EUROPEAN JOURNAL OF CANCER 48 (2012) 396–406

Available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.ejconline.com

The phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 is effective in inhibiting regrowth of tumour cells after cytotoxic therapy

Emma Hernlund [a], *Maria Hägg Olofsson* [a], *Walid Fayad* [a], *Mårten Fryknäs* [b], *Karolina Lesiak-Mieczkowska* [a], *Xiaonan Zhang* [a], *Slavica Brnjic* [a], *Vivien Schmidt* [a], *Padraig D'Arcy* [a], *Tobias Sjöblom* [c], *Angelo De Milito* [a], *Rolf Larsson* [b], *Stig Linder* [a,*]

[a] *Department of Oncology & Pathology, Karolinska Institute, 171 76 Stockholm, Sweden*
[b] *Division of Clinical Pharmacology, Department of Medical Sciences, Uppsala University Hospital, 751 85 Uppsala, Sweden*
[c] *Department of Genetics and Pathology, Uppsala University, SE-751 85 Uppsala, Sweden*

ARTICLE INFO

Article history:
Available online 7 December 2011

Keywords:
Cancer therapy
Regrowth resistance
PI3K
mTOR
NVP-BEZ235

ABSTRACT

Purpose: Regrowth of tumour cells between cycles of chemotherapy is a significant clinical problem. Treatment strategies where antiproliferative agents are used to inhibit tumour regrowth between chemotherapy cycles are attractive, but such strategies are difficult to test using conventional monolayer culture systems.

Methods: We used the *in vitro* tumour spheroid model to study regrowth of 3-D colon carcinoma tissue after cytotoxic therapy. Colon carcinoma cells with wild-type or mutant phosphatidyl inositol 3-kinase catalytic subunit (*PI3KCA*) or *KRAS* alleles were allowed to form multicellular spheroids and the effects of different pharmacological compounds were studied after sectioning and staining for relevant markers of cell proliferation and apoptosis.

Results: Studies using colon cancer cells with gene disruptions suggested that the phosphoinositide 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway was essential for proliferation in 3-D culture. The dual PI3K-mTOR inhibitor NVP-BEZ235, currently in clinical trials, was found to inhibit phosphorylation of the mTOR target 4EBP1 in 3-D cultured cells. The ability of NVP-BEZ235 to inhibit tumour cell proliferation and to induce apoptosis was markedly more pronounced in 3-D cultures compared to monolayer cultures. It was subsequently found that NVP-BEZ235 was effective in inhibiting regrowth of 3-D cultured cells after treatment with two cytotoxic inhibitors of the ubiquitin–proteasome system (UPS), methyl-13-hydroxy-15-oxokaurenoate (MHOK) and bortezomib (Velcade®).

Conclusions: The dual PI3K-mTOR inhibitor NVP-BEZ235 was found to reduce cell proliferation and to induce apoptosis in 3-D cultured colon carcinoma cells, NVP-BEZ235 is a promising candidate for use in sequential treatment modalities together with cytotoxic drugs to reduce the cell mass of solid tumours.

* *Corresponding author:* Address: Cancer Center Karolinska, CCK R8:00, Karolinska Hospital, S-171 76 Stockholm, Sweden.
E-mail address: Stig.Linder@ki.se (S. Linder).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2011.11.013

1. Introduction

Regrowth of tumour cells between cycles of chemotherapy is a distinct clinical phenomenon that has been linked to drug resistance and to poor clinical outcome.[1–3] The probability of tumour eradication is increased if the most effective dose of drug can be administered, either by dose escalation or by increasing dose density. Administration of a high drug doses at the beginning of a chemotherapy cycle is usually not beneficial to the patient since high doses often decrease the therapeutical window of the drugs.[4] A better option is to reduce the time for tumour regrowth between therapy cycles by increasing dose density. If there is a limit to the extent of achievable tumour cell kill, other approaches must be developed to prevent the regrowth of residual tumour cells between cycles of therapy. One possible strategy is to combine cytotoxic agents with agents that have low toxicity to normal tissue and that inhibit cell proliferation of tumour cells.[3] A number of potentially useful 'targeted therapies' have been developed during recent years, therapies which could be used to inhibit cell regrowth of residual tumour cells. Little is known about how such agents should be used to inhibit repopulation, and this problem is difficult to study using conventional *in vitro* cell culture systems.

The phosphoinositide 3-kinase (PI3K) signalling pathway is linked to both growth control and glucose metabolism[5]. Akt (protein kinase B) phosphorylates a number of cellular substrates involved in processes such as cell growth and proliferation, cell survival, and metabolism.[6] One of the mechanisms leading to overactivation of the PI3K signalling pathway is the presence of somatic mutations in the *PIK3CA* gene.[7] Genetic disruption of the PI3K mutant allele in the colon carcinoma cell lines HCT116 and DLD-1 was found to reduce their tumourigenic properties.[15] NVP-BEZ235 is a synthetic small molecular mass compound belonging to the class of imidazoquinolines that potently and reversibly inhibits class 1 PI3K catalytic activity by competing at its ATP-binding site. NVP-BEZ235 also inhibits mammalian target of rapamycin (mTOR) catalytic activity.[8]

Multicellular tumour spheroids (MTS) represent a model for three-dimensional tumour tissue.[9–12] The three dimensional growth of cells in this model simulates the conditions in intervascular microregions of tumours or micro-metastatic foci.[9] MTS follow the Gompertzian growth law, despite their avascular growth.[13] Spheroids have been used as a model to study regrowth after cytotoxic therapy[14] and represent a simple model to evaluate the principles of how cytotoxic and antiproliferative agents can be used to eradicate solid tumours. We here use MTS to examine regrowth of hypoxic tumour cells after administration of a cytotoxic drug and to study the effects of a growth inhibitory agent (NVP-BEZ235) to control regrowth.

2. Materials and methods

2.1. Cell culture

HCT116 and DLD-1 colon carcinoma cells with mutant and wild-type *PI3KCA* and *KRAS* alleles were generously provided by Dr. Bert Vogelstein. Cells were maintained in McCoy's 5A modified medium/10% foetal calf serum at 37 °C in 5% CO_2. MelJuSo cells expressing Ub-GFP were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax/10% foetal calf serum. Multicellular spheroids were formed essentially as described using the hanging drop method.[15] Spheroid growth was determined by photographing spheroids and measuring diameters using the DPXView Pro software (DeltaPix Aps, Maalov, Denmark) followed by volume calculations.

2.2. Immunohistochemistry

Spheroid sections were deparaffinised with xylene, rehydrated and microwaved in citrate buffer (Thermo Scientific, Freemont, CA, USA) and then incubated over-night with the primary antibodies diluted in 1% (w/vol) bovine serum albumin and visualised by standard avidin–biotin–peroxidase complex technique (Vector Laboratories, Burlingame, CA, USA). Counterstaining was performed with Mayer's haematoxylin. Antibody against active caspase-3 was from BD Biosciences (used 1:1000), antibody to Ki67 (MIB-1) was from Dako, Stockholm, Sweden (used at 1:800), antibody to cyclin A from Leica Biosystems Newcastle, Newcastle upon Tyne, (used 1:1000) and antibody to $p27^{Kip1}$ from BD Biosciences (used 1:1000). Hypoxia was studied using the Hypoxyprobe kit (Hypoxyprobe Inc., MA, USA) according to the manufacturer's instructions. In brief, 100 μM Hypoxyprobe was added to the spheroids for 2 h incubation. Antibody directed to Hypoxyprobe was used at 1:50.

