Navitoclax combined with Alpelisib effectively inhibits Merkel cell carcinoma cell growth *in vitro*

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Abstract

Background: Merkel cell carcinoma (MCC) is a highly malignant skin cancer. Despite major treatment improvements during the last decade, up to 50% of patients do not respond to therapy or develop recurrent disease. For these patients, alternative treatment options are urgently needed. Here, we assessed the efficacy of the combination of the BCL-2 inhibitor Navitoclax and the PI3K p110α inhibitor Alpelisib in MCC cell lines.

Methods: The expression of BCL-2 was assessed by immunohistochemistry in MCC and MCC cell lines. Treatment with Navitoclax and Alpelisib alone and in combination was performed on four MCC cell lines. The decrease of cell viability during treatment was assessed by XTT assay and visualized for the combinations by 3D combinatorial index plotting. The increase of apoptotic cells was determined by cleaved PARP Western blotting and Annexin V staining.

Results: Some 94% of MCCs and all three MCPyV-positive cell lines showed BCL-2 expression. Navitoclax monotreatment was shown to be highly effective when treating BCL-2-positive cell lines (IC_{50}-values ranging from 96.0 to 323.0 nM). The combination of Alpelisib and Navitoclax resulted in even stronger synergistic and prolonged inhibitions of MCC cell viability through apoptosis up to 4 days.

Discussion: Our results show that the anti-apoptotic BCL-2 is frequently expressed in MCC and MCC cell lines. Inhibition of BCL-2 by Navitoclax in combination with Alpelisib revealed a strong synergy and prolonged inhibition of MCC cell viability and induction of apoptosis. The combination of Navitoclax and Alpelisib is a novel potential treatment option for MCC patients.

Keywords: apoptosis, BCL-2 inhibitor, Merkel cell carcinoma, Merkel cell polyomavirus, PI3K inhibitor

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Introduction

Merkel cell carcinoma (MCC) is a rare and aggressive skin cancer of the elderly. Some 80% of MCCs are associated with the Merkel cell polyomavirus (MCPyV) and the remaining tumours with UV-induced mutations.[1,2] For a long time, they have been treated by chemotherapeutic regimens including the DNA intercalating drug Doxorubicin (an anthracycline) and the anti-mitotic cytostatic Vincristine (vinca alkaloid). These treatments showed objective response rates (ORRs) ranging from 20 to 61%[3,4] with promising initial responses, but median overall survival rates of approximately 9–9.5 months.[5] Chemotherapeutics also induced adverse events, for example, fatigue, vomiting, mucositis, and in 5–7% of MCC patients, serious events such as neutropenia and sepsis[5] (reviewed by Villani *et al.* [6]).

Recently, immune check point inhibitors (CPIs) targeting the programmed cell death protein ligand 1 (PD-L1) or its receptor PD-1 have been

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introduced in the first-line treatment of patients with MCC.[7–10] It has been assumed that the MCPyV small and large T-antigen (T-Ag) oncoproteins as well as the ultraviolet-induced neoantigens in these tumours elicit immune responses, which may induce PD-L1 expression.[2,7–9] In more than 50% of MCC tissues at least 1–5% of the tumour cells were positive for PD-L1 expression.[10–12] The CPI treatment leads to ORRs of 50–70% in MCC patients. Again, adverse events are frequently reported, such as infusion-related reaction or fatigue.[13,14] The remaining 30–50% of patients with MCC do not respond to CPIs,[13] and alternative treatments thus are urgently required for this group of patients.

Several promising alternative treatments have recently been reported. For example, it is known that the PI3K pathway is activated in 66–88% of MCCs[15,16] and we have recently shown that the phosphatidylinositol-3-kinase (PI3K) inhibitor Alpelisib (Byl719) is a potent inhibitor of MCC cell line growth *in vitro*.[17] Although no data are available on MCC patients, Alpelisib in combination with the synthetic estrogen steroid Fulvestrant has demonstrated efficacy in the treatment of hormone receptor-positive and human epidermal growth factor receptor-2-negative metastatic breast cancer.[18] Known adverse events of Alpelisib comprise hyperglycaemia, diarrhoea and nausea.[19,20]

The B-cell lymphoma 2 (BCL-2) inhibitor Navitoclax offers an additional promising treatment option. BCL-2 expression has been detected in approximately 80% of MCC, irrespective of the presence of MCPyV.[21] Verhaegen and colleagues reported that a MCC mouse xenograft expressing BCL-2 showed a growth arrest after daily treatment with this compound for 18 days.[22] Also, 10 BCL-2 positive University of Michigan (UM)-MCC cell lines, including MCPyV-positive and -negative, showed sensitivity for this inhibitor.[22] Although no data are available for MCC patients, Navitoclax treatment in patients with chronic lymphocytic leukaemia (CLL) in a phase I trial showed an ORR of 35%.[23] Adverse events included dosage-dependent thrombocytopenia.[24] Combined with Rituximab an improved ORR of 55% was observed in CLL patients in phase II studies, and this treatment was well tolerated.[25,26] Thus, combination treatments with Navitoclax may increase outcome without compromising adverse events.

Several studies have shown effective dual inhibition of BCL-2 and PI3K p110 α and δ pathways and induction of apoptosis in different tumour cells *in vitro*, that is, human myeloid leukaemia (HML), glioblastoma as well as small-cell lung cancer (SCLC).[27–29] A combined inhibition of BCL-2 and of the active PI3K pathway in MCC thus may also improve the treatment outcome of MCC. In this study, we first analysed the expression of BCL-2 in 47 MCCs, five MCC cell lines and one B-cell acute lymphoblastic (B-ALL) cell line. Subsequently, the efficacy of the Navitoclax monotreatment and combinations with the PI3K inhibitor Alpelisib were investigated.

