

# Combined targeting of MAPK and AKT signalling pathways is a promising strategy for melanoma treatment

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## Summary

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### Key words

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### Conflicts of interest

None declared.

**Background** In melanoma, several signalling pathways are constitutively activated. Among them, the RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT (AKT) signalling pathways are activated through multiple mechanisms and appear to play a major role in melanoma development and progression.

**Objectives** In this study, we examined whether targeting the MAPK and/or AKT signalling pathways would have therapeutic effects against melanoma.

**Methods** Using a panel of pharmacological inhibitors (BAY 43-9006, PD98059, U0126, wortmannin, LY294002) we inhibited the MAPK and AKT signalling pathways at different levels and evaluated the effects on growth, survival and invasion of melanoma cells in monolayer and organotypic skin culture.

**Results** Antiproliferative and proapoptotic effects of inhibitors alone in monolayer culture were disappointing and varied among the different cell lines. In contrast, combined targeting of the MAPK and AKT signalling pathways significantly inhibited growth and enhanced apoptosis in monolayer culture. To verify our data in a more physiological context we incorporated melanoma cells into regenerated human skin mimicking the microenvironment of human melanoma. Combinations of MAPK and AKT inhibitors completely suppressed invasive tumour growth of melanoma cells in regenerated human skin.

**Conclusions** Combined targeting of MAPK and AKT signalling pathways is a promising strategy for melanoma treatment and should encourage further in-depth investigations.

Malignant melanoma is among the human cancers whose incidence has increased most rapidly in the last few decades. Malignant melanoma presents a therapeutic challenge. When diagnosed early, melanoma is highly curable by surgical excision with adequate safety margins. However, advanced melanoma is highly resistant to all current forms of therapy. One of the major goals of melanoma research has therefore been to identify molecular targets for the development of novel treatment strategies.

A recent discovery has excited the melanoma research community. Davies *et al.*<sup>1</sup> detected BRAF somatic missense mutations in 60–70% of melanoma cell lines and tissues resulting in constitutive activation of BRAF kinase activity. Emerging data further indicated that in melanoma not only the RAS/RAF/MEK/ERK (MAPK) signalling pathway but also the PI3K/AKT (AKT) signalling pathway are constitutively activated through multiple mechanisms.<sup>2</sup> Strong p-AKT expression was

observed in 49% and 77% of the biopsies in primary melanomas and melanoma metastases, respectively.<sup>2</sup> The MAPK and AKT signalling pathways modulate the function of numerous substrates involved in the regulation of cell growth, survival and invasion. Furthermore, adhesion molecules such as  $\alpha\text{v}\beta\text{3}$  integrin and MelCAM, which are critical in melanoma progression, appear to be differentially regulated by MAPK and AKT.<sup>3–5</sup> Therefore, in order to knock out the critical adhesion molecules and signalling pathways, it appears to be necessary to target both the MAPK and AKT signalling pathways at the same time. This hypothesis is supported by the recent observation that BAY 43-9006, a potent RAF kinase inhibitor, did not elicit a sufficient response as a monotherapy in patients with advanced melanoma.<sup>6,7</sup> Herein, we examine the effects of targeting the MAPK and AKT signalling pathways on growth, survival and invasion of melanoma cells in monolayer and organotypic skin culture.

## Materials and methods

### Isolation and culture of human cells

Six metastatic melanoma cell lines (451Lu, SKMel28, 1205Lu, WM852, SKMel19, Mewo) were screened for their susceptibility to single inhibition of the MAPK or AKT pathway. As 451Lu and SKMel28 cells showed similar sensitivities to the other tested melanoma cell lines, we focused on these two well-characterized metastatic melanoma cell lines. Both cell lines harbour the BRAF (V600E) mutation. AKT is activated in both cell lines. Human metastatic melanoma cells (451Lu, SKMel28) were cultured in MCDB153/L15 medium containing 5 µg mL<sup>-1</sup> insulin and 2% fetal bovine serum (FBS) and in RPMI 1640 medium supplemented with 10% FBS, respectively.

After obtaining informed consent, human fibroblasts were isolated from human foreskin following routine circumcision. The skin samples were stored at 4 °C in Hank's balanced salt solution without Ca<sup>2+</sup> or Mg<sup>2+</sup> containing penicillin, gentamicin and amphotericin. Following the method described by Pittelkow and Scott<sup>8</sup> the subcutaneous fat was trimmed off and the remaining cutis cut into pieces and digested in solution B containing 0.25% trypsin as active ingredient for ≈ 19 h at 4 °C. The action of the trypsin was stopped with solution A following which the epidermis was separated from the dermis. Fibroblasts were obtained from dermal explants of human foreskin and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Fibroblasts up to passage 7 were used for organotypic human skin cultures.