2.3. Western blotting

Cell extract proteins were resolved by Bis-Tris PAGE gels or Tris–Acetate gels (for ubiquitin conjugates) (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated overnight with antibodies against phosphorylated 4EBP1 (1:10000), 4EBP1 (1:8000) (Cellsignaling, MA, USA), ubiquitin (1:1000) (Millipore) and actin (1:5000) (SIGMA, Sweden), washed and incubated with HRP-conjugated anti-rabbit Ig (Amersham Biosciences, Little Chalfont, UK) for 1 h. Peroxidase activity was developed by SuperSignal West Pico (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

2.4. Flow cytometry

Cells were treated with the indicated drugs and times, harvested and fixed with formalin for 10 min prior to analysis. A FACSCalibur flow cytometer was used for detection of GFP accumulation as well as for linear analysis of forward scatter for size measurements.

2.5. Viability assessment

Cells were seeded in 96-well plates at a density of 8000 cells/well. At the times indicated, wells were washed with phosphate buffered saline (PBS) and kept at –20 °C until analysed. Acid phosphatase substrate tablets (4-Nitrophenyl phosphate disodium salt hexahydrate) from SIGMA–Aldrich (Sweden) were dissolved in sodium acetate buffer (0.1 M Na acetate, 0.1% Triton-X-100, pH 5). Substrate (100 μl) was added per well and incubated for 2 h at 37 °C. The reaction was stopped by

addition of 10 μl 1 N NaOH. Plates were read in a spectrophotometer at 405 nm. Acid phosphatase activity correlates to cell number in the well[16] and results are not (in distinction to MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays) affected by redox status of the cells.

2.6. Identification of the novel ubiquitin–proteasome system (UPS) inhibitor methyl-13-hydroxy-15-oxokaurenoate (MHOK)

The NCI Mechanistic Set (889 compounds) was screened for agents that induce caspase activation in HCT116 MTS using a previously described assay.[15] Briefly, MTS were cultured in 96-well plates and treated for 24 h with 25 μM of each compound in the library (final solvent concentration was 0.5% DMSO; control wells received DMSO only). One of the compounds identified in the screen was NSC620358 (Supplementary Fig. 1). It was noticed after sectioning that this compound induced apoptosis of outer layer cells, and that these cells detached from the MTS (Supplementary Fig. 1).

3. Results

3.1. Standard chemotherapeutical agents induce limited induction of apoptosis in HCT116 spheroids

We used HCT116 multicellular spheroids as an *in vitro* model for solid tumour tissue. Spheroids were formed in 96 well plates using the principle of hanging drops[17] to achieve spheroids of homogeneous size. The cells in the cores of the spheroids were hypoxic as evidenced by staining for pimonidazole adducts and were negative for the proliferation markers Ki67 and cyclin A (Fig. 1A). Hypoxia-induced cell cycle arrest has previously been shown to be associated with expression of the $p27^{Kip1}$ CDK inhibitor,[18] and we indeed observed strong $p27^{Kip1}$ staining in central areas of spheroids (Fig. 1A). HCT116 cells have a $p53^{wt}$ genotype[19] and it is known that p53 is induced by severe hypoxia.[20] We only observed scattered p53-positive cells in the MTS, and no obvious correlation to hypoxic regions (Fig. 1A).

A number of clinically used chemotherapeutical agents were tested for their ability to induce apoptosis in HCT116 MTS, including cisplatin, doxorubicin, etoposide and vinblastine (Fig 1B). Despite the use of drug concentrations that are at least one order of magnitude higher than the IC50 in monolayer cultures, limited apoptosis was observed in MTS (Fig. 1B) (lower concentrations induce almost no apoptosis, not shown). Cisplatin, doxorubicin and etoposide are known to induce DNA damage and the p53 response, but this response was limited to peripheral cell layers of MTS (Fig. 1B).[21] We conclude, that similar to other models of tightly packed MTS such as the V79 model,[14] HCT116 MTS are relatively resistant to anticancer drugs.

3.2. Regrowth after treatment with an inhibitor of the ubiquitin–proteasome system

Most types of treatment, including the standard agents above, resulted in regrowth of viable cells after several days of incubation in drug-free medium. Regrowth was difficult to study, however, since MTS contained an outer rim of dead cells which made it difficult to estimate the size of the viable portion of the spheroids. However, during screening of drug collections using MTS as targets[15,22] we identified an inhibitor of the ubiquitin–proteasome system, methyl-13-hydroxy-15-oxokaurenoate (Fig. 2A), which was particularly useful for studies of regrowth. This agent is present in the NCI Mechanistic drug library (www.dtp.nci.nih.gov) and is an analogue of kaurenoic acid, a diterpene from the 'kerosene tree' (Copaifera langsdorffii; evidence that MHOK is an inhibitor of the UPS are found in Supplementary Fig. 1). At a concentration of 20 μM, continuous MHOK treatment resulted in a volume reduction of HCT116 spheroids by >50% (Fig. 2B). Treatment with MHOK resulted in activation of caspase-3 in outer cell layers as early as after 4 h, followed by detachment of these cells (Fig. 2C). The remaining MTS cell masses have a defined border of viable cells, facilitating accurate determination of MTS size after drug exposure and further incubation. Cells surviving MHOK treatment remained clonogenic after dispersal of spheroids by trypsin (data not shown). The rapid detachment of outer cell layers at ~4 h created a setting where the effects of exposure of surviving hypoxic and starved cells to oxygen and nutrients could be studied.

When MHOK-treated spheroids were monitored over time, MTS volumes started to increase after a lag period (see below). To examine the kinetics of reproliferation, spheroids were fixed and sectioned at different times after drug treatment and sections were stained for the proliferation markers cyclin A (Fig. 2D) and Ki67 (Fig. 2E). After 9 and 12 h of treatment, cells at the periphery of MTS were negative for both markers (Fig. 2D and E). At 18 h, however, many cells were positive and remained positive at 24 and 48 h (Fig. 2D and E). We conclude from these experiments that the hypoxic cells of the HCT116 spheroid cores were able to reinitiate cell proliferation within 18 h after detachment of the outer cell layers and subsequent reexposure to oxygen and nutrients.