Materials and methods

Patient samples
The MCC tissues were obtained from the Laboratory of Translational Cell and Tissue Research, University of Leuven, Belgium, ($n = 16$) and the Department of Pathology, Maastricht University Medical Centre+ (MUMC+), the Netherlands, ($n = 37$) (Table 1). These tissues are part of the 'Study on the cellular origin of Merkel cell carcinoma – towards innovative treatment', which is validated by the Clinical Trial Centre of the University Hospital Leuven under the reference number 67603 and Local MUMC+, MPTC protocol number: 2013-11. The use of all patient material and patient data was in agreement with the Dutch Code of Conduct for Observational Research with Personal Data (2011) and Tissue (2011, https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf). The MCC tissues from the University of Leuven are here referred to as the Leuven cohort and the tissues from MUMC+ as the Maastricht cohort. Of all tissue specimens of the Leuven cohort, formalin-fixed and paraffin-embedded (FFPE) as well as fresh frozen tissues were available. MCCs were previously diagnosed by histology and immunohistochemistry (CK20, CD56, synaptophysin and chromogranin A) in a routine diagnostic setting and have been reviewed by three experienced pathologists (JVO, VW, AZH).

Cell lines
The MCPyV-positive MCC cell lines MKL-1, MKL-2, WaGa, PeTa; the MCPyV-negative MCC cell line MCC13 and the B-ALL cell line REH were used. All MCC cell lines were kindly provided

Table 1. Summary of clinicopathological data of patients with Merkel cell carcinoma (MCC) as well as immunohistochemistry for MCPyV, BCL-2.

Merkel cell carcinoma clinicopathological data				Immunohistochemistry	
ID	Gender	Primary or metastasis	Tumour localization	MCPyV	BCL-2
L-MCC2	M	met.	para-aortic lymph node	+	+++
L-MCC5	M	prim.	lower leg	+	+
L-MCC6	F	met.	inguinal lymph node	+	+
L-MCC7	M	prim.	face	+	++
L-MCC8	M	met.	upper leg	+	+++
L-MCC10	F	met.	subcutis	(+)	++
L-MCC12	F	met.	skin	-	+++
L-MCC13	F	prim.	face	+	+
L-MCC14	F	prim.	upper arm	-	+++
L-MCC15	M	met.	upper arm	+	++
L-MCC16	M	met.	inguinal lymph node	+	+
L-MCC17	F	prim.	skin	+	+++
L-MCC18	M	met.	axillary lymph node	+	+++
M-MCC2 (14)	M	prim.	outer ear	-	++
M-MCC3 (13)	F	prim.	cheek	+++	++
M-MCC4 (1)	M	prim.	suboccipital	+	+++
M-MCC11 (21)	M	met.	pancreas	+++	++
M-MCC14 (3)	F	prim.	upper eye	+++	+++
M-MCC15 (18)	F	prim.	tongue	+	++
M-MCC20 (4)	F	prim.	cheek	+++	+
M-MCC24 (7)	F	prim.	knee	+++	(+)
M-MCC25 (10)	F	prim.	dorsal upper arm	+++	-
M-MCC26 (8)	F	prim.	upper eye lid	+++	+++
M-MCC27 (16)	F	prim.	glabella	++	+++
M-MCC40 (20)	M	prim.	upper leg	+++	+++
M-MCC46	M	prim.	infra-auricular	-	++
M-MCC47	F	prim.	lower arm	+++	++
M-MCC50	M	prim.	cheek	+	++

(Continued)

Table 1. (Continued)

Merkel cell carcinoma clinicopathological data				Immunohistochemistry	
ID	Gender	Primary or metastasis	Tumour localization	MCPyV	BCL-2
M-MCC52	F	prim.	cheek	+++	+
M-MCC54	M	prim.	gluteus area	+++	++
M-MCC56	F	prim.	cheek	+++	+
M-MCC57	M	prim.	upper leg	+++	+
M-MCC58	M	met.	lymph node	-	++
M-MCC59	F	prim.	upper arm	+++	+++
M-MCC60	F	met.	lymph node	+++	++
M-MCC61	M	met.	lymph node	++	+
M-MCC62	M	prim.	cheek	-	++
M-MCC64	M	prim.	upper lip	++	+++
M-MCC65	F	met.	lymph node	+++	++
M-MCC66	M	prim.	skin	++	++
M-MCC67	F	prim.	skin	-	+++
M-MCC69	M	prim.	skin	-	+++
M-MCC70	M	met.	cheek	+++	(+)
M-MCC71	F	prim.	upper leg	+	+
M-MCC72	F	prim.	cheek	+	++
M-MCC74	M	prim.	gluteus area	-	+
M-MCC78	F	prim.	cheek	-	+++
					46/47
					98.0%

Abbreviations: ID, identity; L-MCC, Merkel cell carcinoma from the Leuven cohort; M-MCC, Merkel cell carcinoma from the Maastricht cohort; M, male; F, female; prim., primary; met., metastasis; -, no expression; (+), weak expression; +, moderate expression; ++, strong expression; +++, very strong expression; in grey marked patient data and ID numbers in brackets are the numbers also reported in zur Hausen *et al.*[30]

by Jürgen Becker (University Hospital Essen, Essen, Germany). REH was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The cell lines were cultured in Gibco® RPMI 1640 medium (Life-science, Eindhoven, the Netherlands) with 10% fetal calf serum (FCS) (Gibco®, Thermo Fisher Scientific, Eindhoven, The Netherlands) in an incubator at 37°C and 5% CO_2.

Immunohistochemistry

All procedures of immunohistochemistry (IHC) were performed on FFPE tissues and FFPE cell lines. The expression of BCL-2 was tested by immunohistochemistry in the Leuven cohort ($n = 16$) and the MUMC+ cohort ($n = 37$). In total, 37 primary and 16 metastatic MCCs were assessed in this study. In addition, BCL-2 expression was tested in the MCPyV-positive MCC cell

lines MKL-1, MKL-2, in the MCPyV-negative MCC cell line MCC13 and in the B-ALL cell line REH. The following antibodies and dilutions were used in this study: anti-Flex BCL-2 Oncoprotein MxH clone 124 (Dako, Amstelveen, the Netherlands), ready to use. The IHC detection was conducted on a Dako Autostainer 48 Link using the EnVision FLEX Visualization Kit K8008 (Dako, Amstelveen, the Netherlands) as described previously and according to standard diagnostic routine protocols and the manufacturer's instructions.[30]

