High-Throughput Screening Identifies Idasanutlin as a Resensitizing Drug for Venetoclax-Resistant Neuroblastoma Cells

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ABSTRACT

Neuroblastoma tumors frequently overexpress the anti-apoptotic protein B-cell lymphoma/leukemia 2 (BCL-2). We previously showed that treating BCL-2-dependent neuroblastoma cells with the BCL-2 inhibitor venetoclax results in apoptosis, but unfortunately partial therapy resistance is observed. The current study describes the identification of drugs capable of resensitizing venetoclax-resistant neuroblastoma cells to venetoclax. To examine these effects, venetoclax resistance was induced in BCL-2-dependent neuroblastoma cell lines KCNR and SJNB12 by continuous exposure to high venetoclax concentrations. Non-resistant and venetoclax-resistant neuroblastoma cell lines were exposed to a 209-compound library in the absence and presence of venetoclax to identify compounds that were more effective in the venetoclax-resistant cell lines under venetoclax pressure. Top hits were further validated in combination with venetoclax using BCL-2-dependent neuroblastoma model systems. Overall, high-throughput drug screening identified the MDM2 inhibitor idasanutlin as a promising resensitizing agent for venetoclax-resistant neuroblastoma cell lines. Idasanutlin treatment induced BAX-mediated apoptosis in venetoclax-resistant neuroblastoma cells in the presence of venetoclax, whereas it caused p21-mediated growth arrest in control cells. In vivo combination treatment showed tumor regression and superior efficacy over single-agent therapies in a BCL-2-dependent neuroblastoma cell line xenograft and a patient-derived xenograft. However, xenografts less dependent on BCL-2 were not sensitive to venetoclax–idasanutlin combination therapy. This study demonstrates that idasanutlin can overcome resistance to the BCL-2 inhibitor venetoclax in preclinical neuroblastoma model systems, which supports clinical development of a treatment strategy combining the two therapies.

Introduction

The intrinsic apoptotic response is tightly regulated through a balance between anti-apoptotic and pro-apoptotic B-cell lymphoma/leukemia 2 (BCL-2) family members (1). Anti-apoptotic members, such as BCL-2, MCL-1, BCL-XL and BCL-W, prevent apoptosis by sequestering pro-apoptotic BH3-only proteins like BIM, PUMA, NOXA, BAD and BID (2, 3). Some BH3-only proteins (e.g., PUMA, NOXA and BAD) interact with anti-apoptotic BCL-2 proteins to prevent them from sequestering other BH3-only pro-

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Materials and Methods

Chemicals
The Sequoia anti-neoplastic library containing 157 approved drugs used in cancer treatment and the SCREEN-Well epigenetics library containing 43 epigenetic compounds were purchased from Sequoia Research Products and Enzo Life Sciences, respectively. Other targeted inhibitors were purchased from Selleck Chemicals, whereas the regular used cytostatics were purchased from Sigma-Aldrich. Both compound libraries were reconstituted to a single 384-well plate. AT7519 (multi-CDK inhibitor), IQ1 (BET bromodomain inhibitor), ceritinib (ALK inhibitor), ribociclib (CDK4/6 inhibitor), idasanutlin (MDM2 inhibitor), trametinib (MEK inhibitor), and YM155 (survivin inhibitor) were added to the reformatted library plate because these compounds target known genomic aberrations in neuroblastoma and were in (pre)clinical development as single-agent therapies for neuroblastoma. Venetoclax and navitoclax (BCL-2 inhibitors) were taken along as controls to confirm the resistant cell lines were resistant to BCL-2 inhibition during the drug screen. For in vivo studies, venetoclax obtained from AbbVie was formulated in 10% ethanol/30% propylene glycol (PEG) 400/600% phosal 50 propylene glycol (PG; v/v/v) in final concentrations of 10 mg/mL. Idasanutlin obtained from Roche was formulated in 2% Klucel LF/0.1% Tween 80/0.09% methyl paraben/0.01% propyl paraben (v/v/v/v) in final concentrations of 15 mg/mL.

Cell culture
Human neuroblastoma cell lines KCNR (RRID: CVCL_7134) and SJNB12 (RRID: CVCL_1442) were chosen as the main cell lines used in this study because both cell lines have shown to express high BCL-2 levels in the absence of TP53 mutations and respond well to venetoclax monotherapy (15, 18). Full profiling of both cell lines is available upon request. KCNR and SJNB12 were cultured as previously described (22) and tested every six weeks for Mycoplasma contamination using the MycoAlert detection kit (LT07–318, Lonza) and MycoAlert assay control set (LT07–518, Lonza). Cell culture protocols are described in detail in the Supplementary Materials and Methods.

Generation of venetoclax-resistant neuroblastoma cell lines
Venetoclax-resistant neuroblastoma cell lines were developed through long-term continuous exposure at the IC50 concentration (7.5 μmol/L for KCNR, 2.75 μmol/L for SJNB12). Following twice weekly routine maintenance splits or medium replenishment, cells were allowed to recover in venetoclax-free medium for 24 hours before adding fresh venetoclax. After three months, KCNR and SJNB12 cell lines cultured under venetoclax pressure replicated similarly to non-exposed control cells. Resistance was confirmed through dose–response curves and cell-cycle analysis (see below).

BCL-2 DNA sequence analysis
Genomic DNA from non-resistant KCNR and SJNB12 cells and venetoclax-resistant KCNR and SJNB12 cells under venetoclax pressure (IC50) was extracted using chloroform and isopropanol. Isolated DNA was purified using a QIAamp DNA mini kit (Qiagen) and PCR-amplified with Taq polymerase (Invitrogen). Gene sequence analysis was then performed using the ABI PRISM 3730 sequencer (Applied Biosystems). Primers used for the gene sequence analysis are: Exon 1: F: GTCCAAGAATGCAAAGCACA, R: GAACCGTCTTGTGGAGAG. Exon 2: F: GCAGGATGCC-TCTTTCTCTG, R: AGCCTGACGTTTTGTTCAT.

mRNA expression profiling
RNA was extracted from KCNR and SJNB12 non-resistant cells and venetoclax-resistant cells in the absence or presence of venetoclax using TRIzol (Invitrogen) following the manufacturer’s protocols. Concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridization to Human Genome U133 plus 2.0, microarrays and scanning were carried out according to the manufacturer’s protocol (Affymetrix Inc.). The mRNA gene expression data were normalized with the MA55.0 algorithm of the GCS program (Affymetrix Inc.) and target intensity was set to 100. All data were analyzed using the R2 genomic analysis and visualization platform (http://r2.amc.nl).