3.3. PI3KCA mutation is required for growth of HCT116 colon cancer cells in multicellular spheroids

We were interested to determine if cytostatic therapy could be used to inhibit regrowth of tumour cells in MTS after cytotoxic treatment. HCT116 colon carcinoma cells have a mutant *PI3KCA* allele and a mutant *KRAS* allele.[19] These mutations are amongst the most frequently occurring in colon carcinomas,[23] and lead to activation of downstream signalling pathways. In order to determine whether *PI3KCA* and/or *KRAS* mutations are important for the proliferation of HCT116 cells in MTS, we examined clonal derivatives where the mutant alleles have been disrupted.[24,25] Disruption of the $PI3KCA^{mut}$ allele had only a minor influence on the rate of proliferation of HCT116 cells in monolayer cultures (Fig. 3A). In contrast, the volume of MTS formed by HCT116 $PI3KCA^{wt/-}$ cells did not increase in size after formation (Fig. 3B), showing that the *PI3KCA* mutation is essential for proliferation of HCT116 cells in 3-D culture. We attempted to test this hypothesis also in DLD-1 cells, another colon cancer cell lines with a mutant *PI3KCA* allele where a derivative with a disrupted mutant allele is available. DLD-1 cells did not form compact spheroids, however. We observed that $PI3KCA^{wt/-}$ DLD-1 cells proliferated significantly slower than $PI3KCA^{mut/wt}$ cells in monolayer cul-

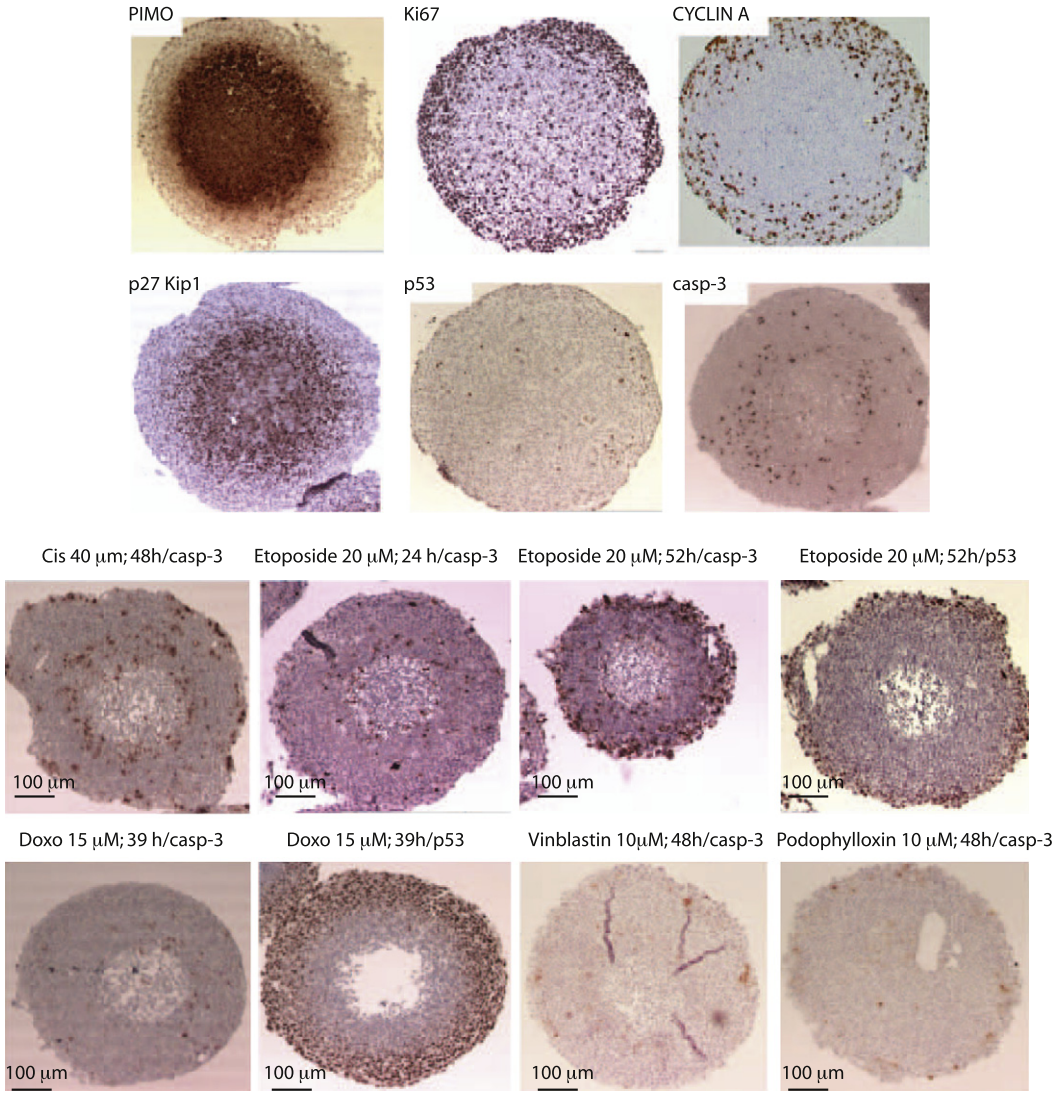

Fig. 1 – Properties of HCT116 multicellular tumour spheroids (MTS) and their response to chemotherapeutical agents. (A) Spheroids prepared in by plating 10,000 HCT116 cells in 96-well plates for 7 days as described,[22] sectioned and stained for pimonidazole adducts (PIMO), Ki67, Cyclin A, $p27^{Kip1}$, p53 or active caspase-3. (B) Spheroids were treated with the indicated agents for the times shown (continuous treatment) and stained for active caspase-3 or p53 as indicated. The diameter of each spheroid was ~500 μm.

ture (Fig. 3C), suggesting that they would do so also in 3-D culture. In contrast to the strong effect on MTS growth by disruption of the mutant *PI3KCA* allele, $KRAS^{wt/-}$ cells showed a similar rate of spheroid growth as the $KRAS^{wt/mut}$ parental cells. In fact, the difference in proliferation of these cells was larger in monolayer culture (Fig. 3D and E).

3.4. *Growth inhibition of HCT116 human colon carcinoma MTS by the dual PI3K/mTOR inhibitor NVP-BEZ235*

The findings using genetically manipulated cells suggested that inhibition of PI3K signalling may be effective in inhibition of regrowth of HCT116 MTS after cytotoxic therapy. We therefore examined the effect of the dual pan-class I PI3K/mTORC1/mTORC2 inhibitor NVP-BEZ235[8] on the proliferation of HCT116 and DLD-1 cells in monolayer and HCT116 cells in spheroids. NVP-BEZ235 was found to strongly inhibit the proliferation of DLD-1 cells, irrespective of the mutational status of the *PI3KCA* gene (Fig. 3C). This result is consistent with reports in the literature showing that NVP-BEZ235 induces growth arrest in many cell lines.[8,26–28] HCT116 cells were also growth inhibited by NVP-BEZ235, but were less sensitive (Fig. 3A). We conclude that NVP-BEZ235 has a growth inhibitory effect on colon cancer cells with $PI3KCA^{mut}$ alleles, but that the anti-proliferative effect is not dependent on *PI3KCA* mutations.