qRT-PCR

RNA was extracted using the RNA isolation kit 'NucleoSpin RNA' according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). RNA concentration was measured with the Nanodrop 2000 (ThermoFisher Scientific, Eindhoven, the Netherlands). RNA was converted into cDNA using the 'iScript™ Select cDNA Synthesis Kit' (Bio-Rad, Lunteren, the Netherlands). A quantitative RT-PCR was performed for the analysis of BCL-2 expression in the cell lines MKL-1, MKL-2, WaGa, PeTa, MCC13, and REH using the forward primer 5'-CTGCACCTGACGCCCTTCACC-3' and the reverse primer 5'-CACATGACCCCACCG AACTCAAAGA-3'.[31] The resulting C_t values were normalized using the housekeeping gene GAPDH which was amplified using the following primers: forward 5'-AGGGCTGCTTTT AACTCTGGT-3' and reverse 5'-CCCCACTTG ATTTTGGAGGGA-3'. The qRT-PCR was performed by using the SYBR Green Supermix (Bio-Rad, Lunteren, the Netherlands) on the CFX96 PCR Detection System (Bio-Rad, Lunteren, the Netherlands) and recorded by the Bio-Rad CFX manager. All used primers were obtained from Eurofins Genomics, Germany.

Western blotting

The MCPyV-positive cell lines MKL-1, MKL-2, WaGa, PeTa and MCPyV-negative cell line MCC13 and the B-ALL cell line REH were grown in T25 flasks (25 cm²). Cells were harvested, pelleted, washed with cold PBS and lysed in RIPA lysis-buffer (Cell Signaling Technology, London, UK) containing phosphatase-protein inhibitor cocktail (Thermo Fisher Scientific, Eindhoven, the Netherlands). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific,

Eindhoven, the Netherlands) according to the manufacturer's instructions. Protein samples of 25 µg were separated by SDS-PAGE in 8% (w/v) polyacrylamide gels and transferred to nitrocellulose membranes according to the manufacturer's instructions using Bio-Rad Mini Protean tetra systems (Bio-Rad Laboratories, Lunteren, the Netherlands), then blocked with 5% BSA (Sigma-Aldrich, Zwijndrecht, the Netherlands), incubated with primary antibodies anti-BCL-2 clone 124 (Dako, Amstelveen, the Netherlands) 1:500, anti-full-length and cleaved PARP clone 46D11 (Cell Signaling Technology, London, UK) 1:1000, anti-α-tubulin clone 236-10501 (Thermo Fisher Scientific, the Netherlands) 1:5000, and monoclonal anti-β-actin antibody clone AC-15 1:10000 (Sigmal-Aldrich, Zwijndrecht, the Netherlands) diluted in blocking buffer and incubated over night at 4°C, washed and incubated with the secondary antibody Alkaline Phosphatase AffiniPure F(ab')2 Fragment Goat Anti-Mouse/Rabbit IgG + IgM (H+L) (Jackson Immuno-Research, USA). The blots were developed by NBT/BCIP (Thermo Fisher Scientific, Eindhoven, the Netherlands) incubation according to the manufacturer's instructions. The blots were scanned using Canon CanoScan 9000F Mark II. The quantification of the protein bands was performed using ImageJ.[32] The protein of interest signals were normalized to the corresponding loading control.

Treatment of cell lines

The cell lines MKL-1, MKL-2, WaGa, MCC13 and REH were treated with Navitoclax (MedChemExpress, Monmouth Junction, USA) and Alpelisib (MedChemExpress, Monmouth Junction, USA). All compounds were dissolved in DMSO. Following concentrations were used for the treatment with Navitoclax: 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nM and 500 nM. The concentrations for Alpelisib were: 10 nM, 100 nM, 250 nM, 500 nM, 1 µM 12.5 µM, 25 µM and 50 µM. The cells were incubated in a 96-well plate (Greiner Bio-One, Kremsmünster, Austria) for 72 h with different concentrations of Navitoclax as well as for 120 h with different concentrations of Alpelisib in Gibco® RPMI 1640 medium (Life-science, Eindhove, the Netherlands) with 10% FCS (Gibco®, Thermo Fisher Scientific, Eindhoven, the Netherlands) in an incubator at 37°C and 5% CO_2. The effect of the drugs on the cell viability

was assessed by the XTT assay (Thermo Fisher Scientific, Eindhoven, the Netherlands) according to the protocol provided by the manufacturer. The read out of the XTT assay was done with the iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Lunteren, the Netherlands). For the combination experiments of Alpelisib with Navitoclax the single effect of all compound concentrations on the cell viability was measured and compared with the combinations. The apoptotic effect of the compounds on the cells was assessed by Western blot detection of cleaved PARP. Cleaved PARP was detected after 24h treatment with some concentrations of all compounds with and without combinations.

Annexin V and PI apoptosis assay

For the apoptosis assay, the REH and WaGa cells were cultured in T75 flasks (Corning, Amsterdam, the Netherlands) in RPMI 10% FCS at a cell concentration of 2×10^5 cells/mL. The cells were treated with Alpelisib alone and in combination with Navitoclax for 96h. At the incubation start and after each 24h the cell number was measured using the TC20 Automated Cell Counter (Bio-Rad Laboratories, Lunteren, the Netherlands). In addition, at every time point a 1mL sample was taken and stained with Annexin V–FITC as well as with propidium iodide (PI) (Sigma-Aldrich, Zwijndrecht, the Netherlands). The Annexin V–FITC was kindly provided by C. Reutelingsperger, Department of Biochemistry, Cardiovascular Research Institute Maastricht and used as described by Vermes *et al.*[33]

Half of the stained cells were used for flow cytometry. As a positive control REH and WaGa cells were treated with 1µM Staurosporin to induce apoptosis. For right gating of the cells unstained cells or stained cells only with Annexin V–FITC or PI were used. As negative controls, unstained cells and single stained cells were used. To limit the spectral overlap, PI staining was measured using PE-Cy7. The gating strategy is shown in Figure S2.

The remaining cells were used to make cytospins of all Annexin V as well as PI-stained cells with the Cyto-Tek Centrifuge (Sakura, Alphen aan de Rijn, the Netherlands). The nuclei of the cells were stained with DAPI mounting medium (Vectashield-Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions. Detection of the fluorescence-stained cells was performed with the Leica microscope DM 5000 B (Leica, Amsterdam, the Netherlands).

