as down tags. cDNA samples were amplified on Applied Biosystems 7900HT Sequence Detection System using standard cycling conditions. The primers were designed by the Primer Express 2.1 program (Applied Biosystems). β-actin was used as internal control. Relative gene expression was calculated by using the 2\textsuperscript{ΔCt} method, whereas the mean fold change + 2\textsuperscript{ΔΔCt} was assessed using the mean difference in the ΔCt between the gene and
Cell viability assay

SK-NBE2C, SK-NAS and SKNFI cells were treated for 48 h with SAHA (Sigma-Aldrich, St. Louis, MO; 0.5–10 μM) and Radicicol (Sigma-Aldrich; 0.5–10 μM). The cells were seeded as six replicates into 96-well plates at a density of 10^4 cells per well. Cell viability was determined after 24 and 48 h of treatment, by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, according to the manufacturer protocol (Promega, Milan, Italy). The experiments have been repeated twice.

Cell cycle analysis

Cells were seeded in cell culture 10 × 20-mm dishes (Corning, Milan, Italy) at a density of 1 × 10^5 cells. After 8 h of serum starvation, the cells were treated with SAHA and/or Radicicol for 48 h. For the cell cycle analysis, 1 × 10^5 cells were washed in PBS and re-suspended in 200 μl propidium iodide (50 μg/ml in PBS; Sigma-Aldrich, St. Louis, MO), plus 50 μl RNase A solution (100 μg/ml in water; Sigma) and 0.04% NP40 in PBS. The cells were incubated at 37°C for 3 h in the dark. The cell cycle distribution was then analyzed using flow cytometry, by fluorescence-activated cell sorting analysis (BD FACS, Canto II, BD Biosciences, Paris, France).

Combined drugs analysis

To test the potential synergistic, additive or antagonistic effects of the combination of HDAC and Hsp90 inhibition, we performed the combination experiments. The cells were seeded as six replicates into 96-well plates at a density of 10^4 cells per well and cell viability was determined after 48 h of treatment, by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega, Milan, Italy). Synergy, additivity and antagonism were defined by a combination index (CI), CI < 1, CI = 1 or CI > 1. CIs were analyzed by the use of CalcuSyn software (version 2.0, Biosoft, Cambridge, UK), that employs the Chou-Talalay method based on the median-effect equation, to simulate the combination effect. These simulations were accurate and statistical significant since the r values for both single agent and their combination are >0.90 (20,21). The experiments have been repeated twice.

Results

Kinome profiling identifies a signature of 27 differentially expressed metagenes between ultra-HR and non-ultra-HR NBLs.

By means of the meta-analysis of kinome expression profiling on 68 ultra-HR NBLs (defined as HR-NBLs who died of disease within 18 months) and an equal number of non-ultra-HR NBLs from two independent freely available data sets (accession no. GSE16476 and GSE45547), we obtained a set of 27 differentially expressed metagenes between ultra-HR and non-ultra-HR termed Kinome-27 signature (Figure 1A). Approximately 14% of these metagenes belong to the mitogen-activated protein kinase signaling pathway (Figure 1B).