High-throughput drug screening
Non-resistant and venetoclax-resistant cell lines were seeded in 384-well plates (8,000 cells/well for KCNR lines, 10,000 cells/well for SJNB12). Resistant cell lines were plated both in the presence or absence of venetoclax (IC50). Cells were allowed to attach overnight before exposure to three different concentrations (10 nmol/L, 100 nmol/L, and 1 μmol/L) of library compounds using the Sciclone ALH 3000 liquid handling robot (Perkin Elmer). As vehicle control, cells were treated with equal volumes of DMSO. Cell viability was determined before and following 72 hours treatment using the MTT colorimetric assay (M2128, Sigma-Aldrich). Viability of vehicle control cells was set to 100%. For each compound in the library, the area under the dose–response curve (AUC) values and the sum of the percentage viable cells (sum %) observed after treatment with 10 nmol/L, 100 nmol/L, and 1 μmol/L were calculated for the non-resistant cells, venetoclax-resistant cells, and venetoclax-resistant cells under venetoclax pressure. Compounds for which the sum % was lower for the venetoclax-resistant cells under venetoclax pressure than for the non-resistant cells were considered resensitizing compounds.

Validation of the resensitizing effects of compounds
Non-resistant and venetoclax-resistant KCNR and SJNB12 neuroblastoma cell lines were seeded in quadruplicate as described above. After overnight incubation, KCNR and SJNB12 non-resistant cells and venetoclax-resistant cells in the absence and presence of venetoclax were treated with either eight concentrations of each compound or vehicle control (DMSO) using the Combi-Bulk Tecan HP D300 digital dispenser (Flewett–Packard).

FACS analysis
To confirm the generation of venetoclax-resistant KCNR and SJNB12 cells, non-resistant and resistant cells were treated with vehicle control (DMSO) or venetoclax using a concentration gradient from 7.5 nmol/L to 10 μmol/L. To study the effects of idasanutlin on sub-G1 induction in SJNB12, non-resistant cells, venetoclax-resistant cells, and venetoclax-resistant cells in the presence of venetoclax (IC50) were treated with DMSO or 15.5 nmol/L to 1 μmol/L idasanutlin. For both FACS experiments, cells were seeded in 6-cm plates and incubated overnight. Subsequently, cells were treated for 72 hours and harvested for FACS analysis to determine the apoptotic sub-G1 fraction. See Supplementary Materials and Methods for a detailed protocol.

In vitro western blotting
The following antibodies were ordered from Cell Signaling Technology (CST) and were used in a 1:1,000 dilution: BCL-2 (D55G8,
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Non-resistant KCNR and SJNB12 cells and venetoclax-resistant cells in the absence and presence of venetoclax were seeded in T175 flasks. For the detection of BIM/BCL-2 and BIM/MCL-1 complexes, cells were harvested suspensions containing 5

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beads (Roche) for 24 hours at 4

antibody followed by adding the solution to protein A- or G-agarose beads (before BCL-2 co-immunoprecipitation) or

15,000 rpm, 4

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recorded twice weekly by caliper measurements (tumor-small side

tumorsmall side

rabbit, #4223), MCL-1 (rabbit, #4572), BCL-XL (54H6, rabbit, #2764), BCL-W (31H4, rabbit, #2724), BIM (C34C5, rabbit, #2933), PARP (rabbit, #9542), BAX (rabbit, #2774), BAK (rabbit, #3814) and PUMA (rabbit, #4976). The β-α-tubulin antibody (DM1A, mouse, #3873) was diluted 1:10,000. Other antibodies used in a 1:1,000 dilution: p21 (rabbit, ab109520, Abcam), p53 (D0–7, mouse, Neomarkers), NOXA (rabbit, ab140129, Abcam), BID (rabbit, ab32060, Abcam), and MDM2 (N-20, rabbit, SC-813, Santa Cruz Biotechnology). Secondary antibodies used in a 1:1,000 dilution: Horseradish peroxidase (HRP)–conjugated goat anti-rabbit (NA9340V) and goat anti-mouse (NA931) antibodies (GE Healthcare). See Supplementary Materials and Methods for a detailed protocol.

In vitro co-immunoprecipitation and immunoblotting

Non-resistant KCNR and SJNB12 cells and venetoclax-resistant cells in the absence and presence of venetoclax were seeded in T175 flasks. For the detection of BIM/BCL-2 and BIM/MCL-1 complexes, cells were harvested using TrypLE express enzyme (12605036, Thermo Fisher Scientific) followed by washing with PBS. For all samples, total cell lysates were prepared in 2% CHAPS buffer [1 mol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 5% (w/v) sodium glycerol phosphate and 2% (w/v) CHAPS]. Samples were allowed to lyse overnight at 4°C, followed by centrifugation for 20 minutes at 15,000 rpm, 4°C to pellet debris. Supernatants were precleared using protein A-agarose beads (before BCL-2 co-immunoprecipitation) or protein G-agarose beads (before MCL-1 co-immunoprecipitation). Equal protein amounts (i.e., 1 mg) were then incubated for 1.5 hours with either a BCL-2 antibody, an MCL-1 antibody or a FlagTag antibody followed by adding the solution to protein A- or G-agarose beads (Roche) for 24 hours at 4°C to immunoprecipitate the desired protein complexes. The following antibodies were used in a 1:140 dilution for immunoprecipitation: BCL-2 (D55G8, rabbit, #4223, CST), MCL-1 (RC13, mouse, #MABC43, Merck Millipore), DYKD DDDDK Tag (rabbit, #2368, CST) or DYKD DDDDK Tag (mouse, #9A3, #8146, CST). Co-immunoprecipitated proteins were isolated by repeatedly spinning down (2 minutes, 8,000 rpm, 4°C) and washing of the sample–antibody–bead complexes. Samples were analyzed by western blot analysis as described above.

