The MYCN inhibitor BGA002 restores the retinoic acid response leading to differentiation or apoptosis by the mTOR block in MYCN-amplified neuroblastoma

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Abstract

Background: Neuroblastoma is one of the deadliest cancers that occur in early childhood and represent 7% of pediatric malignancies [1]. Approximately 25% of patients with a neuroblastoma diagnosis present with MYCN amplification (MNA), which is linked to a poor prognosis, metastasis,
and recurrence [2–5]. MYCN is a key driver of the disease and its overexpression reprograms neuroblastoma cells towards a stem-like phenotype that affects proliferation and cell growth, metabolism, and apoptosis inhibition. It also favors immune escape, invasion, and metastases [6–8].

Different therapeutic approaches have been developed to treat neuroblastoma, but high-risk cases (that are often MYCN amplified) remain critical. Among medical therapies, high-risk cases are treated with 13-cis retinoic acid (RA), which induces neuronal differentiation and leads to cell-growth inhibition [9]. MYCN expression needs to decrease to complete the differentiation program [10]; however, resistance to retinoic acid has been found, which is associated with concomitant relapses and poor survival outcomes [11].

Normally, MYCN expression is restricted during embryogenesis and is not expressed during adulthood [12]. Given its effect on neuroblastoma and its expression profile, N-Myc is a promising therapeutic target [13]. However, drug discovery approaches aimed at blocking N-Myc heterodimerization with MAX or its binding with DNA (without interfering with the highly homologous Myc) has, to-date, largely failed [13]. While indirect strategies have been proposed, due to the broad role, along with the number of pathways affected by its overexpression, N-Myc remains challenging to target. We have previously demonstrated that an alternative approach concerns specific gene expression inhibition. It also favors immune escape, invasion, and metastases [14–16]. The 13-cis retinoic acid (13cis-RA) was purchased from Sigma Aldrich and diluted in ethanol. Cell line expansion was conducted in RPMI-1640, with 10%FBS. Neuroblastoma adherent cells were detached using PBS-EDTA, which was followed by washing and counting with nigrosin using a Burker's chamber. Treatment with BGA002, 13cis-RA and the combination (BGA002 + 13cis-RA) were conducted in OPTI-MEM medium. For the RNA extraction experiment, 5 × 10⁴ cells were plated in a 24-well, flat-bottom plate. For the cell viability assay, 5 × 10⁵ cells were plated in a 96-well, flat-bottom plate. Neuroblastoma cell lines were treated with increasing concentrations ranging from 0.6μM to 10μM. After 6 hours of treatment, up to 4% of FBS was added to the cells.

Quantitative real-time PCR
RNA extraction, retro-transcription, and real-time PCR were performed as previously described [16]. Primers used in this study are listed in Supplementary Table 2.

Cell viability assay and Western blot analysis
Cell viability assays were performed as previously described [16]. Cell viability assays were performed as previously described [16] using Cell Titer Glo Viability Assay (®) kit Promega. A Western blot analysis was conducted using standard methods [19]. Briefly, cells were lysed in radioimmunoprecipitation assay lysis buffer (containing 20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM b-glycerophosphate, 1mM Na₃VO₄, and 0.1% SDS) supplemented with Protease and Phosphatase...
Inhibitor Cocktail (Thermo Fisher Scientific Inc., Rockford, IL, USA). After sonication, cells were centrifuged at 15,000×g at 4 °C for 20 minutes and protein fractions were collected. A total of 30 μg of proteins were separated via SDS-PAGE using Criterion TGX polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Proteins were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, England). The ChemiDoc It2 Imaging System and Vision Works LS Software (UVP, LLC, Upland, CA, USA) were used for the analysis. Bands were uncovered by the Amersham ECL detection system. The expression of specific proteins was assessed using the following antibodies: anti-N-Myc 1:800; anti-Phospho-Akt (Ser473) (#4060) 1:1000; anti-Akt (#9272) 1:1000; anti-Phospho-p70 S6 Kinase (Thr389) (#9206) 1:1000; anti-p70 S6 Kinase (#9202) 1:1000; anti-Phospho-S6 Ribosomal Protein (Ser235/236) (#4858) 1:1000; anti-S6 Ribosomal Protein (#2217) 1:1000; anti-Phospho-4E-BP1 (Thr37/46) (#2855) 1:1000; anti-4E-BP1 (#9452) 1:1000; and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#5174) 1:1000. All antibodies, except N-Myc, were obtained from Cell Signaling Technology (Danvers, MA, USA). N-Myc (sc-53,993) was from Santa Cruz Biotechnology (Dallas, TX, USA).