Prognostic value of Kinome-27 signature

We speculated that the Kinome-27 gene set forms a signature reflecting the activity of the kinase pathways in NBL. As a first analysis of this signature, we investigated the prognostic value of the Kinome–27 gene set (Figure 2 Table I). We used K-means clustering to subdivide the freely available gene expression data of 88 primary NBL tumors of all stages (accession no. GSE16476) in two groups with high or low activity of the Kinome-27 signature (Supplementary Figure 2). One cluster of 38 tumors included the most of HR samples (Supplementary Table 1) and showed high expression of upregulated genes and low expression of downregulated ones, and therefore was assigned Kinome-27-POS. A cluster of 50 NBLs included most of the low-risk samples (Supplementary Table 1) with low expression of upregulated genes and high expression of downregulated genes, which was designated Kinome-27-NEG. Kaplan–Meier
analysis of the two clusters showed that tumors with a Kinome-27-POS signature conferred a poor prognosis (log-rank test, \( P = 8.6 \times 10^{-13} \)) (Figure 2A). When the tumors with MYCN amplification were left out of the Kaplan–Meier analysis, the poor prognosis of the remaining tumors with a Kinome-27-POS signature was still highly significant (\( P = 1.2 \times 10^{-9} \)) (Figure 2B). When we restricted the Kaplan–Meier analysis to HR tumors (defined as INSS stage 4 and age >18 months), Kinome-27-POS
cluster still had a poor prognosis (P = 0.04) (Supplementary Figure 3A). Amplification of MYCN is an established marker for poor prognosis, but it was less significant than the Kinome-27-POS signature (P = 1.2 × 10^{-9}) (Supplementary Figure 3B). This was confirmed in a regression analysis showing a hazard ratio for survival of 3.82 for the Kinome-27 signature versus 6.64 for MYCN amplification (Table I). The multivariate Cox proportional hazard regression model using Kinome-27 signature, age, INSS stage and MYCN status providing the evidence that deregulation of kinome is a prognostic factor in NBL patients (Table I). We confirmed these observations in 498 NBL tumors (accession no. GSE62564) that are also included in GSE45547 set, but profiled by RNAseq. Expression data were used for K-means clustering in two groups. The Kinome-27-POS cluster consisted of 155 patients, including 121 (78%) HR patients. The Kinome-27-NEG cluster consisted of 343 patients, including only 55 HR patients (16%) (Supplementary Table 2). The Kinome-27-POS cluster had a poor prognosis (P = 1.3 × 10^{-9}) (Figure 2C). After removal of the tumors with MYCN amplification in this series, Kaplan–Meier analysis of the remaining tumors still showed a poor prognosis for tumors classified with a Kinome-27-POS signature (P = 4.0 × 10^{-9}) (Figure 2D). Importantly, the analysis restricted to HR patients showed a poor prognosis for tumors classified with the Kinome-27-POS signature (P = 6.4 × 10^{-9}) (Supplementary Figure 3C).

We could validate these observations in an independent data set of 247 NBL tumors (Asgharzadeh: validation set 1), the expression profile of which was determined on Affymetrix Human Exon Array platform. Expression data of these genes were used for K-means clustering in two groups. The Kinome-27-POS cluster consisted of 116 patients, including 114 (98%) HR patients. The Kinome-27-NEG cluster consisted of 131 patients, including 103 HR patients (79%) (Supplementary Table 3). The Kinome-27-POS cluster had a poor prognosis (P = 1.5 × 10^{-9}) (Figure 2E). In MYCN single copy (Figure 2F) and in HR tumors (Supplementary Figure 2D), the Kaplan–Meier analysis still showed a poor prognosis for tumors classified with a Kinome-27-POS signature (P = 1.9 × 10^{-6} and P = 9.4 × 10^{-6}). The Kinome-27-POS signature remained an outcome predictor after multivariate analysis (Table I).

Finally, we validated the prognostic value of Kinome-27 signature in a second independent data set (accession no. GSE3446: validation set 2) of 102 metastatic NBL (INSS stage 4) lacking amplification of the MYCN. Again, after K-means clustering, the Kinome-27-POS signature predicted poor prognosis (P = 1.0 × 10^{-4}) (Supplementary Figure 3E).

We concluded that the Kinome-27 signature predicts clinical outcome in four independent NBL series. Of note, the signature also predicts outcome in HR NBL and identifies a subset of tumors with highly aggressive clinical behavior.

### Identification of HDAC and Hsp90 inhibitors as repressors of Kinome-27 signature and selection of the cell lines reproducing the ultra-HR expression profiling

By using cMap tool, we selected those perturbagens that strongly repressed our gene signature. We particularly focused on two drugs: (i) SAHA, also known as vorinostat, a HDAC inhibitor and (ii) Radicicol, also known as monorden, a Hsp90 inhibitor. Both drugs showed significant permutation P values (P < 0.001) and high negative connectivity scores, indicating that they are able to reverse the expression of the query signature (up and down tags) (Supplementary Figure 4).

To identify both ultra-HR-like and non-ultra-HR-like NBL cell lines to test the selected drugs, we analyzed the Kinome-27 signature in different NBL cell lines from two freely available data sets. We generated a rank score able to define a gene expression-based classification of cellular phenotypes. Particularly, SKNBE2c and SKNAS cells showed the lowest rank score among the 27 cell lines included in the test set (GSE19274); thus, we were selected...
as ultra-HR-like cell lines. Conversely, SKNFI cells exhibited high rank score and were subsequently identified as non-ultra-HR-like NBL cells (Figure 3A). Comparable results were obtained by analyzing the validation set (GSE28019) (Supplementary Figure 5). Quantitative RT-PCR analyses confirmed the upregulation of the up tags and the downregulation of the down tags in SKNE2c and SKNAS cells compared with SKNFI ones (Figure 3B–C). Thus, we concluded that both SKNE2c and SKNAS represent a reliable in vitro model of ultra-HR NBL cells to study the effects of drug treatments.