In vitro efficacy in classical cell line xenograft and patient-derived xenograft models

Experiments involving cell line-derived xenografts were performed with permission from and according to the standards of the Dutch animal ethics committee (DAG102776, DAG103059, DAG309AA and DAG309AB). As in vivo engraftment of SJNB12 did not lead to tumor formation, BCL-2–dependent neuroblastoma cell lines KCNR and KP–NY were selected for the generation of xenograft mouse models. Single-cell suspensions containing 5 × 10⁶ cells were resuspended in Matrigel/PBS (1:1 ratio) and subcutaneously injected into both flanks of female NMRI nu/nu mice (6–15-weeks-old, 20–30 g) obtained from Charles River Laboratories. Tumor volume was recorded twice weekly by caliper measurements (tumorsmall side × tumorsmall side × tumolarge side × π/6). When the tumors reached approximately 1,000 mm³, tumors were excised, chunked, and serially xenotransplanted in recipient mice. Experimental treatment started when transplanted tumors of recipient mice reached approximately 250 mm³. Mice were randomly divided into four groups: (i) vehicle venetoclax + vehicle idasanutin, (ii) 100 mg/kg/day venetoclax + vehicle idasanutin, (iii) 25 mg/kg/day idasanutin + vehicle venetoclax, (iv) venetoclax combined with idasanutin. For KCNR, a fifth group (i.e., delayed combination group) was included, receiving one-week venetoclax monotherapy followed by two weeks combination treatment. Mice (n = 7 per group for KCNR and n = 6 per group for KP–N–YN) were treated daily for up to three weeks to follow treatment effects on tumor growth and regression.

For the patient-derived xenograft (PDX) models, experiments were performed with permission from the Emory institutional animal care and use committee. Viably frozen tumor pieces of CHOA-NBX-4 and COG-N-424x PDXs were disaggregated to generate a single-cell suspension. Cells (4 × 10⁶) were subsequently resuspended in Matrigel at a 1:2 ratio and subcutaneously injected into the flank of female nu/nu athymic mice (5–6-weeks-old, ~18 g) obtained from The Jackson Laboratory. Tumor volume was recorded three times weekly by caliper measurements (L × W × H/π/6). When tumor volume reached approximately 250 mm³, mice were assigned to one of four treatment arms, as described above for the classical in vivo models. Mice (n = 4–6 for CHOA-NBX-4, n = 4 for COG-N-424x) were treated daily for three weeks to follow treatment effects on tumor growth and regression.

Western blotting on xenograft tumor samples

The following antibodies were used: p21 (rabbit, #2947, CST), p53 (mouse, SC-126, Santa Cruz Biotechnology), MDM2 (rabbit, #86934, CST), BCL-XL (rabbit, #2762, CST), BCL-2 (rabbit, SC-492, Santa Cruz Biotechnology), MCL-1 (rabbit, ADI-AAP-240-F, ENZO), BIM (rabbit, AB17003, Millipore), BAX (rabbit, #2772, CST), PARP (rabbit, #9542, CST), GAPDH (rabbit, #2118, CST), anti-rabbit-HRP (goat, HAF008, R&D Systems), and anti-mouse-HRP (goat, 554002, BD Biosciences). All antibodies were used in a 1:1,000 dilution. BAX detection in immunoblotting: BIM (rabbit, #2933, CST, 1:500), MCL-1 (rabbit, ADI-AAP-240-F, ENZO), BIM (rabbit, AB17003, Millipore), BAX (rabbit, #2772, CST), PARP (rabbit, #9542, CST), GAPDH (rabbit, #2118, CST), anti-rabbit-HRP (goat, HAF008, R&D Systems), and anti-mouse-HRP (goat, 554002, BD Biosciences). All antibodies were used in a 1:1,000 dilution. β-Actin (rabbit, #4970, CST) antibody was used as a loading control and diluted 1:10,000. See Supplementary Materials and Methods for detailed protocols. BAX detection in KCNR xenografts was carried out following the protocol for in vitro western blotting.

Co-immunoprecipitation on xenograft tumor samples

Immunoprecipitation was performed using 5 μg of the following antibodies: MCL-1 (mouse, 559027, BD Biosciences) and BCL-2 (mouse, M088729–2, Dako). The following antibodies were used for immunoblotting: BIM (rabbit, #2933, CST, 1:500), MCL-1 (rabbit, ADI-AAP-240-F, Enzo Life Sciences, 1:1,000), BCL-2 (rabbit, SC-492, Santa Cruz Biotechnology, 1:1,000), anti-rabbit-HRP (goat, HAF008, R&D Systems, 1:1,000) and anti-rabbit-HRP (goat, SC-45040 (discontinued), Santa Cruz Biotechnology, 1:5,000). See Supplementary Materials and Methods for detailed protocols. Detection of BIM/BCL-2 complex levels in KCNR was carried out as described previously in the section in vitro co-immunoprecipitation, with minor modifications due to prior tumor homogenization.

Results

Long-term continuous exposure of BCL-2-dependent neuroblastoma cells to high venetoclax concentrations confirms MCL-1–mediated resistance

To investigate whether neuroblastoma cells with acquired resistance to venetoclax can be sensitized again to the compound, wild-type BCL-2–dependent neuroblastoma cell lines KCNR and SJNB12 were made venetoclax-resistant by continuous exposure to IC₅₀ concentrations of venetoclax (7.5 μmol/L for KCNR and 2.75 μmol/L for SJNB12). Resistance to venetoclax was confirmed by quantifiable shifts in venetoclax dose–response curves (Fig. 1A). IC₅₀ values were 22 and 12 times higher, whereas LC₅₀ values were...
mean expression levels of triplicate samples of genes and their expression profiles in control and resistant cells. Venetoclax effects on cell viability were established after 72 hours using the MTT colorimetric assay. mRNA expression analysis was performed for apoptosis-related genes. Immunoblot analysis was used for protein expression in both control and resistant cells. Western blot analysis was used to determine the levels of anti-apoptotic and pro-apoptotic proteins in resistant cells. Venetoclax effects on cell viability were measured using a one-tailed unpaired Student t-test, with P < 0.05 as the minimal level of significance.