Since disruption of the *PI3KCA* mutant allele had a strong influence on the proliferation of HCT116 cells grown in 3-D culture, we examined whether NVP-BEZ235 inhibits the 3-D growth of HCT116 $PI3KCA^{mut}$ cells. We first demonstrated that NVP-BEZ235 reduced the phosphorylation of the mTOR target 4EBP1 in MTS cultures to a similar degree as in monolayer cultures (Fig. 4A), showing that the drug was able to reach the majority of cells in the 3-D culture model at therapeutically

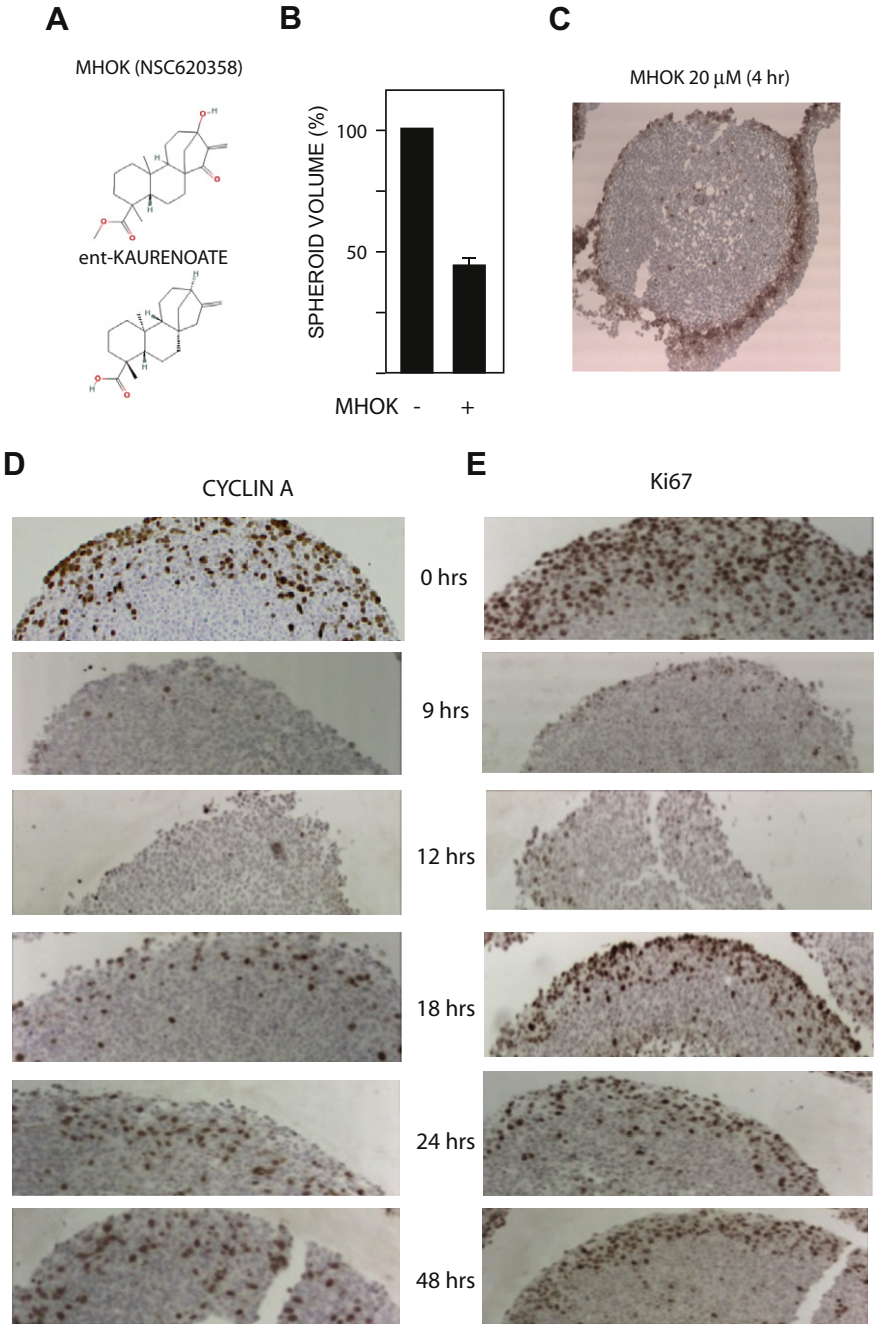

Fig. 2 – Effect of the ubiquitin–proteasome system (UPS) inhibitor methyl-13-hydroxy-15-oxokaurenoate (MHOK) on HCT116 multicellular tumour spheroids (MTS). (A) Structure of methyl-13-hydroxy-15-oxokaurenoate (NSC620358; MHOK) and ent-kaurenoate. (B) Reduction of HCT116 MTS volume after treatment with 20 μM MHOK, data from three independent experiments (+standard deviation). (C) MHOK induces apoptosis and detachment of cells in the peripheral layers of HCT116 MTS. MTS were treated with 20 μM MHOK for 4 h, fixed and stained for active caspase-3. The restriction of cell killing to outer layers appears to be due to instability of the compound in cell medium; adding additional MHOK after 12 h leads to almost complete killing of HCT116 MTS (data not shown). (D, E) Induction of cyclin A (D) and Ki67 (E) positivity in previously quiescent cells following treatment with 20 μM MHOK. MTS were fixed, sectioned and stained after the indicated times. The experiments shown in (C–E) were repeated 3–5 times with similar results.

effective concentrations. Continuous treatment with NVP-BEZ235 resulted in a complete inhibition of the growth of HCT116 MTS, and to decreases in MTS volumes (Fig. 4B). Treatment with NVP-BEZ235 for 4 days, followed by further incubation in drug-free medium, resulted in regrowth of the MTS after a lag period (Fig. 4B), showing that the drug does not induce irreversible growth arrest. Consistent with the finding of inhibition of spheroid growth, the number of Ki67 positive (proliferating) cells in the spheroid peripheral areas decreased after treatment with NVP-BEZ235 (Fig. 4C).