### Reconstruction of human melanomas in organotypic human skin culture

The *in vitro* reconstruction of melanoma is based on the organotypic human skin culture technique.<sup>9</sup> A cell-free buffered collagen solution was prepared consisting of rat tail collagen type I (BD Biosciences, Bedford, MA, U.S.A.) at a final concentration of 1.35 mg mL<sup>-1</sup> in DMEM with 10% FBS; 1.0 mL of the cell-free collagen solution was added to tissue culture inserts (Millicell PC, Millipore, Bedford, MA, U.S.A.) placed in six-well tissue culture plates. While the acellular collagen layer was solidifying, a second collagen solution was prepared similar to the first with the addition of human fibroblasts and 451Lu or SKMel28 human metastatic melanoma cells. Fibroblasts and melanoma cells from subconfluent cultures were trypsinized, washed and resuspended in the second collagen solution at a density of 15 × 10<sup>5</sup> mL<sup>-1</sup> and a fibroblast to melanoma cell ratio of 1 : 1; 3.0 mL of the fibroblast and melanoma cell-containing collagen solution were placed over the solidified acellular collagen layer. After 5 days of incubation at 37 °C, the fibroblast contraction force causes the collagen gel to contract. This structure represents the reconstructed melanoma in organotypic skin culture. For submerged culture conditions, 3 mL of melanoma cell culture medium supplemented with

10% FBS were added beneath the insert and 2 mL inside the insert. The culture medium was changed every 2 days. After 10–14 days of submerged culture, the melanoma reconstructs were harvested. Melanoma reconstructs were fixed with 4% formaldehyde for 8–9 h, dehydrated, and embedded in paraffin. The paraffin sections were stained with haematoxylin and eosin for routine light microscopy.

### Treatment of melanoma cells with signalling pathway inhibitors

For blockade of the MAPK and AKT signalling pathways, the RAF kinase inhibitor BAY 43-9006 (Bayer Corporation, West Haven, CT, U.S.A.), the MEK inhibitors PD98059 and U0126 (Cell Signaling Technology, Beverly, MA, U.S.A.) and the PI3K inhibitors wortmannin (Sigma, Steinheim, Germany) and LY294002 (Cell Signaling Technology) alone or in combination were added directly to the culture medium of melanoma cells in monolayer or organotypic skin culture at the given final concentrations. Melanoma cells treated with culture medium or culture medium with the addition of dimethyl sulphoxide (DMSO) served as controls.

### Validation of signalling pathway inhibition by Western blot analysis

Melanoma cells were plated in T75 tissue flasks in 50% culture medium with 50% fibroblast-conditioned medium. Control cells were cultured in this medium for 2–48 h with or without 0.05% DMSO. Inhibitor-treated cells were incubated immediately after plating with MAPK or AKT signalling pathway inhibitors alone or in combination at the given final concentrations. After 2–48 h adherent cells were washed with phosphate-buffered saline (PBS) and lysed for 10 min on ice in 600 µL buffer containing 10 mmol L<sup>-1</sup> Tris pH 7.5, 100 mmol L<sup>-1</sup> NaCl, 10 mmol L<sup>-1</sup> ethylenediamine tetraacetic acid, 0.5% Triton X-100, 0.5% sodium-deoxycholate. Cell lysates were clarified by centrifugation, 30 µg protein subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking in PBS/0.3% Tween-20/5% dry milk, the membranes were probed with anti-ERK (1 : 1000), anti-phospho-ERK (Thr202/Tyr204; 1 : 1000), anti-AKT (1 : 1000) or anti-phospho-AKT (Ser473; 1 : 1000) primary antibodies (Cell Signaling Technology). Blots were developed with horseradish peroxidase-conjugated secondary antibodies (1 : 10 000; DIANOVA, Hamburg, Germany) and developed by exposure using enhanced chemiluminescence.