Figure 1 shows the dose-response curves of venetoclax for non-resistant and resistant KCNR and SJNB12 cells. Venetoclax effects on cell viability were established after 72 hours of treatment using the MTT colorimetric assay. FACS analysis of the effects of venetoclax on sub-G1 induction in non-resistant and resistant KCNR and SJNB12 cells showed that venetoclax-resistant (red) KCNR and SJNB12 cells had increased sub-G1 populations compared to non-resistant cells (blue). B, FACS analysis of the effects of venetoclax on sub-G1 induction in non-resistant and resistant KCNR and SJNB12 cells showed that venetoclax-resistant (red) KCNR and SJNB12 cells had increased sub-G1 populations compared to non-resistant cells (blue). Data represent the mean percentage of cells in sub-G1 + SD of three replicate experiments.

C, Affymetrix gene expression profiling of anti-apoptotic BCL-2 family members in non-resistant and resistant KCNR and SJNB12 cells in the presence of venetoclax. mRNA expression levels of triplicate samples + SD. Statistical differences between non-resistant and resistant cells in the presence of venetoclax were calculated using a one-tailed paired Student t-test, with P < 0.05 as the minimal level of significance (indicated as *).

D, Western blot analysis of anti-apoptotic and pro-apoptotic BCL-2 family members in non-resistant cells, venetoclax-resistant cells and venetoclax-resistant cells in the presence of venetoclax. Venetoclax effects on cell viability were measured using a one-tailed unpaired Student t-test, with P < 0.05 as the minimal level of significance.

For KCNR, staurosporine was included as a positive control. Data represent the mean percentage of cells in sub-G1 + SD of three replicate experiments.

191 and 51 times higher for resistant versus non-resistant KCNR and SJNB12 cell lines, respectively (Supplementary Table S1). Furthermore, micromolar concentrations of venetoclax (i.e., ≥5 μmol/L) were required to induce an apoptotic response in venetoclax-resistant SJNB12 cells, whereas non-resistant control cells required nanomolar equivalents (i.e., 7.5 nmol/L) to induce a ≥2-fold increase in the sub-G1 population. Venetoclax exposure at the highest concentration tested (10 μmol/L) was insufficient to induce sub-G1 arrest in venetoclax-resistant KCNR cells (Fig. 1B).

Previously, we have shown that MCL-1 plays a pivotal role in venetoclax resistance. Therefore, validation experiments were performed to verify MCL-1–mediated resistance within this study (18). DNA sequence analysis of venetoclax-resistant cells did not identify mutations in the BH3 domain of BCL-2. Gene expression profiling of non-resistant and venetoclax-resistant KCNR and
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SJNB12 cells under venetoclax pressure showed significantly increased MCL-1 mRNA expression in the resistant cell lines (Fig. 1C). This coincided with elevated levels of MCL-1 protein in both models (Fig. 1D). Surprisingly, BCL-2 gene and protein levels were not consistently downregulated. Lower BCL-2 gene expression was observed for resistant SJNB12 cells (Fig. 1C; Supplementary Fig. S1), whereas reduced BCL-2 protein levels were seen for resistant KCNR cells (Fig. 1D). Similar analyses revealed no significant changes in gene and protein levels of the anti-apoptotic BCL-2 family members BCL-XL and BCL-W (Fig. 1C and D). In addition, protein levels of pro-apoptotic BCL-2 family members largely remained unchanged between non-resistant and resistant cell lines, except for BIM and PUMA. Lower levels of BIM were observed in resistant KCNR cells and PUMA was down-regulated in both resistant cell lines under venetoclax pressure (Fig. 1D). BCL-2 immunoprecipitation followed by immunoblotting for BIM, confirmed displacement of BIM from BCL-2 in resistant KCNR and SJNB12 cells with and without venetoclax pressure (Fig. 1E). In line with our previously published observations upon short-term treatment of neuroblastoma cells with venetoclax (18), analysis of BIM/MCL-1 complex levels revealed that BIM released from BCL-2 was sequestrated by MCL-1 in the resistant cells (Fig. 1E). Together, these results support that MCL-1 plays an important role in the acquired resistance induced by long-term exposure of BCL-2-dependent neuroblastoma cells to venetoclax.

High-throughput screening identifies idasanutlin as a resensitizing agent for venetoclax-resistant neuroblastoma cells

To identify compounds that resensitize venetoclax-resistant neuroblastoma cells to venetoclax, we performed a high-throughput drug screen for non-resistant KCNR and SJNB12 cells, venetoclax-resistant KCNR and SJNB12 cells and venetoclax-resistant KCNR and SJNB12 cells in the presence of venetoclax. Cells were exposed for 72 hours to a compound library, containing three concentrations (10 nmol/L, 100 nmol/L and 1 μmol/L) of 157 approved drugs used in cancer treatment, 43 epigenetic regulators and 9 targeted compounds that are currently in (pre)clinical development for neuroblastoma treatment. Compounds that were more effective in the resistant cell lines under venetoclax pressure than in the non-resistant cell lines were considered resensitizing drugs (Fig. 2A).

Hit selection was performed by taking the top 40 resensitizing compounds and subsequently excluding all non-targeted compounds (except cytostatics regularly used in neuroblastoma treatment) and targeted compounds for which the percentage viable cells after treatment with the highest tested concentration (1 μmol/L) was still ≥20%. This yielded 11 and 13 hits for venetoclax-resistant KCNR and SJNB12, respectively (Fig. 2B).

Interestingly, both hit lists contained different inhibitors of the IGFR1/P38K/MtOR axis, including omipalisib (P38K/mTOR inhibitor; KCNR and SJNB12), GSK-1904529A (IGF-1R inhibitor; KCNR), AZD-8055 (mTOR inhibitor; SJNB12), and NVP-BEZ235 (P38K/mTOR inhibitor; SJNB12). In addition, two ALK inhibitors (i.e., ceritinib and NVP-TAE684) were identified as highly ranked resensitizing agents in the F1174 L ALK-mutated model system KCNR. As expected, these ALK inhibitors were not observed as hits in the ALK wild-type cell line SJNB12.

Besides omipalisib, idasanutlin (MDM2 inhibitor), flavopiridol (CDK inhibitor), and vorinostat (HDAC inhibitor) were the only overlapping targeted drugs between the two hit lists. For idasanutlin, omipalisib, and flavopiridol, the increase in efficacy between venetoclax-resistant and non-resistant cells was larger in the presence of venetoclax, confirming the occurrence of resensitization in addition to or instead of overcoming resistance. Overall, the best performing resensitizing compound idasanutlin (23) was selected for further validation in vitro and in vivo, as being the eighth and first hit for venetoclax-resistant KCNR and SJNB12 cells, respectively.