HDAC and Hsp90 inhibition is effective against SKNE2c/SKNAS/SKNFI cell viability

To assess the impact of HDAC and Hsp90 inhibition on NBL cell viability, we measured the effect of escalating doses of SAHA and Radicicol on the previously selected NBL cell lines. We used drug concentrations ranging between 0.5 and 10 \( \mu M \), according to the range reported in the cMap database. The complete dose–response curves are shown in Supplementary Figure 6. The extent of reduction of the cell viability varied depending on the cell line in a concentration- and time-dependent manner.

On SAHA treatment, 50% growth inhibition (GI50) in SKNE2c cells (4.1 \( \mu M \)), SKNAS cells (5.5 \( \mu M \)) and SKNFI (4.9 \( \mu M \)) was reached at 48 h. However, low doses of SAHA affected cell viability of all three NBL cell lines within 24 h. While the viability of ultra-HR-like cells (SKNE2c and SKNAS) further decreased at 48 h at low/medium doses, non-ultra-HR-like cells (SKNFI) viability reduced only at high doses at 48 h (Supplementary Figure 6).

Upon Radicicol treatment in SKNE2c cells, GI50 (6 \( \mu M \)) at 24 h was similar to GI50 (5.4 \( \mu M \)) at 48 h, whereas in SKNAS cells, GI50 (>10 \( \mu M \)) it was not reached, suggesting that these concentrations were not toxic for this cell line. Interestingly, GI50 (8 \( \mu M \)) in SKNFI cells at 24 h was higher than GI50 (2.5\( \mu M \)) at 48 h, suggesting a major drug toxicity in these cells. Thus, low doses of Radicicol affect cell viability of SKNE2c within 24 h, whereas SKNFI cells viability was affected only after 48 h of treatment. These observed differences in cell response to drug treatments might depend on different genomic background. We assumed that the perturbagen effects in ultra-HR-like cells were strictly correlated with the ultra-HR signature, which once reverted could induce the cells toward cell death. Conversely, the affected viability of SKNFI cells could be due more likely to the toxicity of the doses rather than to the biological mechanisms related to the genetic signature. To assess our hypothesis, we investigated if both drugs were able to revert the signature of ultra-HR tumors by in vitro treatment. As the perturbations could directly modulate the transcription of the genes, we selected relatively early time points (6 and 12 h after compound addition) to assess the variations in the expression of the gene-tags. As expected, we observed downregulation of the up tags and upregulation of the down tags in the ultra-HR-like cells, SKNE2c and SKNAS, after 6 and 12 h of treatment with both compounds, whereas no reversion of the signature in non-ultra-HR-like cells SKNFI was observed (Supplementary Figure 7).

Figure 3. SKNE2c and SKNAS cell lines reproduce ultra-HR gene signature. (A) The heat map shows the Kinome-27 signature expression in each cell line from test set (\( n = 27 \), GSE19274). The relative values of each cell are represented by a color shade: dark cells indicate low scores (ultra-HR phenotype), whereas light cells are representative of high scores (non-ultra-HR phenotype). Below, the histogram shows the combined Rank score for each cell line. These latter were divide into two groups, low and high scores, according to the median value of Rank score distribution (dotted line). (B) Gene expression analysis (normalized to \( \beta \)-actin) of up (underlined) and down (italics) tags in SKNE2c versus SKNFI. Data are presented as mean ± SEM (three experiments). \( P \) value has been calculated by Student’s t-test. (C) Gene expression analysis (normalized to \( \beta \)-actin) of up (underlined) and down (italics) tags in SKNAS versus SKNFI. Data are presented as mean ± SEM (three experiments). \( P \) value has been calculated by Student’s t-test.
Synergic effect of SAHA and Radicicol on cell viability and cell cycle