**Apoptosis analysis**
The Kelly, LAN-5, SK-N-BE(2)-C, and TET-21N cell lines were treated as described above. Cells were stained with an Annexin V/FLUOS Staining Kit (F. Hoffmann-La Roche AG, Basel, Switzerland) according to the manufacturer’s instructions. The cell samples were analyzed via CytoFLEX flow cytometer (Beckman Coulter Inc., Brea, CA, USA). The results were analyzed using FlowJo software (Tree Star Inc. Ashland, OR, USA).

**Transmission electron microscopy**
Kelly and LAN-5 cells were seeded at a density of 20,000 cell/cm² in 6-well culture plates. Twenty-four hours after seeding, cells were treated with NaCl 0.9%, BGA002 2.5 μM, 13cis-RA 2.5 μM, and BGA002 2.5 μM + 13cis-RA 2.5 μM in FBS-free culture medium. After 6 hours, up to 4% of FBS was added and treatment proceeded for up to 48 hours at 37 °C with 5% CO₂. Before fixing, cells were treated overnight with 60 μM chloroquine.

At the end of the experiments, the cells were fixed in 2.5% buffered glutaraldehyde directly in 6-well culture plates for 20 minutes at room temperature. They were then detached with a scraper, collected in tubes, pelleted and kept at 4 °C overnight. After washing in phosphate buffer, the cells were post-fixed in 1% buffered osmium tetroxide for 1 hour at 4 °C, washed and dehydrated through graded ethanol followed by embedding in Araldite resin. Samples were sectioned with a ultramicrotome and the ultra-thin sections were collected on grids and counterstained with uranyl acetate and lead citrate. Samples were examined using a Philips CM100 Transmission Electron Microscope (FEI Company, ThermoFisher, Waltham, MA, USA). Digital images were obtained using an Olympus camera (Tokyo, Japan).

**Morphological analysis of differentiation**
The Kelly, LAN-5, SK-N-BE(2)-C, and SH-SY5Y cells were seeded in OPTI-MEM 4% FBS for 24 hours in 6-well plates (Thermo-scientific). Cell number to plate ratio was calculated to avoid confluence. After 24 hours, the cells were treated with 1.25 μM of BGA002, 2.5 μM of 13cis-RA, and 1.25 μM each and BGA002 + 13cis-RA. The treatment was repeated every 48 hours with fresh medium. Images were acquired every 48 hours using an Eclipse TE2000-S microscope (Nikon, Tokyo, Japan). Cells were kept for an additional 9 days (while continuing to change the medium every 48 hours) until day 18. After 12 hours, 9- and 18-day cells were lysed and RNA was extracted as described above. We measured the extension of neurites using Simple Neurite Tracer plug-in in ImageJ software (National Institutes of Health, Bethesda, MD, USA). Neural network analysis is described in detail in the supplementary data.

**Wound healing assay**
The Kelly, LAN-5, SK-N-BE(2)-C, LAN-1, SH-SY5Y, and TET-21N cell lines were seeded in OPTI-MEM 4% FBS to reach confluence after 24 hours in 12-well plates (Thermo-scientific). The day after seeding, a scratch on the cell monolayer was made using a 200 μL tip. Cells were then treated with BGA002 (at 1.25 and 2.5 μM), 13cis-RA (at 1.25 and 2.5 μM) and BGA002 + 13cis-RA (at 1.25 each and 2.5 μM each). From the time of the treatment (day 0) the cells were maintained in culture for up to 72 hours (photos were acquired every 24 hours using an Eclipse TE2000-S microscope (Nikon, Tokyo, Japan)). Cells were then lysed and their RNA extracted as described above. Images were analyzed using the Wound Healing Tool plugin in ImageJ1.46r (NIH). The percentage of the area occupied by the cells was calculated with respect to day 0.

**Lysosome area measurement**
The LAN-5 and Kelly cell lines were seeded in a Nunc Lab-Tek Flask on Slide for live staining. Treatment was administered 48 hours before acquisition. A LysoTracker was added and the cells were incubated for 45 minutes at 37 °C at 5% CO₂. For each condition, z-stacks (at a 200 nm interplane distance) were acquired for up to 48 hours at 37 °C with 5% CO₂. Before fixing, cells were treated overnight with 60 μM chloroquine.
using a Nikon Ti2-E microscope (Nikon, Tokyo, Japan). Images were elaborated using the Fiji plugin in ImageJ software. Z-stacks containing lysosomes were selected using Image>Stack>User Mode. Once selected, all images were binarized using Process>Binary>Make Binary with the Yen method. Lysosomes were then analyzed using Analyze>Measure Particles, with the lower value size set to 0.1 μm².