### Growth assay

Cells were seeded as triplicates in 96-well plates at a density of 1500 cells per well in 150 µL medium (1 × 10<sup>4</sup> cells mL<sup>-1</sup>). MAPK or AKT signalling pathway inhibitors alone or in combination were added directly to the culture medium at the given final concentrations. Cells treated with

culture medium with or without DMSO served as controls. The assay was started following incubation for 72 h. The medium was discarded, each well was washed twice with PBS (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and 100  $\mu\text{L}$  of a solution containing 100  $\mu\text{g}$  MUH (4-methylumbelliferyl-heptanoate)  $\text{mL}^{-1}$  PBS was added. Plates were incubated at 37 °C for 1 h and measured in a Fluoroskan II (Labsystems, Helsinki, Finland), with a  $\lambda_{\text{em}}$  of 355 nm and a  $\lambda_{\text{ex}}$  of 460 nm. The intensity of fluorescence indicates the number of vital cells in the wells.<sup>10</sup>

## Apoptosis assays

### Annexin staining

Melanoma cells were treated with 50% culture medium plus 50% fibroblast-conditioned medium with or without DMSO as controls, inhibitors (6  $\mu\text{mol L}^{-1}$  BAY 43-9006, 50  $\mu\text{mol L}^{-1}$  PD98059, 20  $\mu\text{mol L}^{-1}$  U0126, 4  $\mu\text{mol L}^{-1}$  wortmannin, 50  $\mu\text{mol L}^{-1}$  LY294002) or inhibitor combinations (BAY 43-9006 + wortmannin, PD98059 + LY294002, U0126 + LY294002) and incubated for 48 h. Cells were washed twice in cold PBS and resuspended in 1X binding buffer (BD Biosciences, Heidelberg, Germany) at a concentration of  $1 \times 10^6$  cells  $\text{mL}^{-1}$ ; 100  $\mu\text{L}$  of each solution ( $1 \times 10^5$  cells) were transferred to a 5-mL culture tube; 5  $\mu\text{L}$  of Annexin V-FITC and 5  $\mu\text{L}$  of propidium iodide (both, BD Biosciences, Heidelberg) were added. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Then 400  $\mu\text{L}$  of the 1X binding buffer were added to each tube. Cells were analysed by flow cytometry on a FACScalibur using CellQuest software (BD Biosciences, Heidelberg). The number of apoptotic cells was determined as the percentage of annexin-positive/propidium iodide-negative cells.

### Cell death detection enzyme-linked immunosorbent assay

After treatment of melanoma cells with culture medium or culture medium plus DMSO as controls, 6  $\mu\text{mol L}^{-1}$  BAY 43-9006, 4  $\mu\text{mol L}^{-1}$  wortmannin or 6  $\mu\text{mol L}^{-1}$  BAY 43-9006 combined with 4  $\mu\text{mol L}^{-1}$  wortmannin for 48 h, apoptosis was quantified by a cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates was detected by biotinylated antihistone- and peroxidase-coupled anti-DNA antibodies and was calculated according to the formula: absorbance of sample cells/absorbance of control cells. This factor was used as a parameter of apoptosis and is given as the mean  $\pm$  SD of duplicates.

### Migration and invasion assay

Migration assays were performed in Boyden chambers containing polycarbonate filters with a pore size of 8  $\mu\text{m}$  (Control