Statistics

The half maximal inhibitory concentration (IC_{50}) values were determined with Graphpad Prism 8. By using the One-Way ANOVA test the significance of the dose–response curves was determined; *p*-values < 0.5 are considered as significant. Further, the combinatory index (CI) for the different combinations was determined to state if the combinatory effect for a range of combinations is synergistic, additive or antagonistic. First, the HillSlope (H) and the IC_{50} values were used to calculate IC_ns in the range from IC_{10} to IC_{90}.

$$IC_n = \left(\frac{n}{100 - n} \right)^{\frac{1}{H}} * IC_{50}$$

From the different IC_ns the CIs for the different combinations are calculated. For every calculated IC one Navitoclax concentration stays constant and the concentration of the second compound varies. For the calculation of the CIs the corresponding IC of Navitoclax (IC_{nC1}) and of the second compound (IC_{nC2}) are used.

$$CI = \frac{conc_{C1}}{IC_{C1}} + \frac{conc_{C2}}{IC_{C2}}$$

The different calculated CIs for the different combinations ($conc_{C1}$ and $conc_{C2}$) are used to generate a 3D isobologram plot. The 3D isobologram generation was performed using the R package 'scatterplot3d'.[34,35] Red-marked region illustrates a synergistic effect (CI < 1) of both compounds, whereas white and blue represent an additive (CI $= 1$) and antagonistic (CI > 1) effect, respectively.

Results

Cytoplasmic expression of BCL-2 in MCC

BCL-2 expression was assessed in 47 FFPE MCC tissues and five MCC cell lines by IHC, and semi-quantitatively scored from negative (−) to very strong positive (+++) as illustrated in Figure 1 (A–E). In total, 98% (46 out of 47) of MCCs as well as all the MCPyV-positive cell lines (MKL-1, MKL-2, WaGa and PeTa) revealed a specific cytoplasmic expression of BCL-2. The intensity of expression varied between the MCC tissues: 34% (16 out 47) very strong, 36% (17 out of 47) strong, 23% (11 out
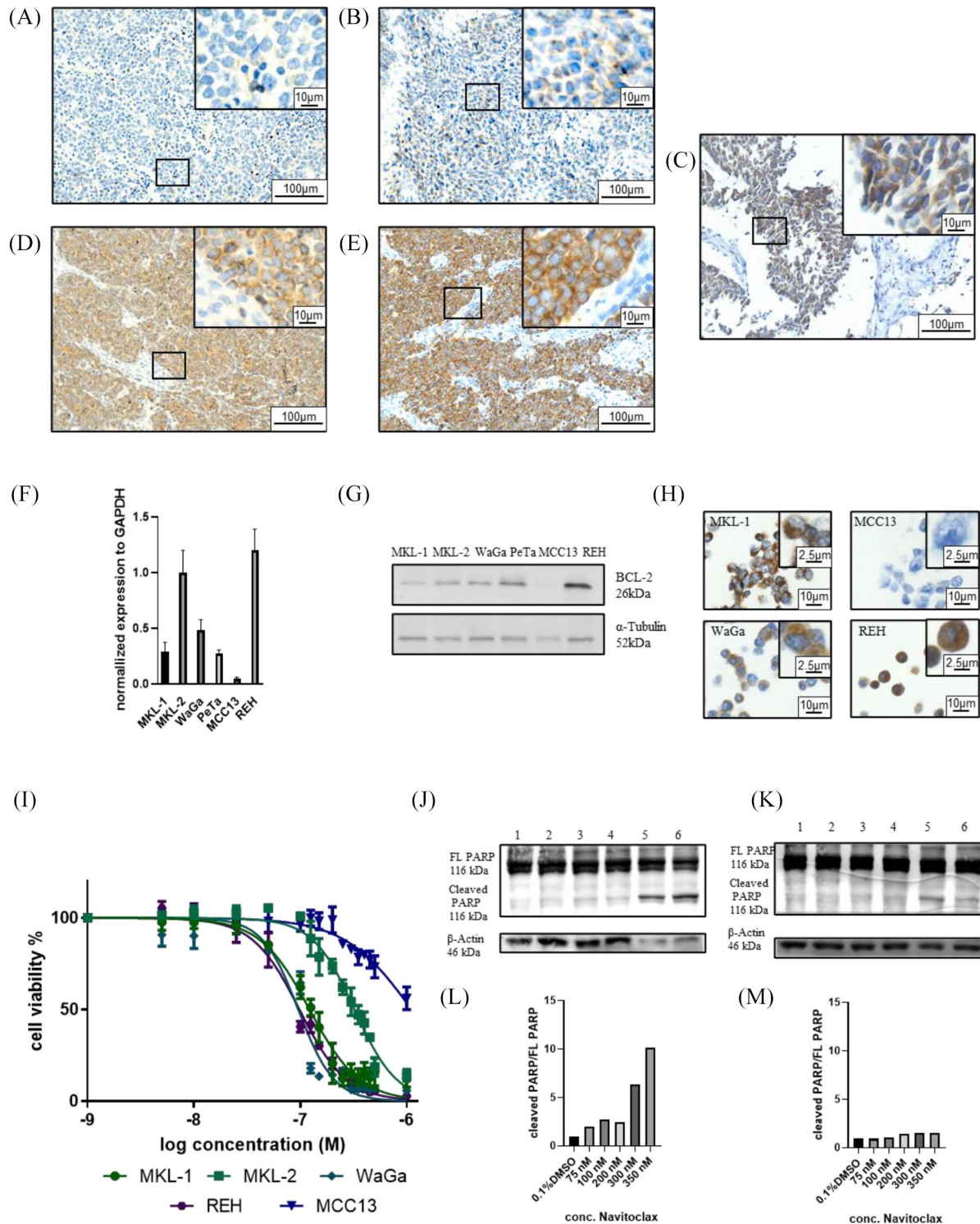
Figure 1. BCL-2 expression in MCC tissues as well as in cell lines and BCL-2 inhibition of MCC cell lines induces apoptosis.
Examples of negative (A), weak (B), moderate (C), strong (D), very strong (E) cytoplasmic expression of BCL-2 in the MCC tissues M-MCC25, M-MC70, M-MCC20, M-MCC3 and M-MCC14, respectively, are shown by immunohistochemistry (IHC) staining. The expression of BCL-2 in MCPyV-positive cell lines MKL-1, MKL-2, WaGa and PeTa is confirmed on RNA level by q-RT-PCR (F) and on protein level by Western blot (G). Exemplarily, the moderate BCL-2 expression is shown in the MCC MKL-1 and WaGa cells assessed by IHC (H). The treatment of the MCC cell lines with the specific BCL-2 inhibitor Navitoclax gained in dose–response curves for all assessed cell lines except MCC13 (I). The cell viability was assessed by the XTT-viability assay. Exemplarily, the apoptotic effect of Navitoclax is assessed for MKL-2 (J) and MCC13 (K) by the detection of cleaved PARP normalized to full length PARP. The normalization against β-Actin and full-length (FL) PARP of these Westerns blots is shown in (L) for MKL-2 and in (M) for MCC13. The tissue microphotographs were taken at 20× magnification; the squares in the right corner of the images were a 40× magnification of the tissue cells. The cell line microphotographs were taken at 40× magnification.