**Neuroblastoma luminescent cells and the xenograft ectopic neuroblastoma mouse model**

CHP-134-luc was prepared as described previously [16]. CHP-134-Luc cell line was chosen because shows a better engraftment ratio in comparison with previous cell line tested for such as Kelly-Luc previously used. All experiments with mice were approved by the Scientific Ethical Committee of Bologna University (protocol no. 07/73/2013 and 564/2018-PR). Six-week-old mice (NOD/SCID CB17; both sexes) were inoculated with CHP-134-luc (10 x 10⁶ cells for each animal) in the dorso–poste- rio–lateral position. Prior to injection, mice were sedated with isoflurane. Luminescence was used to monitor the growth of tumors (D-Luciferin was administered via intraperitoneal injection, and luminescence was monitored using the UviTec Imaging System (Clever Scientific, Ltd., Rugby, UK). Treatment administration began after a predefined starting point during bioluminescent acquisition and was conducted daily for 28 days with an injection of 100 μL of vehicle, 10 mg/kg/day of BGA002, 10 mg/kg/day of 13cis-RA, and 10 mg/kg/day each of BGA002 and 13cis-RA. Vehicle and BGA002 were administered via subcutaneous injection while 13cis-RA was given via intraperitoneal injection. Animals were monitored until they reached the endpoint (10 mm linear tumor or 60 days post treatment). Tumor size and volume was calculated using a caliper. After reaching the endpoint, the mice were sacrificed. The tumors were removed, measured, weighed, and fixed in 4% formalin. Immunohistochemistry was conducted as previously described [16].

**Statistical analysis**

Statistical analysis was performed with the Prism software version 6 (GraphPad) or with R software version 3.5 or Python software version 3.7. The different analyses and tests were specifically designed for each experiment.

**Data availability**

The data generated in this study are available within the article and its supplementary data files. Expression profile data analyzed in this study were obtained from Gene Expression Omnibus (GEO) at GSE9169, GSE80151 and GSE80153, from Array Express at E-MTAB-1781, from the TARGET repository at the official website, from Cancer Cell Line Encyclopedia.

**Results**

BGA002 in combination with RA cooperates to inhibit MYCN activity

In a previous article, we demonstrated that BGA002 was able to block MYCN expression in neuroblastoma cell lines [16]. Therefore, here we investigated how the combined treatment of BGA002 and RA would affect MYCN expression in a broad panel of neuroblastoma cell lines (17 cell lines, which recapitulated the neuroblastoma landscape: MNA cell lines (n = 10), MNA p53-mutated (n = 3), non-MNA (n = 3), and non-MNA p53-mutated (n = 1)). Treatment with RA alone achieved poor inhibition of MYCN mRNA expression. Treatment with BGA002 showed a marked reduction in all neuroblastoma cell lines, and combined treatment with RA further strengthened MYCN inhibition (Fig. 1A) in a dose-dependent manner (Supplementary Fig. S1A). We also tested cell-viability inhibition after...
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treatment in the same neuroblastoma cell line panel. While RA alone showed a modest effect, BGA002 strongly inhibited cell viability in all cell lines in a dose-dependent manner (Fig. 1B and Supplementary Fig. S1B), and the combined treatment led to a significantly stronger effect as demonstrated by a lower GI_{50} (Fig. 1B-C). Moreover, the combined treatment of BGA002 and RA was found to be synergic (Supplementary Fig. S2A-D). We also verified that the MYCN mRNA inhibition translated to a decrease in proteins and that BGA002-RA treatment led to a stronger N-Myc decrease in three MYCN-amplified neuroblastoma (MNA-NB) cell lines (Fig. 1D-E and Supplementary Fig. S3A). Furthermore, RA failed to induce apoptosis in the neuroblastoma cell lines, while BGA002 alone induced apoptosis and the combined treatment with RA reinforced this effect, especially in MNA-NB Kelly cells (Fig. 1F and Supplementary Fig. S3B-C).