In vitro validation confirms the resensitizing potential of idasanutlin

The top resensitizing hit idasanutlin was tested more extensively in vitro, along with other resensitizing compounds, to validate the screening results using a low-throughput methodology. Targeted compounds for which synergy with BCL-2 inhibition has been previously described (20, 24–31) and regularly used cytostatics for neuroblastoma treatment (Supplementary Table S2) were also tested. For all compounds, IC50 values in non-resistant KCNR and SJNB12 cells and in venetoclax-resistant KCNR and SJNB12 cells in the absence and presence of venetoclax were determined from dose-response curves after 72 hours treatment (Supplementary Table S3).

Figure 3A and B shows the top 15 resensitizing compounds for KCNR and SJNB12, determined by the largest fold decrease in IC50 value between resistant cells under venetoclax pressure and non-resistant cells. Results confirm the resensitizing potential of idasanutlin, with a 1.5-fold decrease in IC50 value for KCNR (i.e., 30 vs. 20 nmol/L) and an almost 20-fold decrease in IC50 value for SJNB12 (i.e., 1,011 vs. 52 nmol/L; Fig. 3C). For both neuroblastoma cell lines, only limited differences were observed in idasanutlin IC50 values between the venetoclax-resistant cells in the absence of venetoclax and the non-resistant cells. This supports the hypothesis that idasanutlin resensitizes venetoclax-resistant neuroblastoma cells to venetoclax, rather than overcoming venetoclax resistance. In line with the screening results, omipalisib and vorinostat were also found in the top 15 list of most potent resensitizing drugs for venetoclax-resistant KCNR and SJNB12 cells.

Idasanutlin induces growth arrest in non-resistant cells and apoptosis in venetoclax-resistant cells in the presence of venetoclax

Idasanutlin acts by inhibiting the interaction between MDM2 and the tumor-suppressor p53, thereby preventing p53 degradation (32, 33). For this reason, we first studied the effects of idasanutlin on p53 and MDM2 proteins levels. As expected, idasanutlin dose-dependently increased p53 and MDM2 in the non-resistant cells as well as the venetoclax-resistant cells in the absence and presence of venetoclax for both KCNR and SJNB12 (Fig. 4A). Because p53 is involved in both cell-cycle arrest and apoptosis, we also studied the effects of idasanutlin on p53 target genes p21 and BAX (34–36). Interestingly, in the non-resistant cells and venetoclax-resistant cells in the absence of venetoclax a stronger p21 upregulation was observed, whereas in the venetoclax-resistant cells in the presence of venetoclax a stronger BAX upregulation was observed upon idasanutlin treatment. These findings correspond with apoptosis induction in the venetoclax-resistant lines under venetoclax pressure demonstrated by the observed PARP cleavage (Fig. 4A) and higher sub-G1 fraction after cell-cycle analysis (Fig. 4B; Supplementary Fig. S2). Taken together, in vitro results indicate that treating BCL-2-dependent neuroblastoma cells with a combination of venetoclax and idasanutlin is more effective in inducing an apoptotic response than either of the compounds alone.
Venetoclax and idasanutlin combination therapy causes tumor regression in a BCL-2–dependent neuroblastoma xenograft model

The additional value of combining idasanutlin with venetoclax therapy was subsequently validated in vivo using BCL-2–dependent p53 wild-type KCNR xenografts. Mice were treated for three weeks with daily oral doses of 100 mg/kg venetoclax and/or 25 mg/kg idasanutlin. Both single-agent therapies and combination therapy with venetoclax and idasanutlin significantly inhibited tumor growth, with average changes in tumor volume of +78% (venetoclax), +186% (idasanutlin), and −80% (venetoclax + idasanutlin) versus +512% for the vehicle control (Fig. 5A; Supplementary Fig. S3B). Combination therapy demonstrated superior efficacy over either of the drugs alone, with 1/6 complete remission (CR), 2/6 very good partial responses (VGPR), and 3/6 partial regressions (PR) versus no response (NR in 1/6 and 3/7 mice, respectively) or progressive disease (PD in 5/6 and 4/7 mice, respectively) for venetoclax and idasanutlin mono-therapy. Mice receiving one-week venetoclax monotherapy followed by two weeks combination therapy showed slightly less favorable responses compared with immediate addition of both compounds, with one VGPR, three PRs and three NRs. However, comparing immediate combination therapy with delayed combination did...
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**Figure 3.** In vitro validation studies confirm resensitization of venetoclax-resistant neuroblastoma cells to venetoclax by idasanutlin treatment. For combined hits from the high-throughput drug screen as well as targeted compounds for which synergy with BCL-2 inhibition has been described in literature and the regularly used cytostatics in neuroblastoma treatment, dose-response curves were established in non-resistant KCNR and SJNB12 cells and venetoclax-resistant KCNR and SJNB12 cells in the absence and presence of venetoclax to establish IC50 values. A and B, Fold difference in IC50 values between non-resistant cells and either venetoclax-resistant cells (green bars) or venetoclax-resistant cells under venetoclax pressure (red bars). Results are shown for the top 15 compounds showing the largest fold decrease in IC50 value between the non-resistant cells and the resistant cells in the presence of venetoclax for KCNR (A) and SJNB12 (B), respectively. C, Dose-response curves of the MDM2 inhibitor idasanutlin in non-resistant KCNR and SJNB12 cells (blue) and venetoclax-resistant KCNR and SJNB12 cells in the absence (green) or presence (red) of venetoclax. +V = venetoclax IC85 concentration (KCNR, 7.5 µmol/L and SJNB12, 2.75 µmol/L).

not lead to significant changes in the average tumor volume. Co-immunoprecipitation studies on tumor material harvested 4 hours after administration of the last doses, showed complete BIM release from BCL-2 in mice that received venetoclax alone or the immediate combination with idasanutlin (Fig. 5B). Interestingly, residual BIM/BCL-2 complex levels were observed in mice that received delayed combination therapy, which might explain the slightly less favorable response observed in this group (Fig. 5B). Additional western blot analysis revealed increased BAX levels in idasanutlin-treated tumors and an even more pronounced BAX upregulation in both combination arms (Supplementary Fig. S3D). Together, these results indicate that the observed tumor regression upon combination therapy might be the result of a dual effect on the intrinsic apoptotic pathway: BIM release from BCL-2 caused by venetoclax and p53-mediated activation of the pro-apoptotic BCL-2 family member BAX caused by idasanutlin.