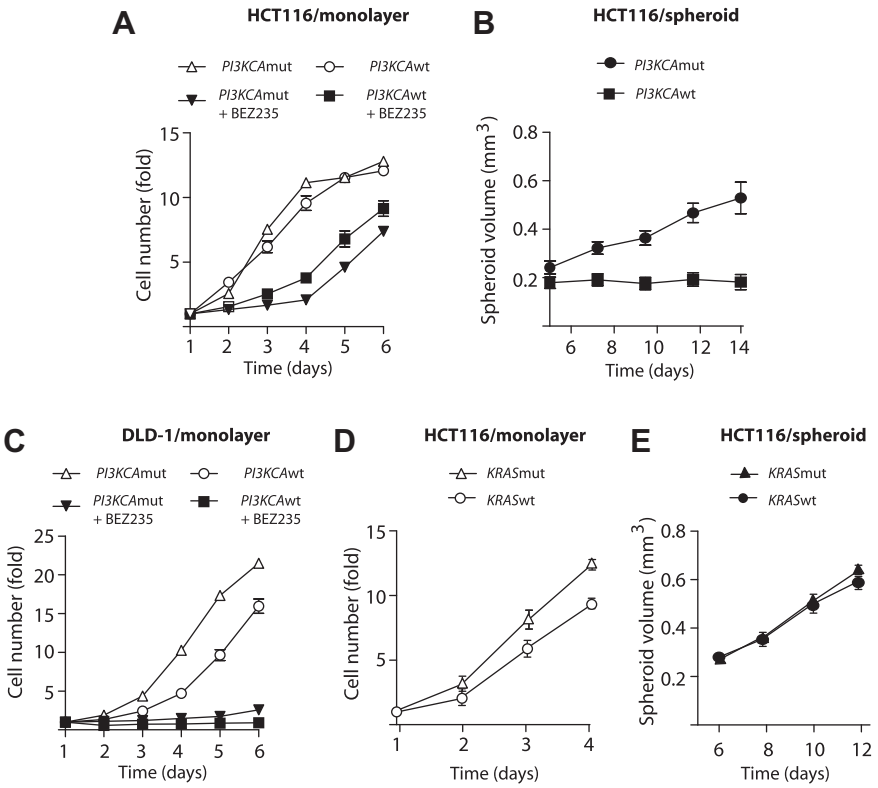

Fig. 3 – Role of the PI3KCA/mammalian target of rapamycin (mTOR) pathway for proliferation of HCT116 and DLD-1 colon cancer cells under 2-D and 3-D conditions. (A) Monolayer growth of *PI3KCA*$^{mut/wt}$ and *PI3KCA*$^{wt/-}$ HCT116 colon carcinoma cells. Cells were seeded in 96 well plates and treated with 0.2 μM NVP-BEZ235 where indicated. Cell proliferation was determined by the acid phosphatase viability test (16). (B) Volumes of HCT116 *PI3KCA*$^{mut/wt}$ and *PI3KCA*$^{wt/-}$ MTS after formation in 96 well plates. (C) Monolayer growth of *PI3KCA*$^{mut/wt}$ and *PI3KCA*$^{wt/-}$ DLD-1 colon carcinoma cells. Treatment with 0.2 μM NVP-BEZ235 and viability testing as in (A). Symbols used are the same as in (A). (D) Monolayer growth of HCT116 colon carcinoma cells with *KRAS*$^{mut/wt}$ and *KRAS*$^{wt/-}$ alleles. Treatment with 0.2 μM NVP-BEZ235 and viability testing as in (A). (E) Volumes HCT116 *KRAS*$^{mut/wt}$ and *KRAS*$^{wt/-}$ MTS after formation in 96 well plates. All measurements were performed in triplicate and mean values + standard deviation are presented.

The PI3K/Akt/mTOR pathway is known to be important for regulation of cell proliferation and NVP-BEZ235 has previously been shown to inhibit proliferation of monolayer tumour cells[8,29] and able to retard tumour growth.[8] The decrease in HCT116 MTS volume at early phases of treatment was surprising since Ki67 positivity was not affected at 24 h (Fig. 4C). One possibility was that volume reduction was associated with increased apoptosis, although no signs of apoptosis were observed after treatment with NVP-BEZ235 in monolayer cultures (consistent with the lack of effects on cell proliferation, Fig. 3A). NVP-BEZ235 was also previously reported not to induce apoptosis in various cell lines.[8,29–31] We did, however, observe increased active caspase-3 staining in sections of NVP-BEZ235-treated spheroids (Fig. 4D). We also examined apoptosis in HCT116 spheroids generated from *PI3KCA*$^{wt/-}$ and *PI3KCA*$^{wt/mut}$ cells (Fig. 4D). Higher numbers of cells staining for active caspase-3 were observed in *PI3KCA*$^{wt/-}$ MTS, consistent with the NVP-BEZ235 results. We also considered the possibility that inhibition of the PI3K/AKT/mTOR pathway by NVP-BEZ235 could reduce HCT116 cell size.[32,33] We did not, however, observe any effects of NVP-BEZ235 on the size of HCT116 cells as determined by analysis of forward scatter data from flow cytometry (Fig 4E). We conclude from these experiments that NVP-BEZ235 reduces cell proliferation and induces limited apoptosis of HCT116 cells in MTS. NVP-BEZ235 does not, however, eradicate spheroids and surviving cells retain proliferative capacity.

3.5. *Sequential treatment with proteasome inhibitors and NVP-BEZ235*

The experiments presented above suggested that sequential treatment with MHOK and NVP-235 should be effective in reducing 3-D cell mass. Indeed, treatment with MHOK (day 4) followed by NVP-BEZ235 treatment (day 5–12) was found to lead to a spheroid volume ~15% of that prior to MHOK treatment (Fig. 5A). In comparison, omitting NVP-BEZ235 post-treatment lead to regrowth of MTS. MHOK was found to have a strong cytotoxic effect also on MTS that had been pretreated with NVP-BEZ235 (Fig. 5B). Spheroid volumes were reduced to 29.2 ± 2.5% compared to the volume before MHOK treatment and decreased further during the next few days to <5% of the volume at day 1. These experiments show that combining continuous NVP-BEZ235 treatment with one cycle of treatment with the cytotoxic agent MHOK leads to an almost complete eradication of HCT116 MTS.