Treatment with BGA002-RA strengthened BGA002-induced mitochondria alteration in Kelly cells, as demonstrated by electronic microscope ultrastructural analysis, size, and perimeter reduction. RA alone had no activity (Fig. 1G). The ultrastructural analysis also showed that BGA002-RA led to the consistent appearance of macrovacuoles in MNA-NB cells (Kelly) (Fig. 1G). Interestingly, we found a lower apoptotic effect in other MNA-NB cells (LAN-5 and SK-N-BE(2)-C) (Fig. 1F and Supplementary Fig. S3B), while Tet-21N cells did not undergo apoptosis (Supplementary Fig. S3C). We also noticed that the mitochondria in MNA-NB cells (LAN-5) with a lower apoptosis effect after BGA002-RA treatment were similar to untreated cells (Fig. 1G). Confocal image analysis also showed a dramatic reduction in mitochondrial volume in Kelly cells, however, the same extent of damage in LAN-5 cells was not observed (Supplementary Figs. S4–5 and Supplementary Fig. S6A-B).

BGA002-RA treatment induces differentiation in MNA-NB cells

Undifferentiated neuroblastomas are considered high risk and are associated with poor survival outcomes. Therefore, we separated neuroblastoma patient expression profiles into 2 clusters for differentiation status using a differentiation signature (1557 genes, based on gene ontology pathways) (Supplementary Fig. S7A-D). We also investigated the transcription regulation of differentiation and used a dataset of neuroblastoma cell lines treated with RA to identify putative transcription factors involved in differentiation. We used this list of transcription factors to identify regulons that were differentially active in two different neuroblastoma cohorts (Supplementary Fig. S8A-B). We identified 3 clusters of neuroblastoma patients, according to the selected regulon activity implied in differentiation, which showed differential survival (Supplementary Fig. S8C-D).

As RA treatment is known to induce neuron differentiation, and MYCN inhibition is necessary to fully achieve differentiation, we tested whether BGA002-RA could lead to MNA-NB cell differentiation. Specifically, we used SH-SY5Y cells as a control for differentiation (Supplementary Fig. S9A-B). We treated the MNA-NB cell line LAN-5 with BGA002 and/or RA (2.5 μM) for 9 days and obtained optical microscope images at different time points (Supplementary Fig. S9C-E). We also tested the differentiation activity of a lower concentration of BGA002 and/or RA (1.25 μM) in MNA-NB cell lines (LAN-5, SK-N-BE(2)-C, and Kelly) for 9 days. Microscopic images showed that RA alone was sufficient to induce differentiation in SH-SY5Y but not in MNA-NB cells (Fig. 2A, Supplementary Fig. S9A and Supplementary Fig. S10A-B). Conversely, we observed an increase in neurite length with BGA002 treatment only in MNA-NB cells (Fig. 2B and Supplementary Fig. S10C). The combined treatment of BGA002-RA showed a significant
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increase in neurite length (Fig. 2B and Supplementary Fig. S10C).

To measure the differences between the different conditions, we trained a convolutional neuron network as a feature extractor. On the new feature vectors, we calculated the Euclidean distance between the control condition and the other conditions (Supplementary Fig. S11A-D). This complementary approach confirmed that MNA-NB cells treated with BGA002 and BGA002-RA were different from untreated cells, while RA-treated MNA-NB cells were similar to untreated cells (Fig. 2C and Supplementary Fig. S11E). The Kelly MNA-NB cell line, which exhibited high levels of apoptosis following BGA002 or BGA002-RA treatment, failed to undergo differentiation. Remarkably, an evaluation of the differentiated phenotype of BGA002-RA treated LAN-5 cells performed after an additional 9 days without treatment showed a persistence of the differentiation status (Supplementary Fig. S12A). In LAN-5 cells, MYCN mRNA expression also resulted in inhibition after 9 days of treatment (Fig. 2D and Supplementary Fig. S12B), and this inhibition persisted after an additional 9 days without treatment (Fig. 2D). These data were further confirmed by confocal microscopy analysis, performed using synapsin-1 as marker for differentiation in LAN-5 MNA-NB cells. After 9 days, neuron-like structures resulted well established only in cells treated with BGA002-RA, in which the cytoplasm showed long, ramified protrusions and the cells clustered together (Fig. S13A). Synapsin-1 quantification in these cells treated with BGA002-RA showed a higher value compared to untreated cells or to single treatments (Fig. S13B), reinforcing the data on the differentiation involvement.