Effects of venetoclax–idasanutlin combination therapy were also tested in mice with high BCL-2–expressing p53 wild-type KP–N–YN cell line–derived xenografts. Although average changes in tumor volume after three weeks treatment with venetoclax, idasanutlin or combination therapy (i.e., +119%, +138%, and +97%, respectively) were lower compared with the vehicle control (+236%), differences were not statistically significant (Fig. 5A; Supplementary Fig. S3B). Co-immunoprecipitation studies showed less pronounced BIM release from BCL-2 after venetoclax monotherapy or combination treatment compared with KCNR xenografts (Fig. 5B). Additional analysis of BIM/MCL-1 complex levels furthermore showed that BIM was already bound to MCL-1 in untreated xenografts and complex levels even raised upon combination treatment (Supplementary Fig. S3E). This indicates that KP–N–YN neuroblastoma xenografts are not solely BCL-2-dependent.
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Venetoclax and idasanutlin combination therapy causes tumor regression in a patient-derived BCL-2-dependent neuroblastoma xenograft model

As PDX models have shown to better recapitulate the biological and molecular characteristics of the original patient tumor (37), the effects of venetoclax–idasanutlin combination treatment were tested against a BCL-2–dependent PDX, CHOA-NBX-4. Single-agent treatment with venetoclax or idasanutlin significantly inhibited the growth of CHOA-NBX-4 tumors after 10 days of treatment, with average changes in tumor volume of +232% and +96%, respectively, versus +597% for vehicle control mice (Fig. 6A; Supplementary Fig. S4B). Although the p53 status of CHOA-NBX-4 is still under investigation, its sensitivity toward idasanutlin indicates that this model is p53 wild-type. In line with the classical xenograft model KCNR, combination treatment of CHOA-NBX-4 PDXs was superior to single-agent treatment with an average percentage change in tumor volume of −72% (Supplementary Fig. S4B). 10 days of combination therapy resulted in five PRs (5/6), whereas the best response for both monotherapies was one case of stable disease (1/5 for venetoclax and 1/4 for idasanutlin; Supplementary Fig. S4C). BIM/BCL-2–binding patterns were similar to the patterns observed in classical KCNR xenografts, showing BIM release following addition of venetoclax as a single-agent therapy, as well as, in combination with idasanutlin (Fig. 6B). Interestingly, CHOA-NBX-4 tumors treated with venetoclax monotherapy were not affected by MCL-1–mediated resistance, as demonstrated by the lack of BIM/MCL-1 complex levels in any of the treatment arms (Fig. 6B). In line with in vitro observations, idasanutlin increased p53 and MDM2 levels when given alone and in combination with venetoclax (Fig. 6C). Increased p53 levels were accompanied by upregulated levels of BAX, a known activation target of p53. BIM release upon venetoclax binding to BCL-2 and elevated levels of p53 and BAX upon idasanutlin binding to MDM2 explain the strong apoptotic response confirmed upon combination therapy by increased cleaved PARP levels (Fig. 6B and C).

Effects of venetoclax–idasanutlin combination therapy were additionally studied in a second BCL-2–dependent p53 wild-type PDX model, COG-N-424x. Unfortunately, none of the treatment strategies
Idasanutlin monotherapy (25 mg/kg/day) or venetoclax in combination with idasanutlin for three consecutive weeks. For the KCNR xenograft model, an extra volume in the KCNR (left) and KP-N-YN (right) neuroblastoma xenograft model. Mice were treated with either vehicle, venetoclax monotherapy (100 mg/kg/day), idasanutlin monotherapy (25 mg/kg/day) or venetoclax in combination with idasanutlin for three consecutive weeks. For the KCNR xenograft model, an extra group was included, in which mice first received one-week venetoclax monotherapy followed by two weeks combination treatment (delayed combination). The percentage change in tumor volume was calculated using the following formula: 

\[ \text{percentage change in tumor volume} = \left( \frac{\text{tumor volume at the end of treatment} - \text{tumor volume at the start of treatment}}{\text{tumor volume at the start of treatment}} \right) \times 100\% \]

**Figure 5.**

In vivo effects of venetoclax in combination with idasanutlin in classical neuroblastoma xenograft mouse models. A, Waterfall plots of the percentage change in tumor volume in the KCNR (left) and KP-N-YN (right) neuroblastoma xenograft model. Mice were treated with either vehicle, venetoclax monotherapy (100 mg/kg/day), idasanutlin monotherapy (25 mg/kg/day) or venetoclax in combination with idasanutlin for three consecutive weeks. For the KCNR xenograft model, an extra group was included, in which mice first received one-week venetoclax monotherapy followed by two weeks combination treatment (delayed combination). The percentage change in tumor volume was calculated using the following formula: 

\[ \text{percentage change in tumor volume} = \left( \frac{\text{tumor volume at the end of treatment} - \text{tumor volume at the start of treatment}}{\text{tumor volume at the start of treatment}} \right) \times 100\% \]

B, In vivo effects on BIM release from BCL-2 for the KCNR (left) and KP-N-YN (right) neuroblastoma xenograft model. BIM/BCL-2 complex levels were detected by anti-BCL-2 immunoprecipitation (IP), followed by western blotting for BIM. BIM/BCL-2 complex levels were established for n = 2 mice per group after treatment completion (KCNR) or after 7 days of treatment (KP-N-YN), using tumor material harvested 4 hours after administration of the last dose. WCL, whole-cell lysate.