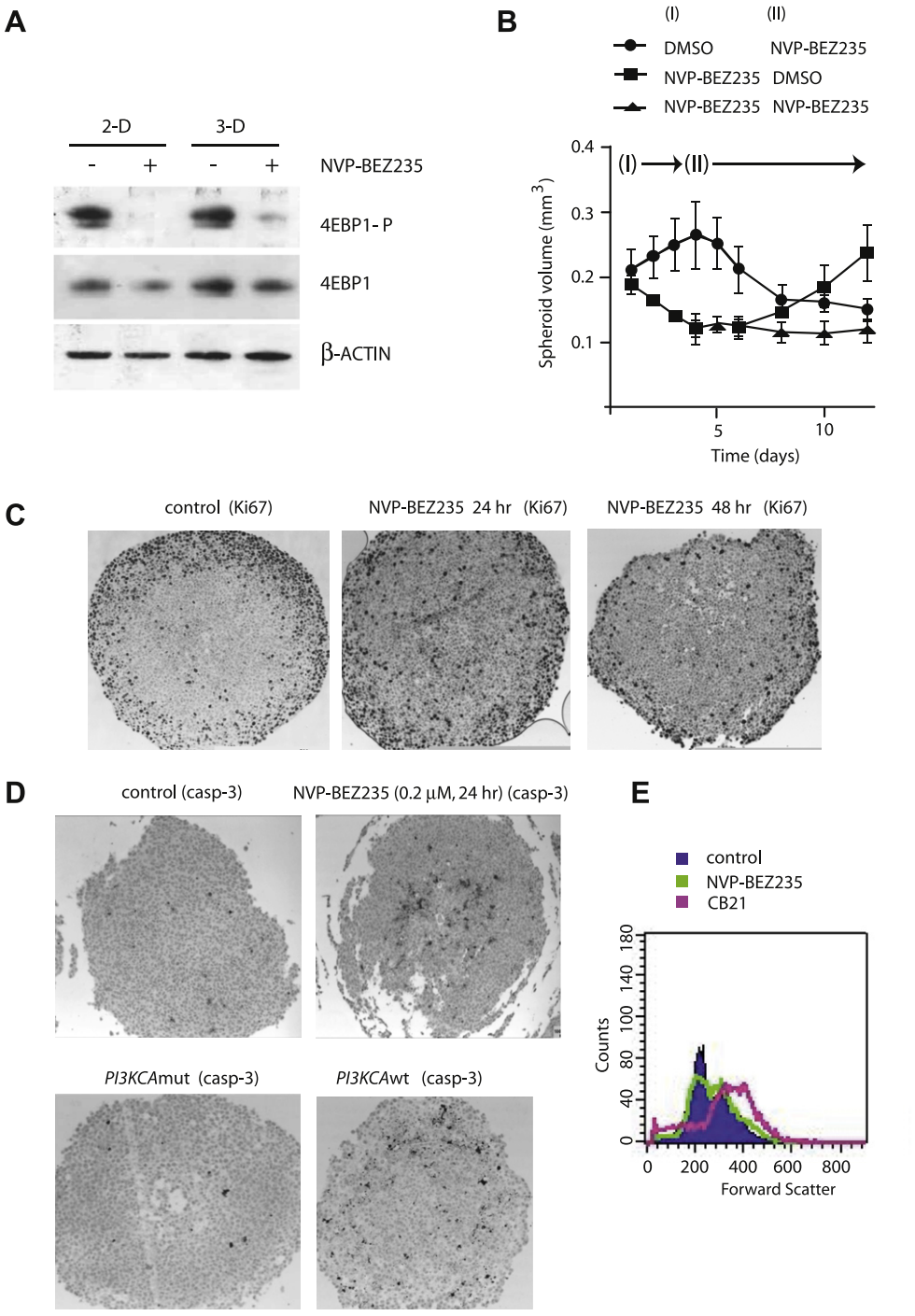

Fig. 4 – Effect of NVP-BEZ235 on HCT116 multicellular tumour spheroids (MTS). (A) NVP-BEZ235 (0.2 μM) inhibits phosphorylation of the mammalian target of rapamycin (mTOR) target 4EBP1 in both monolayer and spheroid cultures. Lysates were prepared after 24 h of treatment and subjected to Western blotting using the indicated antibodies. (B) Effect of NVP-BEZ235 on HCT116 MTS volumes. Cells were treated with NVP-BEZ235 (0.2 μM) or vehicle (0.5% DMSO) as indicated. Measurements were performed in triplicate and mean values + standard deviation are presented. (C) Decrease of Ki67 staining of HCT116 MTS after treatment with NVP-BEZ235. MTS were treated with 0.2 μM NVP-BEZ235 for the indicated times, fixed and stained for Ki67. (D) Increase in caspase-3 staining after treatment with 0.2 μM NVP-BEZ235 and in *PI3KCA*$^{wt/-}$ cells. MTS were treated with 0.2 μM NVP-BEZ235 for 24 h, fixed, sectioned and stained with an antibody to active caspase-3. A similar result was observed in three independent experiments. In the lower panel, HCT116 cells with the indicated genotypes were allowed to form spheroids for 7 days, fixed and stained with an antibody to active caspase-3. (E) HCT116 cells treated for 24 h with 0.2 μM NVP-BEZ235, fixed and analysed on forward scatter by flow cytometry. CB21 is an iron chelator that causes cell cycle arrest and increases in cell size (Hernlund et al., unpublished) and was included as a positive control.

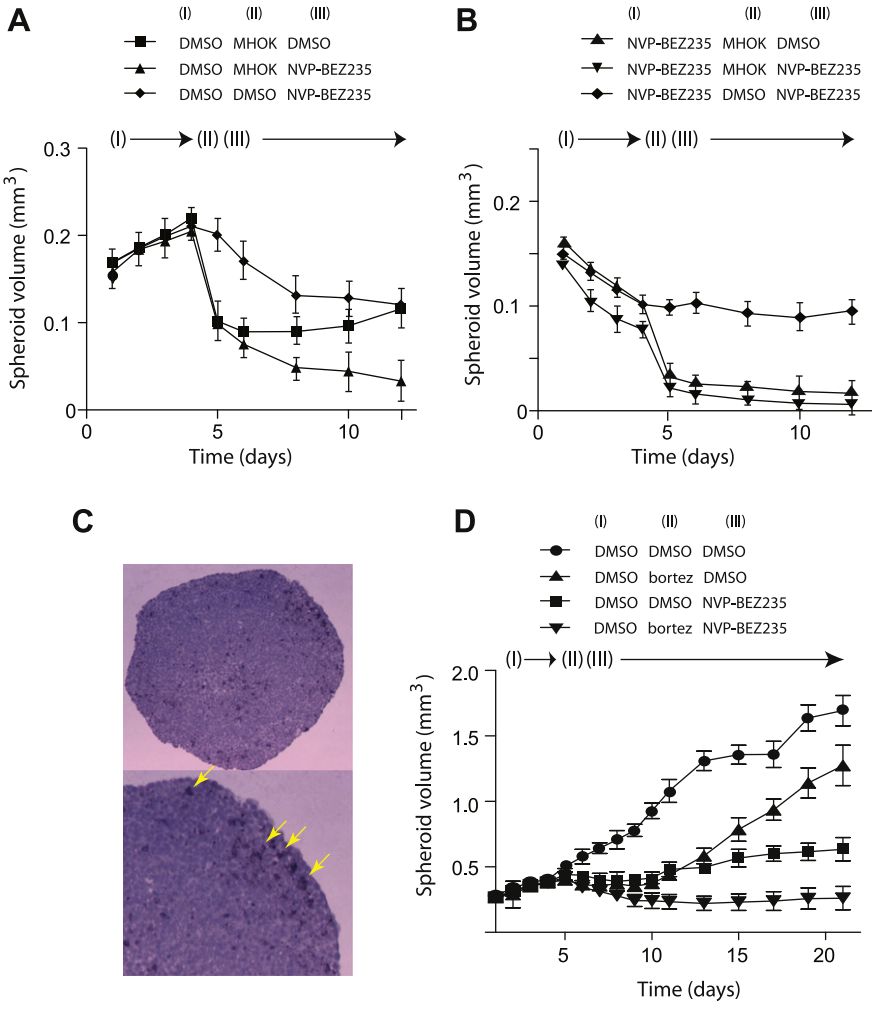

Fig. 5 – NVP-BEZ235 inhibits multicellular tumour spheroids (MTS) regrowth after treatment with ubiquitin–proteasome system (UPS) inhibitors. (A, B) HCT116 MTS were treated with methyl-13-hydroxy-15-oxokaurenoate (MHOK) and NVP-BEZ235 in sequence as indicated. MTS volumes were determined at different time points. (C) MTS were treated with bortezomib for 24 h, fixed, sectioned and stained for active caspase-3. Cells staining positive for active caspase-3 are preferentially observed in peripheral cell layers (examples shown indicated by arrows). (D) HCT116 MTS were treated with bortezomib (0.1 μM) and daily NVP-BEZ235 (0.2 μM) in sequence as shown and volumes were determined. All measurements were performed in triplicate and mean values + standard deviation are presented.

Although MHOK is a useful agent for *in vitro* studies, this agent does not inhibit tumour growth *in vivo* (our unpublished data). Another inhibitor of the UPS, bortezomib (Velcade®) is in clinical use against multiple myeloma. Bortezomib induces apoptosis in several human tumour cell lines,[34–37] but has poor efficiency against human solid tumours.[38] We were therefore interested in determining whether the effect of bortezomib would be potentiated by NVP-BEZ235 in a sequential treatment schedule. As shown in Fig. 5C, and similar to the drugs tested in Fig. 1B, bortezomib induced limited induction of caspase-3 in HCT116 MTS. Spheroids treated with 0.1 μM bortezomib for 24 h followed by a change to drug free-medium decreased in size over the first 3 days by ~50% compared to control (Fig. 5D). Regrowth of the spheroids occured at later times. The regrowth is efficiently inhibited by the daily addition of 0.2 μM NVP-BEZ235 (Fig 5D).