BGA002-RA treatment rebalances cellular retinoic acid-binding protein 1/2 in RA-resistant neuroblastoma

The high expression level of cellular retinoic acid-binding protein (CRABP)1 and the low expression level of CRABP2 play a role in resistance to RA treatment in breast and pancreatic tumors; however, currently, no specific studies have investigated their roles in neuroblastoma [20–22]. In this study, we found that neuroblastoma had the highest CRABP1 expression (Fig. 3A). Moreover, we found that CRABP1 had a significantly higher expression in MNA-NB patients (Figs. 3B and S3B), and a higher expression was linked to a worse prognosis (Fig. 3C). It is known that CRABP1 sequesters RA in the cytoplasm. Its elevated expression in neuroblastoma could cause RA resistance by limiting RA access to the nucleus, which is mediated by binding to CRABP2 [23, 24]. Therefore, we tested how specific MYCN inhibition by BGA002 and RA treatment affected CRABP1/2 expression in neuroblastoma cell lines. Interestingly, only the combined BGA002-RA treatment induced a concomitant downregulation of CRABP1 and upregulation of CRABP2 expression (Fig. 3D). When used as single agents, RA upregulated CRABP1 and CRABP2 while BGA002 downregulated CRABP1 and CRABP2 (Fig. 3D). We tested the effect of CRABP1 and CRABP2 inhibition in MNA and p53mut neuroblastoma cell lines (SK-N-BE(2)-C), which are known to be resistant to RA. Treatment with RA alone showed no prominent effect on viability, while the addition of siRNA against CRABP1 overcame RA resistance (Fig. 3E). Interestingly, the addition of siRNA against CRABP2 blocked CRABP1-restored RA susceptibility (Figs. 3E and 4).

BGA002-RA treatment inhibits the migration capacity of MNA-NB

MYCN expression levels correlate with metastatic behavior, which leads to decreasing adhesion and increasing motility, invasion, and matrix degradation [25]. On the one hand, N-Myc downregulates integrins (α1, β1) and E-cadherin. On the other hand, it leads to increases in focal adhesion kinase and the expression of metalloproteases [26–30]. Here we found that pathways related to cell adhesion were negatively enriched in the differentially expressed genes between MNA and non-MNA-NBs in two different datasets (Supplementary Fig. S14). Furthermore, these pathways were enriched...
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in the genes that were inversely correlated with MYCN in the two datasets (Supplementary Fig. S14). Therefore, we investigated how BGA002-RA affected invasion and migration through a wound-healing assay in MNA-NB (Supplementary Fig. S15A). We observed a modest effect following RA treatment with 3 different concentrations and only at late time points (48 or 72 hours) (Supplementary Fig. S15A and B). In contrast, we found the inhibition of migration capacity after treatment with BGA002 (2.5 μM) at the earliest time point (24 hours), which then increased at later time points (Supplementary Fig. S15A and B). The combined treatment with BGA002-RA further increased the effect of migration inhibition. Specifically, we observed an inhibitory effect at the earliest time-point, which increased over time, with the inhibition also observable at lower doses (0.6 and 1.25 μM) (Supplementary Fig. S15B). At the molecular level, we investigated whether genes involved in migration were downregulated following MYCN inhibition [30–33] and found that BGA002 or BGA002-RA treatments were able to downregulate genes involved in MNA-NB migration. A substantial effect after RA treatment alone was not observed (Supplementary Fig. S16A).
BGA002-RA treatment leads to mTOR complex inhibition in MNA-NB

In MYCN-related mice models, phosphatidylinositol 3-kinase (PI3K)/mTOR pathway inhibition is reported to destabilize N-Myc and be effective against tumors [34, 35], while other studies have reported that N-Myc could regulate the mTOR pathway in neuroblastoma [36, 37]. However, it has been shown that, in other tumors, RA is capable of inhibiting mTOR [38]. In the present study we found that neuroblastoma presented the highest level of mRNA expression of genes involved in the mTOR pathway (small cell lung cancer ranked second) and presented low promoter methylation (Supplementary Fig. S17A). Moreover, neuroblastoma and small cell lung cancer clustered together for mTOR gene expression (Supplementary Fig. S17B-D), which could be related to the fact that these two highly aggressive tumors derive from peripheral nervous system cells.