Discussion

Preclinical studies using the BCL-2–specific inhibitor venetoclax demonstrate antitumor activity in neuroblastoma tumors with high venetoclax monotherapy, idasanutlin monotherapy or combination therapy) led to statistically significant differences in the average change in tumor volume (+193%, +185%, and +178%, respectively) compared with the vehicle-treated mice (+216%; Fig. 6A; Supplementary Fig. S4B). Because COG-N-424x mice did not complete the 21-day treatment cycle due to tumor burden, group comparison was performed after a suboptimal period of only four days of treatment to have sufficient mice available in each treatment arm. Additional waterfall plots with tumor volumes at the last day of treatment confirm no responses to treatment (Supplementary Fig. S4D). Despite the lack of significant effects on COG-N-424 tumor sizes, some of the observed biochemical effects were surprisingly similar to responding tumors. Combination therapy led to enhanced PARP cleavage, and both p53 and MDM2 were upregulated after idasanutlin monotherapy and combination therapy (Fig. 6C). In addition, BIM/BCL-2 co-immunoprecipitation studies demonstrated BIM displacement after venetoclax monotherapy and combination therapy. In line with tumors non-responsive to venetoclax therapy, overall levels of BIM bound to BCL-2 were very low (Fig. 6B; Supplementary Fig. S5B). In addition, BIM was not bound to MCL-1 in vehicle-treated and monotherapy-treated mice, but high BIM/MCL-1 complex levels were observed after combination therapy (Fig. 6B).

BCL-2 and BIM/BCL-2 complex levels (18, 38). However, acquired resistance develops following venetoclax therapy, as is commonly seen with highly specific targeted compounds, which ultimately limits its usefulness as a single-agent treatment strategy (18, 39).

We previously established that acquired venetoclax resistance in neuroblastoma cells coincides with upregulation of MCL-1 and BIM relocation to MCL-1 (18). Similar effects were confirmed for the venetoclax-resistant neuroblastoma cell lines in this study. Upregulation of other anti-apoptotic BCL-2 family members was shown to play a pivotal role in neuroblastoma resistance to venetoclax. This was further supported by the absence of mutations in the BH3 domain of BCL-2. The development of MCL-1–mediated resistance to venetoclax suggests that a combination with MCL-1 inhibitors will enhance therapeutic effectiveness. Indeed, preliminary studies have revealed promising apoptotic effects upon simultaneous inhibition of BCL-2 and MCL-1 in BCL-2-dependent neuroblastoma models (18). However, MCL-1 inhibitors are still in early clinical development, limiting rapid translation of the combined strategy into the clinic. Considering the promise of venetoclax, which is already under clinical investigation (NCT03236857), we aimed to discover alternative combinatorial strategies for fast clinical implementation. To this end, we performed a screen with multiple approved drugs and promising targeted drugs in clinical or late preclinical development to identify compounds that resensitize neuroblastoma cells with acquired resistance to venetoclax.

Interestingly, we found numerous compounds targeting the IGF-1R/P13K/mTOR axis in the drug screen hits, indicating potential for combining venetoclax with an inhibitor of this drug class. These compounds demonstrate antitumor activity in neuroblastoma tumors with high venetoclax monotherapy, idasanutlin monotherapy or combination therapy) led to statistically significant differences in the average change in tumor volume (+193%, +185%, and +178%, respectively) compared with the vehicle-treated mice (+216%; Fig. 6A; Supplementary Fig. S4B). Because COG-N-424x mice did not complete the 21-day treatment cycle due to tumor burden, group comparison was performed after a suboptimal period of only four days of treatment to have sufficient mice available in each treatment arm. Additional waterfall plots with tumor volumes at the last day of treatment confirm no responses to treatment (Supplementary Fig. S4D). Despite the lack of significant effects on COG-N-424 tumor sizes, some of the observed biochemical effects were surprisingly similar to responding tumors. Combination therapy led to enhanced PARP cleavage, and both p53 and MDM2 were upregulated after idasanutlin monotherapy and combination therapy (Fig. 6C). In addition, BIM/BCL-2 co-immunoprecipitation studies demonstrated BIM displacement after venetoclax monotherapy and combination therapy. In line with tumors non-responsive to venetoclax therapy, overall levels of BIM bound to BCL-2 were very low (Fig. 6B; Supplementary Fig. S5B). In addition, BIM was not bound to MCL-1 in vehicle-treated and monotherapy-treated mice, but high BIM/MCL-1 complex levels were observed after combination therapy (Fig. 6B).
Figure 6. In vivo effects of venetoclax in combination with idasanutlin in neuroblastoma patient-derived xenograft (PDX) mouse models. A, Waterfall plots of the percentage change in tumor volume in the CHOA-NBX-4 (left) and COG-N-424x (right) PDX model. Mice were treated with either vehicle, venetoclax monotherapy (100 mg/kg/day), idasanutlin monotherapy (25 mg/kg/day) or venetoclax in combination with idasanutlin. The percentage change in tumor volume was calculated using the following formula: [(tumor volume at the end of treatment − tumor volume at the start of treatment)/tumor volume at the start of treatment] × 100%. Waterfall plots were established using tumor volumes obtained 10 days after treatment initiation (CHOA-NBX-4) or 4 days after treatment initiation (COG-N-424x). B, In vivo effects on BIM release from BCL-2 and BIM capturing by MCL-1 for the CHOA-NBX-4 (left, day 3) and COG-N-424x (right, day 7) PDX model. BIM/BCL-2 and BIM/MCL-1 complex levels were established by immunoprecipitation of BCL-2 or MCL-1, respectively, followed by western blotting for BIM. C, Western blot analysis of the in vivo effects of venetoclax and idasanutlin alone or in combination on p21, p53, MDM2, BCL-XL, BCL-2, MCL-1, BIM, BAX and cleaved PARP in the CHOA-NBX-4 (left, day 3) and COG-N-424x (right, day 7) PDX model at 4 hours after drug administration. GAPDH was used as a loading control. WCL, whole-cell lysate.