of 47) moderate and 4% (2 out of 47) weak (Table 1). The MCPyV-negative cell line MCC13 was negative for BCL-2 (Figure 1F). The BCL-2 expression in cell lines was confirmed on RNA level by quantitative RT-PCR and on protein level by Western blot (Figure 1G and H).

BCL-2 inhibition induces apoptosis in MCC cells

The MCC cell lines WaGa, MKL-1, MKL-2 and MCC13 as well as the B-ALL cell line REH were treated with different concentrations (range 1 nM to 1 µM) of the specific BCL-2 inhibitor Navitoclax (Figure 1I). With increasing concentrations, the cell viability decreased. For all cell lines except for BCL-2, negative MCC13 dose–response curves and IC_{50} values could be generated. MKL-1, WaGa and REH demonstrated similar high sensitivity towards Navitoclax treatment (IC_{50} around 100 nM). Surprisingly, MKL-2 was less sensitive ($IC_{50} = 323.3$ nM), although this cell line showed a high BCL-2 expression.

Subsequently, we assessed if BCL-2 inhibition promotes apoptosis in MCC cells, as assessed by the detection of cleaved PARP protein by apoptosis-activated caspases. Indeed, all cell lines except MCC13 (Figure 1L and M) showed different levels of increase of cleaved PARP with incremental Navitoclax concentrations.

Effect of the PI3K p110α inhibitor Alpelisib with Navitoclax on cell growth and apoptosis

Since we recently reported that the PI3K p110α inhibitor Alpelisib is a potent inhibitor of MCC cell growth in vitro, we investigated the efficacy of the combination of Alpelisib with Navitoclax in the four MCC cell lines and REH. Treatment with Alpelisib alone (AN0) resulted in IC_{50} values ranging from 2.2 µM in REH to 32.2 µM in MCC13 (Figure 2A–C, Figure S1 and Table 2). Subsequently, the cell lines were treated with increasing concentrations of Navitoclax added to the eight Alpelisib concentrations used to produce the AN0 dose–response curve, that is, WaGa, MKL-1 and REH (more sensitive to Navitoclax) were treated with Navitoclax concentrations ranging from 25 to 100 nM (AN25 to AN100), and MKL-2 and MCC13 (less sensitive to Navitoclax) with concentration ranging from 200 to 400 nM (AN200 to AN400) (Figure 2 and Figure S2).

In comparison to Alpelisib alone, the combination treatment resulted in stronger inhibitions of cell growth, but differed in sensitivity when comparing WaGa, MKL-1, MKL-2 and REH. The sensitivity of WaGa and MKL-1 increased 100-fold, REH and MKL-2 10-fold. This is also reflected in the IC_{50}-values of these treatments which decreased with increasing Navitoclax concentrations (Figure 2D–F and Figure S1 C and D). MCC13 did not show any significant increase in cell growth inhibition, which is in line with the lack of response to Navitoclax (Figure S1).

Interestingly, WaGa cells showed only at the two higher Navitoclax concentrations (75 nM and 100 nM) a higher sensitivity for the combined treatment than for Alpelisib alone (Figure 2A). This observation indicates that some combinations of Navitoclax and Alpelisib complement each other and other combinations counteract each other. Such effects can also be visualized using 3D isobologram plotting according to the Chou–Talalay method[36] (Figure 2G–I). With this method one can visualize if combinations of inhibitors at certain concentrations are synergistic, additive or antagonistic. This can be represented by the CI: CI < 1, synergistic; CI = 1, additive; CI > 1, antagonistic. In WaGa cells synergy was observed when the Navitoclax concentration was ⩾60 nM and the Alpelisib concentration was ⩽10 µM (Figure 2G; red-coloured area). At other combinations, the inhibitory effect on the cell viability was additive or even antagonistic (Figure 2G; white and blue-coloured areas, respectively). In contrast, in MKL-1 cells the combined treatment showed a large range of possible concentrations with which synergy could be obtained (Figure 2H). In MKL-2 cells synergy again was seen at smaller range of concentrations, that is, 200 nM of Navitoclax combined with Alpelisib concentrations between 1 µM and 12.5 µM (Figure 2C and I). In REH cells maximal synergy of the combined treatment could be observed compared with the other cell lines (Figure 2). It is clear from the dose–response curves and the 3D plots that synergy especially is obtained at concentrations where inhibition is at its maximum (Figure 2).