Neuroblastoma cell lines presented high expressions for different genes of the mTOR pathway (Supplementary Fig. S18A) and a higher expression was found in MNA versus non-MNA patients (Fig. 5A). Remarkably, these genes were also significantly predictive for overall survival (Fig. 5B), and strongly correlated with MYCN expression (Fig. 5C). Interestingly, MNA-NB cell lines showed a higher G1\textsubscript{S} when treated with mTOR inhibitors (Supplementary Fig. S19A-B), and a ChiP-seq public data analysis showed that N-Myc directly regulated different mTOR pathway genes (Supplementary Fig. S19C-D). Therefore, we tested whether BGA002-RA could inhibit the mTOR pathway in MNA-NB. We found that BGA002 or BGA002-RA strongly inhibited the expression of genes involved in the mTOR pathway, while RA alone failed to downregulate their expression (Fig. 5D). We also evaluated mTOR pathway activity through protein phosphorylation. The results showed a reduction in protein kinase B (AKT), p70S6K, and 4E-BP1 phosphorylation after treatment with BGA002, which was strengthened by BGA002-RA; thus, demonstrating mTOR pathway inhibition in MNA-NB (Fig. 5E-F).

We noticed that neuroblastoma patients with high mTOR pathway activity had significantly worse survival outcomes (Supplementary Fig. S20A). Therefore, we combined mTOR activity, differentiation score, and MYCN status into a single score to predict the survival of neuroblastoma patients. Using receiver operating characteristic curve analysis, we tested the predictive ability and accuracy of our model. The combined score showed a high predictive ability at 1 year (area under the curve = 0.914) and satisfactory predictive ability at 3 and 5 years (Supplementary Fig. S20B). The combined score was superior to the single components (Supplementary Fig. S20C) and had superior accuracy in comparison to commonly used clinical characteristics (Supplementary Fig. S20D).

BGA002-RA treatment leads to autophagy reactivation

Metabolic reprogramming is a cancer hallmark [39] and the mTORC1 complex plays an important role in metabolic control while suppressing autophagy [40]. In our investigation, we found that pathways related to autophagy were negatively enriched in differentially expressed genes in MNA with respect to non-MNA in two different neuroblastoma datasets (Supplementary Fig. S21A-C). In addition, MYCN silencing in an inducible MYCN model exhibited autophagy signature re-expression in concomitant mTOR downregulation datasets (Supplementary Fig. S22A-B). As we found mTOR pathway downregulation following BGA002-RA treatment, we evaluated whether this event resulted in autophagy reactivation. Treatment with BGA002-RA showed an increase in lysosomes after the treatment in MNA-NB cell lines (Fig. 6A and Supplementary Fig. S23A-B), and in particular higher diameter lysosomes (>2 μm) resulted more numerous in this condition respect the single treatments (Fig. 6B). Electron microscopy analysis also uncovered the appearance of a large number of macrovacuoles after BGA002-RA treatment in MNA-NB cells (Fig. 1G).
Fig. 5 (See legend on previous page.)
**BGA002-RA shows an in vivo anti-tumor effect against MNA-NB**

We evaluated the anti-tumor capacity of systemic treatment with BGA002-RA in comparison to the vehicle, or BGA002 or RA alone in an MNA-NB xenograft mouse model (CHP-134 cells). BGA002 treatment alone or RA alone had already demonstrated survival augmentation (Fig. 7A), and we found that combined BGA002-RA treatment also showed a significant increase in survival (Fig. 7A and Supplementary Fig. S24A), and a significant hazard ratio reduction in comparison to the vehicle (0.28, p-value = 0.004) (Supplementary Fig. S24B). BGA002-RA treatment also reduced tumor growth during treatment in comparison to the vehicle (Supplementary Fig. S24C).

We conducted a histological analysis of tumors 60 days after the end of treatment. Tumor vascularization was strongly present in vehicle and remained in RA treated mice; however, it was not present in BGA002 and BGA002-RA treated mice (Fig. 7B). Moreover, immunohistochemistry analysis showed that N-Myc protein expression in RA-treated tumors was similar to vehicle. While BGA002 treatment was already capable of reducing N-Myc protein, BGA002-RA treatment consistently strengthened this effect (Supplementary Fig. S25A-B). In addition, we found that the trend in results observed for N-Myc protein staining in tumors was similar to the results found with Ki67 staining (Fig. 7B).

**Discussion**

While retinoic acid (RA) treatment has shown high efficacy in the treatment of acute promyelocytic leukemia, numerous clinical trials are exploring the efficacy for a wide range of human malignancies [20]. Moreover, RA treatment has also been shown to be beneficial in high-risk neuroblastoma for controlling minimal residual disease therapy [41]. However, approximately 50% of neuroblastoma patients have intrinsic or acquired resistance to RA treatment, particularly patients with MNA-NB [42, 43]. As RA treatment is less toxic than chemotherapy and is able to induce differentiation in malignant cells, there is intensive research to overcome these efficacy limitations [20]. In this respect, different studies have proposed chemical RA modification, different formulations, or the use of RA in combination with other treatments [44]. Nevertheless, the exact mechanism of acquired resistance to RA treatment is still debated.