combinations have indeed been described before to result in synergistic or additive effects on cell death in various types of cancers, including chronic lymphocytic leukemia, non-Hodgkin lymphoma, multiple myeloma, and acute myelogenous leukemia (40). However, these favorable combined effects still have to be confirmed in neuroblastoma. Our drug screen furthermore revealed that ALK inhibitors could restore the sensitivity to venetoclax in resistant neuroblastoma cells with an ALK-mutated background. Because ALK mutations are frequently detected in patients with neuroblastoma, our data provide further support for combining venetoclax with an ALK inhibitor (41, 42). Despite these interesting findings, we decided to focus on drugs resensitizing both venetoclax-resistant neuroblastoma model systems, to ensure that a larger patient population might benefit from treatment with the identified drug combination. Targeted compounds idasanutlin, omipalisib, flavopiridol, vorinostat, and the cytoplastic doxorubicin demonstrated superior antitumor activity in venetoclax-resistant KCNR and SJNB12 cells compared with their non-resistant ancestor cell lines, thereby confirming previously reported favorable combinations with BCL-2 inhibitors (20, 25–31). Further validation of number one hit idasanutlin confirmed its potential in restoring sensitivity of neuroblastoma cells to venetoclax. On the basis of the differences in idasanutlin dose–response curves and IC50 values for resistant neuroblastoma cell lines in the absence versus presence of venetoclax, resensitizing effects of idasanutlin seem to be much more pronounced for SJNB12 compared with KCNR. However, western blot analysis showed that idasanutlin monotherapy led to cell-cycle arrest in venetoclax-resistant cells in the absence of venetoclax, while causing a strong apoptotic response for both venetoclax-resistant KCNR and SJNB12 cells in the presence of venetoclax. These results indicate that resensitization is also occurring in KCNR, despite the limited differences observed in the percentage viable cells. However, our in vitro validation studies did show a surprising difference in the mechanism of action for idasanutlin between KCNR and SJNB12 cells. Although causing MCL-1 downregulation in normal KCNR cells and
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Venetoclax-resistant KCNR cells in the absence and presence of venetoclax, idasanutlin treatment of SJNB12 only resulted in a modest decrease in MCL-1 expression in the non-resistant cells with no effects in both venetoclax-resistant conditions. These inconsistent effects of idasanutlin on MCL-1 protein levels are in line with previously published observations. Although Lehmann and colleagues (24) and Pan and colleagues (43) reported that idasanutlin promotes MCL-1 degradation, no effects of idasanutlin on MCL-1 were observed by Van Goethem and colleagues (44). Differences in response to idasanutlin treatment might be due to variations in the genetic background of the tested model systems and/or more complex interactions between multiple involved pathways. All model systems used in the current study are p53 wild-type, which is crucial for the anticancer activity of idasanutlin. In addition, KCNR is a MYCN-amplified neuroblastoma cell line harboring an activating mutation in ALK, whereas SJNB12 is MYCN and ALK wild-type. Both MYCN and ALK are well-known oncogene drivers in neuroblastoma.

Superior apoptotic responses upon venetoclax–idasanutlin combination therapy were confirmed in vivo. These findings are supported by a study of Van Goethem and colleagues (44) in which venetoclax was identified as a synergistic interaction partner of idasanutlin. Our study revealed that antitumor effects observed after venetoclax–idasanutlin combination therapy were caused by a dual effect on the intrinsic apoptotic pathway: BIM release from BCL-2 caused by venetoclax and p53-mediated BAX activation by idasanutlin. This combination of apoptotic effects led to tumor regression, including one CR (Supplementary Figs. S3B, S3C, S4B, and S4C) and increased survival chances (Supplementary Figs. S3A and S4A) in two neuroblastoma xenograft models. Longer studies investigating repeated cycles of venetoclax–idasanutlin combination therapy are required to estimate whether combination therapy with two targeted compounds is sufficient to achieve sustained tumor regression (Supplementary Figs. S3C and S4C).

Combination therapy with venetoclax and idasanutlin was not effective in two other neuroblastoma xenograft models tested. This may be explained by their lower BCL-2 dependency and the occurrence of MCL-1-mediated resistance. KP–N–YN is a BCL-2-dependent cell line (Supplementary Fig. S6), but the xenograft model generated from these cells showed unexpected variation in baseline levels of BCL-2 and BIM/BCL-2 complex (Fig. 5B; Supplementary Fig. S3A). Similarly, only limited BIM/BCL-2 complex levels could be detected in COG-N-424x PDXs, despite the observation that BIM was only bound to BCL-2 and not to the anti-apoptotic proteins MCL-1 or BCL-XL (Fig. 6B; Supplementary Fig. S5B and S5C). Previously, we and others found that high BCL-2- and BIM/BCL-2 complex levels are biomarkers of efficacy for venetoclax therapy (18, 38). Observed variable or low levels of BCL-2 in KP–N–YN and COG-N-424x xenografts might therefore be one explanation for their lack of response to venetoclax–idasanutlin combination therapy. Baseline BIM/MCL-1 complex levels in KP–N–YN xenografts and massive increases in BIM/MCL-1 complex levels observed in both non-responsive xenograft models upon combination therapy with venetoclax and idasanutlin might be another explanation (Fig. 6B; Supplementary Fig. S3E). Finally, as shortly addressed above, the in vitro observations that idasanutlin led to variable effects on MCL-1 and induced apoptosis in venetoclax-resistant cells kept under venetoclax pressure while only causing cell-cycle arrest when venetoclax was removed, suggest the involvement of more complex interactions that might explain the differences in therapy response.

In conclusion, our results indicate that idasanutlin is a potential drug candidate for resensitizing venetoclax-resistant neuroblastoma cells to venetoclax. This preclinical data provide further rationale for the ongoing clinical trial testing the combination of venetoclax and idasanutlin in pediatric cancers, including neuroblastoma (NCT04029688). On the basis of our data, we predict that a subset of BCL-2–dependent patients with neuroblastoma will benefit from a combination of venetoclax and idasanutlin. However, the current study also underlines the importance of finding relevant biomarkers of efficacy for this combination to prevent unnecessary clinical failures and offer potential non-responders better alternatives.

Authors’ Disclosures

D. Lelieveld reports other from Gemtab outside the submitted work. D.A. Egan reports other from Core Life Analytics outside the submitted work. M. Kerstjens reports grants from Stichting KiKa during the conduct of the study; personal fees from Janssen Vaccines Leiden outside the submitted work. No disclosures were reported by the other authors.

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L.T. Bate-Eye: Formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing.
I.K. Allos: Formal analysis, validation, investigation, methodology;
J.Y. Lee: Formal analysis, validation, investigation, visualization, writing—original draft.
B. Koopmans: Validation, investigation, methodology.
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K.C. Goldsmith: Conceptualization, resources, methodology, writing—review and editing.
J.J. Molenaar: Conceptualization, supervision, funding acquisition, methodology, writing—review and editing.
M.E.M. Dolman: Conceptualization, formal analysis, supervision, funding acquisition, methodology, writing—original draft, project administration, writing—review and editing.

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