4. Discussion

A major problem in treatment of solid tumours is that hypoxic and quiescent cell populations distant from tumour vessels may not be reached by anticancer drugs at effective concentrations. Combining different drugs may not necessarily solve this problem. In fact, drugs that inhibit cell proliferation may antagonise the activity of cytotoxic drugs,[39–41] an expected effect since the proliferative state of the tumour cell population is of key importance for determining sensitivity to many anticancer agents.[42] Even in situations where drug combinations are synergistic on cells cultured in monolayers, results may not apply *in vivo* since drugs may not necessarily reach the populations of cells in 3-D tumour tissue at the necessary concentrations at the same point in time. Testing modalities such as sequential treatment is difficult to perform on monolayer cultures, and results are difficult to interpret since monolayer cultures do not

contain populations of hypoxic and quiescent cells present in solid tumours. We here studied the sequential treatment of 3-D multicellular colon cancer spheroids with cytotoxic proteasome inhibitors and a PI3K/mTOR inhibitor. The identification and use of MHOK made it possible to study the kinetics of cell cycle entry of cells in previously hypoxic areas in HCT116 MTS. We found that previously quiescent tumour cells became positive for cyclin A and Ki67 within 18 h after exposure to oxygen and nutrients, showing that reproliferation of tumour cells can – at least under some conditions – be initiated rapidly. The PI3K/mTOR inhibitor NVP-BEZ235 was found to be effective in blocking regrowth of MTS after MHOK treatment. The combination of MHOK and NVP-BEZ235 reduced the volume of HCT116 MTS to <5% of the initial volume; a quite dramatic effect considering numerous reports of resistance of MTS to chemotherapeutical agents (see[11,21,43]). MHOK is not effective *in vivo* (our unpublished data) and we also examined whether NVP-BEZ235 would inhibit regrowth after treatment with the proteasome inhibitor bortezomib (Velcade®), approved for treatment of multiple myeloma. Bortezomib induces apoptosis in several human tumour cell lines[34–37] (see www.dtp.nci.nih.gov for activity on the NCI60 panel), but has poor efficiency against human solid tumours.[38] We indeed found that sequential treatment with NVP-BEZ235 and bortezomib was effective in reducing the size of HCT116 MTS, encouraging further testing of this treatment modality in animal models.

Issues of whether anticancer drugs penetrate tissue efficiently and reach cells at effective concentrations are often neglected.[44,45] NVP-BEZ235 strongly inhibited mTOR signalling in MTS, showing that the drug is able to penetrate into 3-D tissue and reach cells that overexpress the pathway. NVP-BEZ235-treatment resulted in decreased Ki67 and cyclin A labelling. The inhibitor also caused an initial decrease in MTS volume, most likely due to induction of apoptosis. NVP-BEZ235 did not show detectable induction of apoptosis in HCT116 monolayer cultures (not shown), and has generally not been reported to induce apoptosis in other cell types.[8,29–31] It is therefore possible that tumour cells in 3-D tissue are more sensitive to NVP-BEZ235 compared to cells in exponential monolayer culture. The observation of increased apoptosis in MTS formed by HCT116 *PI3KCA*$^{wt/-}$ cells is consistent with this hypothesis. However, despite the induction of apoptosis, spheroid regrowth was observed when NVP-BEZ235 was no longer added, showing that a population of cells remained viable and retained proliferative capacity.

We here show that NSC620358, methyl-13-hydroxy-15-oxokaurenoate, is an inhibitor of the UPS as evidenced gene expression profiling, accumulation of high-molecular-weight ubiquitin complexes and functional blocking of the proteasome. Methyl-13-hydroxy-15-oxokaurenoate is chemically closely related to *ent*-kaurenoate, commonly found it many plants. NSC620358 is cytotoxic to most cell lines in the NCI60 panel, but is particularly active on leukaemia cell lines (average GI50 on leukaemia cell lines: 0.27 μM, average GI50 on all cell lines: 1.5 μM; www.dtp.nci.nih.gov). MHOK was found to induce apoptosis of an FaDu xenograft model *in vivo* as evidenced by increases in levels of the caspase-cleaved keratin 18 (ccK 18) plasma biomarker,[46] but tumour growth was not significantly affected (data not shown). The compound may therefore affect a minor fraction of cells also *in vivo*, leading to shedding of apoptosis products into blood from cells situated close to tumour vessels, but not to a major response in terms of tumour regression. MHOK is, however, useful as a reagent for studies of MTS due to its ability to induce cell death of peripheral cells.

Multicellular spheroids have been used as a model to study regrowth after cytotoxic therapy[14] and represent simple models to evaluate the principles of how drugs can be combined to eradicate solid tumours. A limitation of the MTS system is that although a number of different cell lines form MTS,[47] not all cell lines do so. We here aimed to examine the growth of HCT116 and DLD-1 colon carcinoma spheroids with wild-type and mutant *PI3KCA* alleles, but only obtained compact spheroids using HCT116 cells. DLD-1 cells have been reported to form spheroids,[48] but the strain tested here did not generate compact spheroids. HCT116 cells, on the other hand, form compact spheroids containing hypoxic and quiescent cells in the cores. These cells expressed the cyclin-dependent kinase inhibitor p27^{Kip1}, previously found to be induced by hypoxia[18,49,50] and essential for blocking cell cycle progression in hypoxic cells.[18]

Preclinical drug testing should routinely involve monolayer cultures, 3-D culture systems and animal models. Our results suggest that 3-D multicellular spheroids may be more sensitive to PI3K/mTOR inhibition compared to monolayer cells and suggest that treatment modalities where bortezomib (or other cytotoxic therapies) are used in sequence with NVP-BEZ235 may be worth exploring.

Role of the funding source

The funding sources had no role in the study design, collection, analysis and interpretation of data.

Conflict of interest statement

None declared.

Acknowledgements

We are grateful to Bert Vogelstein and Victor Velculescu at the Howard Hughes Medical Institute, The Johns Hopkins University, Baltimore, for the kind gifts on HCT116 cells with a disrupted *PI3KCA* mutant allele. The work was supported by Cancerfonden, Radiumhemmets Forskningsfonder, Vetenskapsrådet, Chemores (EU FP6, Grant number: LSHC-CT-2007-037665), VINNOVA and SSF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.11.013.