Beyond neuroblastoma, MYCN amplification has also been found in different neoplasias, and the list of malignancies where it plays a role is expanding [45]. Targeting N-Myc has high potential due to its role in cancer development, its association with a poor prognosis, its wide control of expression, and its restricted expression at the embryonic stage. Here we have demonstrated that combined BGA002 and RA treatment was able to inhibit MYCN expression and cell viability in both MNA- and non-MNA-NB cell lines showing a synergistic effect. Furthermore, in MNA/p53mut neuroblastoma cell lines (which are, on average more resistant to treatment) this effect was even more relevant—as indicated by a lower EC50. BGA002-RA showed improved efficacy in inhibiting N-Myc protein expression and in inducing apoptosis in comparison with the single treatment. Previously, we showed that MYCN inhibition by BGA002 led to the reactivation of mitophagy and cell death via mitochondria damage due to reactive oxygen species increases [16]. In line with this, we have now found mitochondria alteration following BGA002-RA administration, which is associated with apoptosis in MNA-NB cells.

Neuroblastoma pathogenesis has also been associated with differentiation failure and, especially in MNA-NB, the persistence of cancer cells in an undifferentiated, embryonic-like state [46]. However, we found that, at low doses, BGA002-RA was able to induce differentiation in MNA-NB cells. Results showed that untreated or RA-alone treated MNA-NB cell lines failed to undergo differentiation. Conversely, MYCN inhibition by BGA002 was already capable of inducing neurite length increases, and we found a stronger effect after combined BGA002-RA treatment in MNA-NB cell lines. Hence, the block of MYCN by BGA002 reverted the differentiation resistance to RA in MNA-NB cells. In this context, BGA002-RA did not alter mitochondria in MNA-NB cells that underwent differentiation. Our analysis showed that MYCN inhibition remained 9 days after the end of treatment. Thus, there was a persistence of the MYCN inhibition and of the differentiated phenotype after suspension of BGA002-RA treatment in MNA-NB cells. Furthermore, here we have presented a new complementary approach.
Fig. 6 (See legend on previous page.)
to quantify neuroblastoma differentiation that is based on a convolutional neural network. This method is scalable and can be used to monitor differentiation in neuroblastoma cell lines with different drug combinations. A similar approach can also be used to monitor changes in cellular morphology in high-throughput screening.

With the aim to elucidate the mechanisms of RA resistance in neuroblastoma, we examined the balance between cellular retinoic acid-binding protein (CRABP)1 and CRABP2. Remarkably, our analysis uncovered that CRABP1 had a significantly higher expression in MNA-NB patients that was linked to a worse prognosis. As it is known that CRABP1 sequesters RA in the cytoplasm, its elevated expression in neuroblastoma could cause RA resistance by limiting RA access to the nucleus mediated by its binding to CRABP2 [23, 24]. In this context, it is of relevance our finding that only the combined BGA002-RA treatment induced a concomitant downregulation of CRABP1 and upregulation of CRABP2 expression, reverting the CRABP1/2 balance in neuroblastoma cells.

The ability to migrate and invade is a cancer hallmark, and cancer cells with an undifferentiated and mesenchymal phenotype are more prone to metastasize [47]. Indeed, MNA-NBs have a high metastatic capacity in different target sites in the body [48, 49]. Our results showed that blocking MYCN led to impairment of MNA-NB cell line migration with concomitant downregulation of genes involved in the migration.

Previous studies have demonstrated mTOR pathway activation in two thirds of neuroblastoma patients, with AKT and mTOR phosphorylation in primary neuroblastoma, and this pathway activation correlated with reduced event-free and overall survival [50–52]. Furthermore, the mTOR pathway is often involved in resistance to cancer therapies [53]. MYCN amplification leads to the activation of many downstream pathways, including mTOR, and the mTOR pathway leads to N-Myc protein stabilization [54, 55]. Therefore, the use of mTOR pathway inhibitors in combination with other agents for neuroblastoma therapy has been proposed [56]. However, the mTOR pathway is not a cancer specific pathway and is widely used by non-cancerous cells as well. Thus, mTOR pathway inhibitors are not specific for neuroblastoma cells and present different side effects in normal cells, limiting their potential clinical use. Here we showed that MYCN silencing by BGA002 or BGA002-RA led to the inhibition of mTOR pathway gene expression and an overall reduction in pathway activity in MNA-NB cells. MYCN expression is mainly restricted to cancer cells (and especially in MNA-NB cells where it is highly expressed), while it has a very limited pattern of expression in normal cells [6]. Thus, our approach of specific MYCN targeting by BGA002 may result in the inhibition of the mTOR pathway only in cancer cells, leaving healthy cells unaffected [49].