SKNBE2c, SKNAS and SKNFI cells were exposed to both single and combined treatment with SAHA and Radicicol; subsequently, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay test after 48 h of treatment. To determine whether the cytotoxic effects obtained with different SAHA and Radicicol combinations were synergistic, we calculated the CI according to the Chou–Talalay method (22). A CI of 1 indicates an additive effect of the two drugs, whereas CI values <1 indicate synergy and CI values >1 indicate antagonism. We treated our three cell lines with both SAHA and Radicicol at non-constant combination ratios with the purpose to identify which ratios of the two drugs are the best to obtain a synergistic effect. Dose–response curves (Supplementary Figure 8–10) and CI values (Figure 4) showed that in SKNBE2c cells, we obtained a strong synergism at high concentration of Radicicol, whereas in SKNAS, this effect was observed at low concentration of Radicicol. Instead, a more broad effect was observed with SKNFI cells. We evaluated the effect of the treatment with either single-drug concentrations or synergic concentration on cell cycle. We treated the three cell lines with single-drug concentrations, which affect cell viability within the first 24 h (respect to vehicle-treated cells) as described above and with the lowest drugs synergic concentrations for 48 h (Figure 5). Cell cycle analysis highlighted that combined treatment at synergic concentration leads to a marked accumulation of SKNBE2c and SKNAS cells in G2-phase, suggesting that the cells are not more proliferating. Of note, G2 block of cell cycle is a prerequisite for good candidate drugs in preclinical and clinical studies. Conversely, in SKNFI cells, we observed an accumulation in S-phase and a reduction of cells in G2-phase (Figure 5). In combined treatment, we also observed a strong increment of cell death in SKNBE2c and SKNFI cells.

Discussion

Despite multimodal therapies, a high percentage of HR NBLS become refractory to current treatments. Among these, ultra-HR NBLS represent a subset of patients who will not respond to or relapse early after standard HR treatment. At the time of diagnosis, it is currently not possible to distinguish the subset of HR patients who will achieve long-term survival from those who will develop progressive disease. Gene expression profiling has provided clinically useful biomarkers of risk stratification in HR NBL. Our aim was to provide a gene signature that identifies the subset of ultra-HR patients in diagnostic setting and which can provide alternative therapeutic targets. In this study, we searched for a specific kinome gene signature with genes differentially expressed between ultra-HR and non-ultra-HR NBLS. Our analysis led to the identification of a gene signature of 27 differentially expressed metagenes, named Kinome-27 signature, which is able not only to predict clinical outcome in HR NBLS but also to identify a subset of tumors with highly aggressive clinical behavior.

The use of kinase inhibitors is a fascinating way to block cancer growth. Most of these drugs block signaling processes that cancer cells use to divide but frequently trigger the production of a complex secretome that activates multiple survival pathways in the remaining drug-sensitive cancer cells (23). Particularly, multiple cancers treated with ALK inhibitors unfortunately develop therapeutic resistance. Acquired drug resistance is a major obstacle to the success of chemotherapy. Of note, among the genes included in Kinome-27 signature, alterations in at least three of them, namely CDK4, PLK1 and RET, have been already associated with cancer drug resistance (24–26). However, we did not look for signaling pathways with therapeutic potential enriched in our kinase gene lists, but we directly submitted our gene lists to the cMap database, as described elsewhere (27). Indeed, application of this tool on the subgroup-specific signatures directly allowed the identification of new candidate therapeutic small molecules able to destroy Kinome-27 signature in ultra-HR NBLS.

HDAC and Hsp90 inhibitors have been extensively studied as options for cancer therapy (28,29). HDAC inhibitors prevent deacetylation of histones and other proteins that can arrest the cell cycle at the G2/M phase, thereby inducing differentiation and apoptosis of tumor cells, while exhibiting a low toxicity toward normal cells. Particularly, SAHA is the first HDAC inhibitor that has been approved by the US Food and Drug Administration and a phase II clinical trial in children with relapsed solid tumors, lymphomas and leukemias is ongoing (30). Although a number of clinical trials have been undertaken with SAHA, the efficacy of this drug as a single agent is low in NBL. Early-phase trials and pre-clinical data suggested that HDAC inhibitors could be most effective when used in combination with retinoids (31,32). Indeed, retinoid signal activation enhances the HDAC inhibitor anticancer signal (33,34) and their combination is well-tolerated in a phase I/II pediatric trial (32).

Figure 4. CI for proliferation studies testing SAHA and Radicicol combined together. SKNBE2c (A), SKNAS (B) and SKNFI (C) cell lines were treated in combination with SAHA and radicicol at non-constant molar ratio and after 48 h of incubation cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The effectiveness of various drug combinations was analyzed by the CalcuSyn Version 2.1 software (Biosoft). The CI was calculated according to the Chou–Talalay method. A CI of 1 indicates an additive effect of the two drugs, CI values <1 indicate synergism, and CI values >1 indicate antagonism.
Treatment with HDAC inhibitors can repress MYC expression and its oncogenic function in a broad range of tumor types, and this is an undervalued effect of HDAC inhibitor treatment, particularly in tumors driven by aberrant MYC expression or activity. Of note, SAHA has been described to decrease the viability of NBL cell lines with a concomitant decrease in MYCN expression (35).