REFERENCES

1. Preisler HD, Venugopal P. Regrowth resistance in cancer: why has it been largely ignored? *Cell Prolif* 1995;**28**(6):347–56.
2. Preisler HD, Raza A, Bonomi P, et al. Regrowth resistance as a likely significant contributor to treatment failure in drug-sensitive neoplastic diseases. *Cancer Invest* 1997;**15**(4):358–68.
3. Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nat Rev Cancer* 2005;**5**(7):516–25.
4. Simon R, Norton L. The Norton–Simon hypothesis: designing more effective and less toxic chemotherapeutic regimens. *Nat Clin Pract Oncol* 2006;**3**(8):406–7.
5. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* 2008;**27**(41):5497–510.
6. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;**129**(7):1261–74.
7. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the *PIK3CA* gene in human cancers. *Science* 2004;**304**(5670):554.
8. Maira SM, Stauffer F, Brueggen J, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther* 2008;**7**(7):1851–63.
9. Sutherland RM, Durand RE. Growth and cellular characteristics of multicell spheroids. *Recent Results Cancer Res* 1984;**95**:24–49.
10. Mueller-Klieser W. Multicellular spheroids. A review on cellular aggregates in cancer research. *J Cancer Res Clin Oncol* 1987;**113**(2):101–22.
11. Friedrich J, Ebner R, Kunz-Schughart LA. Experimental anti-tumor therapy in 3-D: spheroids – old hat or new challenge? *Int J Radiat Biol* 2007;**83**(11):849–71.
12. Hirschhaeuser F, Menne H, Dittfeld C, et al. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 2010;**148**(1):3–15.
13. Demicheli R, Foroni R, Ingrosso A, et al. An exponential-Gompertzian description of LoVo cell tumor growth from in vivo and in vitro data. *Cancer Res* 1989;**49**(23):6543–6.
14. Durand RE. Multicell spheroids as a model for cell kinetic studies. *Cell Tissue Kinet* 1990;**23**(3):141–59.
15. Herrmann R, Fayad W, Schwarz S, Berndtsson M, Linder S. Screening for compounds that induce apoptosis of cancer cells grown as multicellular spheroids. *J Biomol Screen* 2008;**13**(1):1–8.
16. Friedrich J, Eder W, Castaneda J, et al. A reliable tool to determine cell viability in complex 3-d culture: the acid phosphatase assay. *J Biomol Screen* 2007;**12**(7):925–37.
17. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng* 2003;**83**(2):173–80.
18. Gardner LB, Li Q, Park MS, et al. Hypoxia inhibits G1/S transition through regulation of p27 expression. *J Biol Chem* 2001;**276**(11):7919–26.
19. Ikediobi ON, Davies H, Bignell G, et al. Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. *Mol Cancer Ther* 2006;**5**(11):2606–12.
20. Graeber TG, Peterson JF, Tsai M, et al. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol* 1994;**14**(9):6264–77.
21. Fayad W, Brnjic S, Berglind D, et al. Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model. *Int J Cancer* 2009;**125**(10):2450–5.
22. Fayad W, Rickardson L, Haglund C, et al. Identification of agents that induce apoptosis of multicellular tumour spheroids: enrichment for mitotic inhibitors with hydrophobic properties. *Chem Biol Drug Des* 2011;**78**(4):547–57.
23. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science* 2007;**318**(5853):1108–13.
24. Samuels Y, Diaz Jr LA, Schmidt-Kittler O, et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* 2005;**7**(6):561–73.
25. Yun J, Rago C, Cheong I, et al. Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* 2009;**325**(5947):1555–9.
26. Marone R, Erhart D, Mertz AC, et al. Targeting melanoma with dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitors. *Mol Cancer Res* 2009;**7**(4):601–13.
27. Liu TJ, Koul D, LaFortune T, et al. NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. *Mol Cancer Ther* 2009;**8**(8):2204–10.
28. Baumann P, Mandl-Weber S, Oduncu F, Schmidmaier R. The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma. *Exp Cell Res* 2009;**315**(3):485–97.
29. Manara MC, Nicoletti G, Zambelli D, et al. NVP-BEZ235 as a new therapeutic option for sarcomas. *Clin Cancer Res*;**16**(2):530–40.
30. Konstantinidou G, Bey EA, Rabellino A, et al. Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. *Cancer Res* 2009;**69**(19):7644–52.
31. Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. *Proc Natl Acad Sci USA* 2009;**106**(46):19503–8.
32. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev* 2002;**16**(12):1472–87.
33. Kozma SC, Thomas G. Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. *BioEssays* 2002;**24**(1):65–71.
34. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999;**59**(11):2615–22.
35. Ling YH, Liebes L, Jiang JD, et al. Mechanisms of proteasome inhibitor PS-341-induced G(2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clin Cancer Res* 2003;**9**(3):1145–54.
36. Yin D, Zhou H, Kumagai T, et al. Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene* 2005;**24**(3):344–54.
37. Milano A, Iaffaioli RV, Caponigro F. The proteasome: a worthwhile target for the treatment of solid tumours? *Eur J Cancer* 2007;**43**(7):1125–33.
38. Caravita T, de Fabritiis P, Palumbo A, Amadori S, Boccadoro M. Bortezomib: efficacy comparisons in solid tumors and hematologic malignancies. *Nat Clin Pract Oncol* 2006;**3**(7):374–87.
39. Xu JM, Azzariti A, Colucci G, Paradiso A. The effect of gefitinib (Iressa, ZD1839) in combination with oxaliplatin is schedule-dependent in colon cancer cell lines. *Cancer Chemother Pharmacol* 2003;**52**(6):442–8.
40. Troiani T, Lockerbie O, Morrow M, Ciardiello F, Eckhardt SG. Sequence-dependent inhibition of human colon cancer cell growth and of prosurvival pathways by oxaliplatin in combination with ZD6474 (Zactima), an inhibitor of VEGFR

and EGFR tyrosine kinases. *Mol. Cancer Ther* 2006;**5**(7): 1883–94.
41. Morelli MP, Cascone T, Troiani T, et al. Sequence-dependent antiproliferative effects of cytotoxic drugs and epidermal growth factor receptor inhibitors. *Ann Oncol* 2005;**16**(Suppl. 4):iv61–8.
42. Valeriote F, van Putten L. Proliferation-dependent cytotoxicity of anticancer agents: a review. *Cancer Res* 1975;**35**(10):2619–30.
43. Olive PL, Durand RE. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev* 1994;**13**(2):121–38.
44. Erlanson M, Daniel-Szolgay E, Carlsson J. Relations between the penetration, binding and average concentration of cytostatic drugs in human tumour spheroids. *Cancer Chemother Pharmacol* 1992;**29**(5):343–53.
45. Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer* 2006;**6**(8):583–92.
46. Olofsson MH, Cummings J, Fayad W, et al. Specific demonstration of drug-induced tumour cell apoptosis in human xenografts models using a plasma biomarker. *Cancer Biomark* 2009;**5**(3):117–25.
47. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. *Nat Protoc* 2009;**4**(3):309–24.
48. Mellor HR, Ferguson DJ, Callaghan R. A model of quiescent tumour microregions for evaluating multicellular resistance to chemotherapeutic drugs. *Br J Cancer* 2005;**93**(3): 302–309.
49. Krtolica A, Krucher NA, Ludlow JW. Molecular analysis of selected cell cycle regulatory proteins during aerobic and hypoxic maintenance of human ovarian carcinoma cells. *Br J Cancer* 1999;**80**(12):1875–83.
50. Masuda A, Osada H, Yatabe Y, et al. Protective function of p27(KIP1) against apoptosis in small cell lung cancer cells in unfavorable microenvironments. *Am J Pathol* 2001;**158**(1):87–96.