Furthermore, compared with classical parameters, here we showed that the survival of neuroblastoma patients can be more efficiently predicted by combining mTOR activity, MYCN-status, and differentiation.

We also found that autophagy pathways were down-regulated in MNA-NB patients, while MYCN silencing resulted in autophagy reactivation. BGA002-RA treatment resulted in large increases in lysosomes and macro-vacuoles in MNA-NB cells, particularly in cells that proceeded with apoptosis instead of undergoing differentiation.

Conclusions
As different studies have highlighted, MYCN amplification reshapes the neuroblastoma landscape by creating undifferentiated, aggressive, highly vascularized, disseminating, and nearly untreated tumors. In this study we showed that specific MYCN inhibition by BGA002 led to the reversion of different MNA-NB hallmarks. In combination with RA, BGA002 was able to inhibit migration capacity and induce differentiation or apoptosis, thus overcoming RA-resistance in MNA-NB cells. We also showed that blocking a single cancer-specific gene (MYCN) is a more sustainable method for inhibiting the mTOR pathway in neuroblastoma cells only, avoiding side effects of mTOR inhibition in healthy cells. Moreover, while N-Myc has been reported to induce angiogenesis, we found that, in a mouse model of highly vascularized MNA-NB, in vivo treatment with BGA002-RA had a dramatic effect on vascularization with absence of tumor blood vessels, which remained after treatment suspension. There was also a significant increase in survival.

Our study shows that it is possible to realize precision medicine, even for the worst type of neuroblastoma (MNA-NB), by the identification of optimal combinated.
Fig. 7 (See legend on previous page.)
drugs that can achieve potent and selective block of cancer pathways only in tumor cells, preserving the impact of side effects to normal cells. MYCN amplification is not restricted to neuroblastoma, and restoration of RA treatment could be beneficial in different MNA-tumors. Therefore, BGA002-RA could potentially be administered to a wide range of aggressive MNA-related malignancies.

Abbreviations
MNA: MYCN amplified; NB: Neuroblastoma; RA: Retinoic Acid; PNA: Peptide Nucleic Acid, agPNA: Antigene PNA; BGA002-RA: Combination of BGA002 and RA.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13046-022-02367-5.

Additional file 1.
Additional file 2.

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Authors’ contributions
SL, carried out molecular, in vitro and in vivo studies, data acquisition and participated in the writing, in the conceptualization and the design. SR conducted statistical, bioinformatic, machine learning and deep learning analysis, wrote and revised the manuscript, and designed bioinformatics / machine learning analysis. LM participated in developing the methodology, data acquisition and in vitro molecular studies, and reviewed the manuscript. DM conducted in vitro and in vivo studies, participated in writing and in administrative, technical, or material support. CA conducted in vitro and in vivo studies, participated in figure assembling and in administrative, technical, or material support. SB and SA conducted in vitro and in vivo studies. ALS aided in administrative, technical, and material support, revising the original draft. GN and LC developed the chemical synthesis of the compound, and participated in administrative, technical, and material support. FP conducted in vitro proteomics studies and participated in the original draft writing. SV conducted the electronic microscope experiments and analysis. MF provided access to patient data and revised the manuscript. AMA supervised proteomics experiments and revised the manuscript. GP supervised the electronic microscope experiments and analysis, and revised the manuscript. AP and PH participated in funding acquisition, manuscript revision and study supervision. RT participated in study design and conceptualization, funding acquisition, manuscript revision and study supervision. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article (and its additional files). Datasets used are described in the material and method section and public available (accession number is provided).

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the University of Bologna.

Consent for publication
The authors provide consent for publication.

Competing interests
R. Tonelli and A. Pession are BIOGENERA shareholders. S. Raieli, C. Amadesi, D. Bartolucci, S. Bortolotti, S. Angelucci, S. Lampis, A. Scardovi, G. Nieddu, and L. Cerisoli are employed at BIOGENERA. The authors declare no potential conflicts of interest.

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