Similarly, the inhibition of Hsp90 function is emerging as a novel strategy for cancer therapy. More than 200 substrates of Hsp90, so called client proteins, have been identified and many of them are involved in critical function for cancer cells, such as growth, proliferation and survival. Indeed, client proteins include receptor type tyrosine kinases, constitutively activated tyrosine kinases, signaling proteins, mutated oncogenic proteins, steroid receptors and cell cycle regulators. Hsp90 inhibitors have proved to be promising antmyeloma agents in pre-clinical settings and >17 of them are currently being evaluated in clinical trials (29,36). Particularly, Radicicol has been described to exhibit growth suppressive effects in EBV-positive lymphomas, especially natural killer (NK) lymphoma (37), to induce apoptosis in colon cancer cells (38) and in human epithelial ovarian carcinoma cell lines (39). Moreover, both HDAC and Hsp90 inhibitors seem to act on Hypoxia-inducible factor 1-alpha (HIF-1α) pathway inhibition. Although the exact mechanism of HDAC inhibitors is still unclear, the Hsp90 inhibitor Radicicol prevents the interaction between HIF-1α/HIF-1β heterodimers and DNA attenuating the HIF-1 activation process (40,41). In agreement with our previous observations, HIF-1α inhibition represent an additional therapeutic advantage in HR NBL promoting cell differentiation into more benign phenotype and cell senescence in vitro (42).

Figure 5. Cell cycle analysis in NBL cell lines treated with SAHA and Radicicol alone and combined together. SKNBE2c, SKNAS and SKNFI cell lines were treated with single drugs or with both drugs at concentrations shown in the sketch above. Cells were stained with propidium iodide and analyzed using flow cytometry. Cells in apoptosis have fewer DNA content. The percentages of cells populations in different cell cycle stages are shown in the bar graphs. Data are presented as mean ± SEM (three experiments). P value has been calculated by Student’s t-test. *P < 0.05.
Our results showed that HDAC and Hsp90 inhibitors are able to switch back the Kinome-27 signature in ultra-HR-like cells by modulation of diverse biological targeted pathways. In light of the differences in the molecular targets, regimens combining these compounds could extend the benefits observed. Acetylation of HSP90 has been observed in cells treated with HDAC inhibitors or after silencing of HADAC6, and this correlates with the destabilization of several oncogenic Hsp90 client proteins (28). Thus, drug combinations centered on both HDAC and Hsp90 inhibitors could represent a promising approach for anti-tumor therapies in ultra-HR NBL patients. Drug combinations may exhibit synergistic or antagonistic effects depending on the interaction of their targets within a network (43). Particularly, SAHA and Radicicol may synergize in ultra-HR-like cells leading to a cell cycle block in G2/M phase, which represents an actionable effect of anticancer drugs and could depend on modulation of Kinome-27 pathways. Conversely, SAHA and Radicicol synergistic effect in non-ultra-HR-like cells may be reversible because cells accumulated in S-phase might repopulate and act on the mechanism for the cell cycle-mediated drug resistance (44).

Conclusions

We generated a specific kinome gene signature (Kinome-27) able to identify a subset of HR NBL patients who will not adequately respond to standard treatments (ultra-HR NBL). We speculate that two drugs (SAHA and Radicicol) are able to selectively target Kinome-27 and block cell cycle in G2/M phase. Although the synergy at low concentrations suggests the potential use of combined SAHA and Radicicol in clinical practice with reduced dose-dependent side effects, more investigations are needed to further elucidate the synergism and antagonism at certain molar ratio. Preclinical in vivo studies and prospective clinical trials will provide the effective relevance and the clinical benefit of this treatment to improve the quality of life in ultra-HR NBL patients.

Supplementary material

Supplementary data are available at Carcinogenesis online.

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Authors’ contributions

R.R., F.C. and M.C. designed and conducted the study, and prepared the manuscript; L.P. and M.A. performed in vitro drug treatment; F.M. performed gene expression analysis; G.V. performed combined drugs analysis; C.L. performed bioinformatic analyses; J.C. contributed to Kinome-27 signature analysis; M.R. performed cell cycle analysis; F.C., M.F. and A.I. contributed to critical review of the manuscript.

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