Since we have earlier seen that Navitoclax can induce apoptosis, we investigated if the combined Alpelisib–Navitoclax treatment within 24 h resulted in higher levels of cleaved PARP assessed by Western blotting (Figure 3). For WaGa, MKL-1 and REH an increase of cleaved PARP
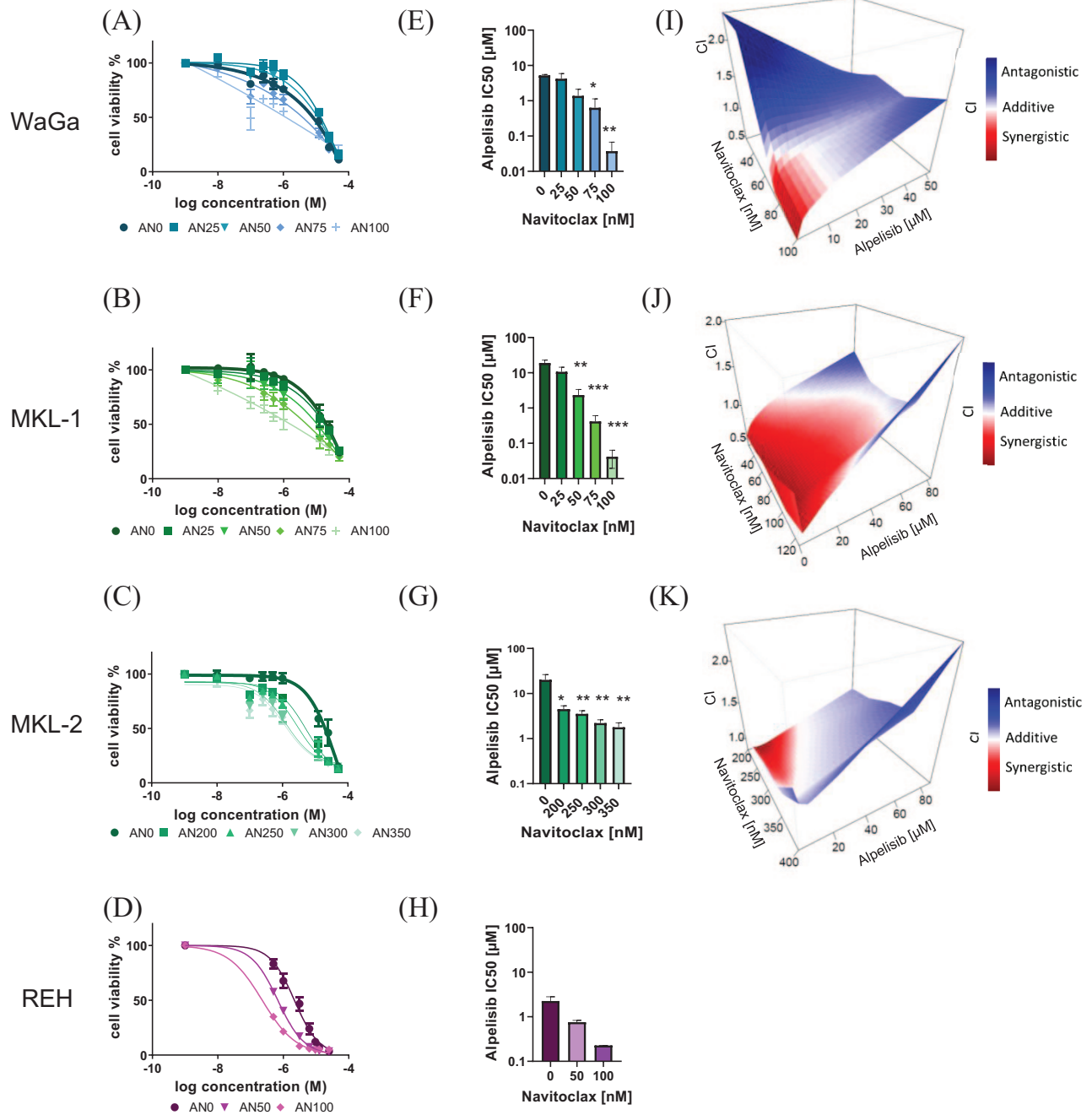
Figure 2. Significant synergistic combinatorial effect of Alpelisib and Navitoclax on the MCC cell line cell viability.
The dose–response curves for Alpelisib without Navitoclax for the MCPyV-positive MCC cell lines [MKL-1 (A), MKL-2 (B), WaGa (C) and REH (D)] are illustrated by the AN0 curves. The increasing concentrations (nM) of Navitoclax are illustrated by the numbers AN25, BN50, AN75, AN100 etc. The calculated IC_{50} of Alpelisib alone and in combination with different Navitoclax concentrations (nM) are shown for the corresponding cell line (E–H). The 3D combinatory index (CI) plots visualize the combined concentrations of Navitoclax and Alpelisib which are synergistic (red), additive (white) or antagonistic (blue) (I–L). The red circle in the dose–response curves illustrates the determined synergistic range. The standard errors are illustrated by the standard error of the mean (SEM). (*: $p < 0.05$); (**: $p < 0.01$); (***: $p < 0.001$).

could be detected at the highest concentrations used. Treatment of WaGa cells showed that in particular Navitoclax promotes apoptosis that only can be increased in combination with the highest Alpelisib concentration (synergistic effect)

(Figure 3). In contrast, in REH cells the combination of Navitoclax with the highest Alpelisib concentration resulted in increased cleaved PARP (synergistic effect). In MKL-2 cells an increase of cleaved PARP was observed with increasing

Table 2. Summary of the BCL-2 immunostaining results in the four MCC cell lines and REH, as well as the calculated IC$_{50}$ values when treated with Navitoclax.

Cell lines	Derived from	Immunohistochemistry		IC$_{50}$ treatment	
		MCPyV	BCL-2	Navitoclax	Alpelisib
WaGa	MCC	+	++	95.6 nM	5.1 µM
MKL-1	MCC	+	+++	122.0 nM	18.2 µM
MKL-2	MCC	+	+	323.3 nM	18.4 µM
MCC13	MCC	-	-	n.d.	32.2 µM
REH	B-ALL	-	+++	98.0 nM	2.2 µM

Abbreviations: B-ALL, B-cell acute lymphoblastic leukaemia; MCC, Merkel cell carcinoma; MCPyV -, no expression; +, expression; IC$_{50}$, the half maximal inhibitory concentration; n.d., could not be determined based on the dose–response curve.

Alpelisib concentrations but was more optimal at the 300 nM than the 350 nM Navitoclax concentration (Figure 3D). This is in agreement with the observation that the first concentration is in the synergistic and the second is in the additive range (Figure 2I). No apoptosis induction was detected in MCC13 cells (Figure 3E).

In order to assess the long-term effect of the combined treatment on apoptosis induction, we incubated WaGa and REH cells with 100 nM Navitoclax and 12.5 µM Alpelisib alone and in combination for four days and analysed apoptosis using the Annexin V and PI flow cytometry. MKL-1 and MKL-2 cells were omitted in this experiment because of their different growth pattern (cell clumps) which is a limitation for this assay. Flow cytometry analyses separated WaGa as well as REH cells into live (PI, Annexin V negative), necrotic (PI single positive), early apoptotic (Annexin V single positive) and late apoptotic (PI as well as Annexin V positive) cells (Figure 4). The increase or decrease of these cell populations was recorded for four consecutive days. The level of necrotic WaGa cells untreated and treated was low (<8.9%) and did not increase during time (Figure 4), whereas the number of Annexin V-positive apoptotic cells increased significantly (Figure 4). As previously seen for cleaved PARP, Navitoclax alone but in particularly in combination with Alpelisib strongly induced apoptosis (14.2 and 44.6%, respectively) already within 24 h of treatment. While after 24 h the percentage of apoptotic cells decreased for Navitoclax and slightly increased for Alpelisib, it remained significantly higher for the Alpelisib–Navitoclax-treated cells. Remarkably,
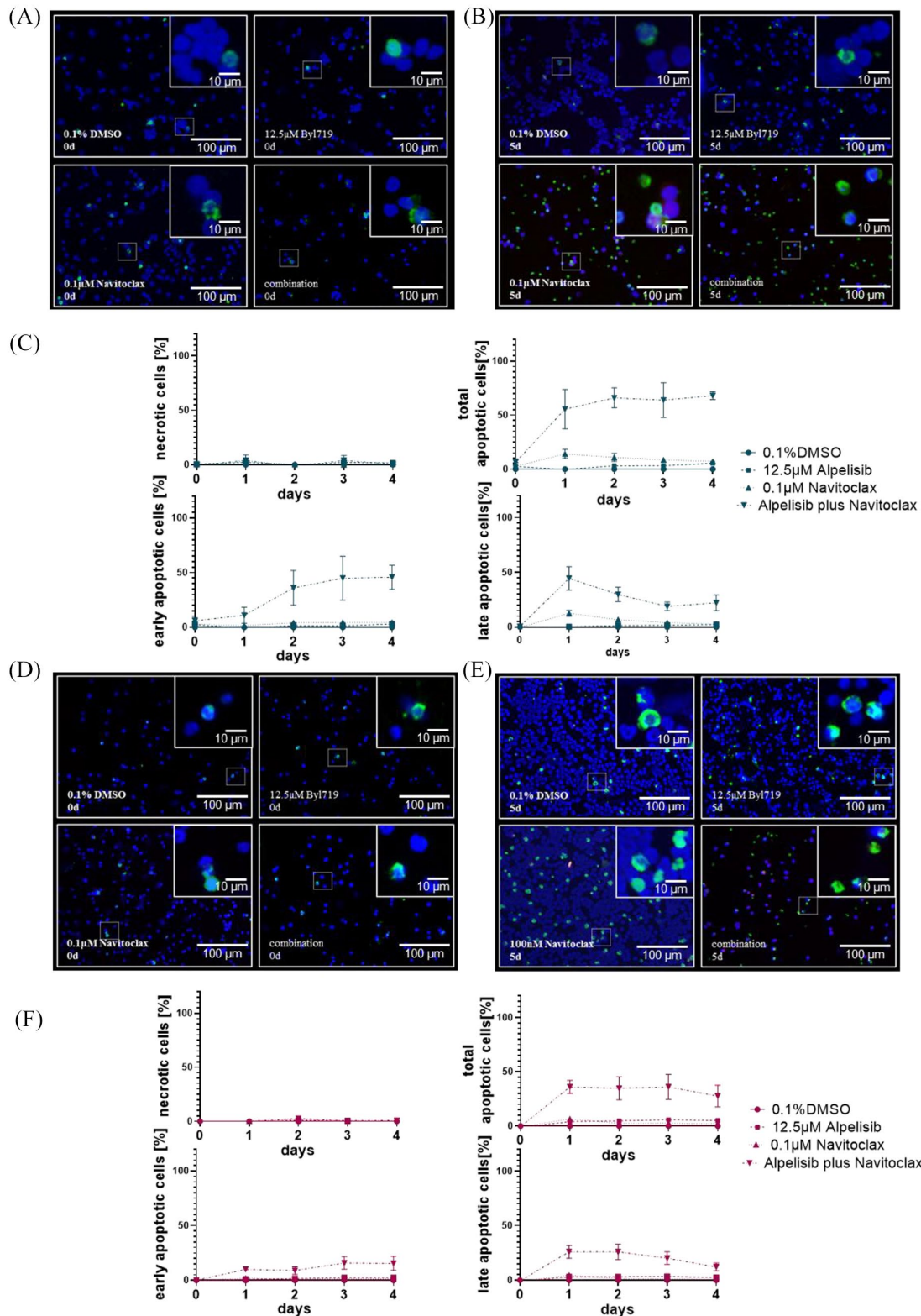
the combined treatment resulted in a strong increase of late apoptotic cells and a more slow increase of early apoptotic cells. In comparison to Navitoclax or Alpelisib alone, these data suggest that in WaGa cells Navitoclax boosts apoptosis within the first 24 h, which is slowly continued by the synergistic effect of Alpelisib in the next three incubation days. In REH cells a similar effect on apoptosis induction was observed but to a lesser extent (Figure 4). Thus, compared with the single treatments the apoptotic effect of the Alpelisib–Navitoclax combination persisted and resulted in higher apoptotic cell numbers in both cell lines.

Discussion

The aim of this study was to investigate the efficacy of a combination treatment of the BCL-2 inhibitor Navitoclax and the PI3K inhibitor Alpelisib on cell growth inhibition and apoptosis induction in MCCs. Our results demonstrate that almost all MCCs express the anti-apoptotic BCL-2 protein and MCC cell lines are sensitive for Navitoclax. The combination of Navitoclax with Alpelisib further decreased cell line viability through induction of apoptosis. At concentrations at which both inhibitors acted synergistically, the apoptosis induction continued for up to 4 days.

A prerequisite for effective treatment with Navitoclax is the expression of the anti-apoptotic BCL-2 protein in tumour cells. For this purpose, we investigated the expression of these proteins in 47 MCCs and five MCC cell lines. Some 98% of MCCs and all five cell lines were positive for

Figure 3. Combinatorial apoptotic effect of Alpelisib and Navitoclax on the MCC cell lines.
Different concentrations of Navitoclax and Alpelisib alone and in combination are used to treat the MCC cell lines MKL-1, MKL-2, WaGa and MCC13 as well as the B-ALL cell REH. The MCC cell lines and REH except MCC13 reveal a stronger cleaved band abundance compared with Navitoclax and Alpelisib without combination as seen by Western blot and quantification using ImageJ.

Figure 4. Combined Alpelisib–Navitoclax treatment induces increased apoptosis in WaGa and REH cells. Only the Alpelisib–Navitoclax combination inhibits cell growth.
WaGa and REH cells were treated with Alpelisib in combination with and without Navitoclax for 5 days. After each day, the early apoptotic cells were detected by Annexin V (GFP/green). The late apoptotic cells were detected with nuclear PI staining (red) and membranous Annexin V (green). A part of the treated cells was stained additionally with DAPI and visualized using fluorescence microscopy. Exemplarily, the cell staining of day 0 and day 4 were shown for, the 0.1% DMSO control Alpelisib, Navitoclax and combinations of Alpelisib with Navitoclax (A, B for WaGa and D, E for REH). Further, flow cytometry was used to determine the percentage of necrotic cells (PI singl. pos), the percentage of early apoptotic cells (Annexin V single pos.) and the percentage of late apoptotic cells (PI pos. Ann pos.) after treatment of the WaGa (C) and REG (F) cell line.

BCL-2, which is in agreement with an overall occurrence of approximately 80% (325 of 403) BCL-2 positivity in MCC tissues based on the current literature.[21,37–43] High expression BCL-2 expression has been also reported for epithelial cancers and melanomas, and was thought to induce resistance towards chemotherapy or immunotherapy.[44,45] It is tempting to speculate that such a mechanism is also responsible for the resistance in MCCs treated by these therapies.[3,4,13,14]

Navitoclax treatment was shown to be highly effective when treating MCC cell lines MKL-1, MKL-2 and WaGa (IC_{50} values ranging from 96.0 to 323.0 nM), which express high levels of BCL-2. In contrast, the BCL-2-negative MCC13 cell line showed a lower sensitivity to Navitoclax. In general, it is accepted that the inhibition of BCL-2 might increase the induction of apoptosis in tumour cells.[46] Indeed, we observed that the single Navitoclax treatment reduced the cell viability through apoptosis induction in the cell lines and this effect was lowest in the MCC13 cell line. Our data are in agreement with a previous study showing that Navitoclax was a potent inhibitor of viability in other MCC cell lines.[22] It might be that BCL-2 expression can serve as predictive biomarker for BCL-2 inhibitor treatments.

Although Navitoclax treatment of patients with several forms of leukaemia has resulted in strong apoptosis induction in tumour cells, 17% of patients also suffered from severe adverse events such as grade 4 thrombocytopenia.[25,47] The risk for Navitoclax-induced thrombocytopenia is dosage dependent, and could be decreased by using lower Navitoclax concentrations, for example when applied in combination with other compounds in patients with leukaemia.[23,25,26,48,49] In the current study, we have investigated the combination of Navitoclax with the FDA-approved PI3K inhibitor Alpelisib, which has been previously described as a potent cell viability inhibitor of MCC cell lines.[17,18] This treatment combination further improved the inhibiting effect on the cell viability of the MCC cell lines WaGa, MKL-1 and MKL-2 cells through strong apoptosis induction. This also demonstrates that the concentrations of both compounds could be reduced to achieve the same effects of single Navitoclax application. Our data further add to the knowledge on effective double inhibition of BCL-2 and PI3K pathways in other tumours *in vitro*, that is, HML and glioblastoma as well as SCLC.[27–29,50,51]

In this light, also the B-ALL cell line REH tested in the current study proved to be sensitive for the combination of Navitoclax and Alpelisib.

In addition to the potent inhibitory effect of the combined treatment in MCC and REH cell lines, we also examined if the induction of apoptosis continues during longer incubations. For this purpose, the WaGa and REH cells were treated for 4 days, resulting in a rapid increase of apoptosis within 24 h and stabilization of the percentage of apoptotic cells for up to 4 days. In comparison with incubations of Navitoclax alone, this inhibitor appears to be responsible for the rapid increase of apoptotic cells within the first day (regular apoptotic events due to, for example, stress in addition to the effect of the treatment), after which the combination with Alpelisib extends this effect over a longer time period. This can be measured by a high percentage of late apoptotic cells (both PI and Annexin V positive) already after 1 day that decreases afterwards, whereas the percentage of early apoptotic cells (only Annexin V positive) indicates apoptosis induction still increases and maximizes at day three. Because treatments containing Navitoclax have so far always been given in daily regimens in cell lines studies and clinical trials,[47,51–53] our data indicate that regimens of the here tested combination with Alpelisib might be effective over a longer timeframe and should be further investigated for clinical use.

A limitation of this study is the fact that we used a limited number of MCC cell lines to investigate the Navitoclax–Alpelisib treatment. It will be interesting to assess the treatment effect in a MCC xenograft mice model, for example the model reported by Verhaegen *et al.*[22] Our results obtained in the BCL-2-negative MCC13 cell line suggest that if BCL-2 is not expressed the combination treatment of Navitoclax and Alpelisib is not significantly effective. An additional consideration may concern the efficacy of one dose of the combined treatment to inhibit the MCC cell lines for up to 4 days, taking into account that in humans the half-life values of Navitoclax and Alpelisib have been reported to be 17 h and 7.6 h, respectively.[19,25] Although this information is not indicative for the time these compounds are active within tumour cells, further studies are needed to investigate this subject. A technical limitation concerned the fact that the Annexin V assay was not compatible with the spheroid growth of MKL-1 and MKL-2, but an impression of apoptosis could be established using the cleaved PARP Western blot assay.

In conclusion, our results reveal that Navitoclax and Alpelisib show strong synergy in the inhibition of cell viability and induction of apoptosis in MCPyV-positive cell lines. The treatment response showed to last for up to 4 days with application of one dose. The combination of Navitoclax with Alpelisib should therefore further be explored, and could be a potential new treatment for patients with MCC.

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Supplemental material
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