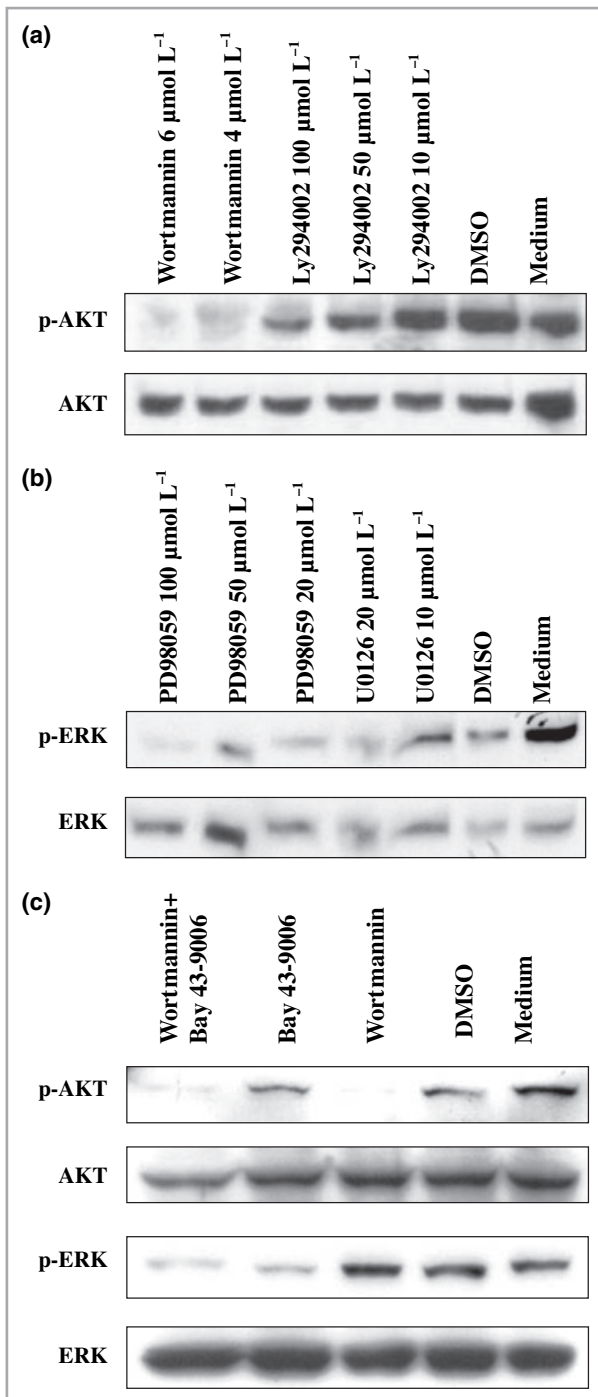
inserts, BD Biosciences, Heidelberg). For analysis of cell invasion polycarbonate filters with the same pore size were used coated with a Matrigel basement membrane matrix (BD Bio-coat Matrigel invasion chambers, BD Biosciences, Heidelberg). Metastatic SKMel28 melanoma cells were plated at a cell density of  $8 \times 10^4$  cells/chamber in fibroblast-conditioned medium with or without DMSO and inhibitors (6  $\mu\text{mol L}^{-1}$  BAY 43-9006, 4  $\mu\text{mol L}^{-1}$  wortmannin, 6  $\mu\text{mol L}^{-1}$  BAY 43-9006 + 4  $\mu\text{mol L}^{-1}$  wortmannin, 50  $\mu\text{mol L}^{-1}$  PD98059, 50  $\mu\text{mol L}^{-1}$  LY294002, 50  $\mu\text{mol L}^{-1}$  PD + 50  $\mu\text{mol L}^{-1}$  LY294002), respectively. After 2 h, the lower compartment, filled with RPMI 1640 medium without FCS, was replaced with fibroblast-conditioned medium containing 10% FCS as a chemoattractant. After incubation for 24 h at 37 °C noninvading cells remaining on the upper surface of the chamber were removed by scrubbing with a cotton-tipped swab and the invaded cells adhering to the bottom surface of the chamber membrane were fixed and counted after cell staining with haematoxylin and eosin. The assays were performed in triplicate; at least six fields were counted per filter and mean cell numbers with their SDs were calculated.

## Results

### Combined inhibition of MAPK and AKT signalling pathways significantly inhibits melanoma cell growth

For blockade of the MAPK and AKT signalling pathways a panel of pharmacological inhibitors was used: the RAF kinase inhibitor BAY 43-9006, the MEK inhibitors PD98059 and U0126 and the PI3K inhibitors wortmannin and LY294002. The efficacy of the selected signalling pathway inhibitors in melanoma cells was verified by Western blot analysis for total and phosphorylated ERK and AKT, respectively. The PI3K inhibitor LY294002 was effective at a concentration of 50  $\mu\text{mol L}^{-1}$  (Fig. 1a). Testing different dosages of the MEK inhibitors PD98059 and U0126 we found that the lowest dosage effective in inhibition of phosphorylation of ERK was 50  $\mu\text{mol L}^{-1}$  PD98059 and 20  $\mu\text{mol L}^{-1}$  U0126 (Fig. 1b). As previously described, 6  $\mu\text{mol L}^{-1}$  BAY 43-9006 or 4  $\mu\text{mol L}^{-1}$  wortmannin effectively inhibited phosphorylation of ERK and AKT, respectively (Fig. 1c).<sup>3,6</sup> The combined blockade of both pathways using 6  $\mu\text{mol L}^{-1}$  BAY 43-9006 and 4  $\mu\text{mol L}^{-1}$  wortmannin resulted in inhibition of both ERK and AKT (Fig. 1c).

The effects of inhibition of MAPK and AKT signalling pathways on growth of metastatic melanoma cells (451Lu, SKMel28) in monolayer culture were determined by a fluorimetric assay using MUH (Fig. 2). Whereas both cell lines lack NRAS mutations they harbour the BRAF (V600E) mutation. AKT is activated by phosphorylation in both cell lines after stimulation. Little or no effect on the number of proliferating cells was seen when comparing monolayer cultures from control melanoma cells and melanoma cells treated with the PI3K inhibitors wortmannin at dosages ranging from 4  $\mu\text{mol L}^{-1}$  to 10  $\mu\text{mol L}^{-1}$  or LY294002 at dosages from 1  $\mu\text{mol L}^{-1}$  to



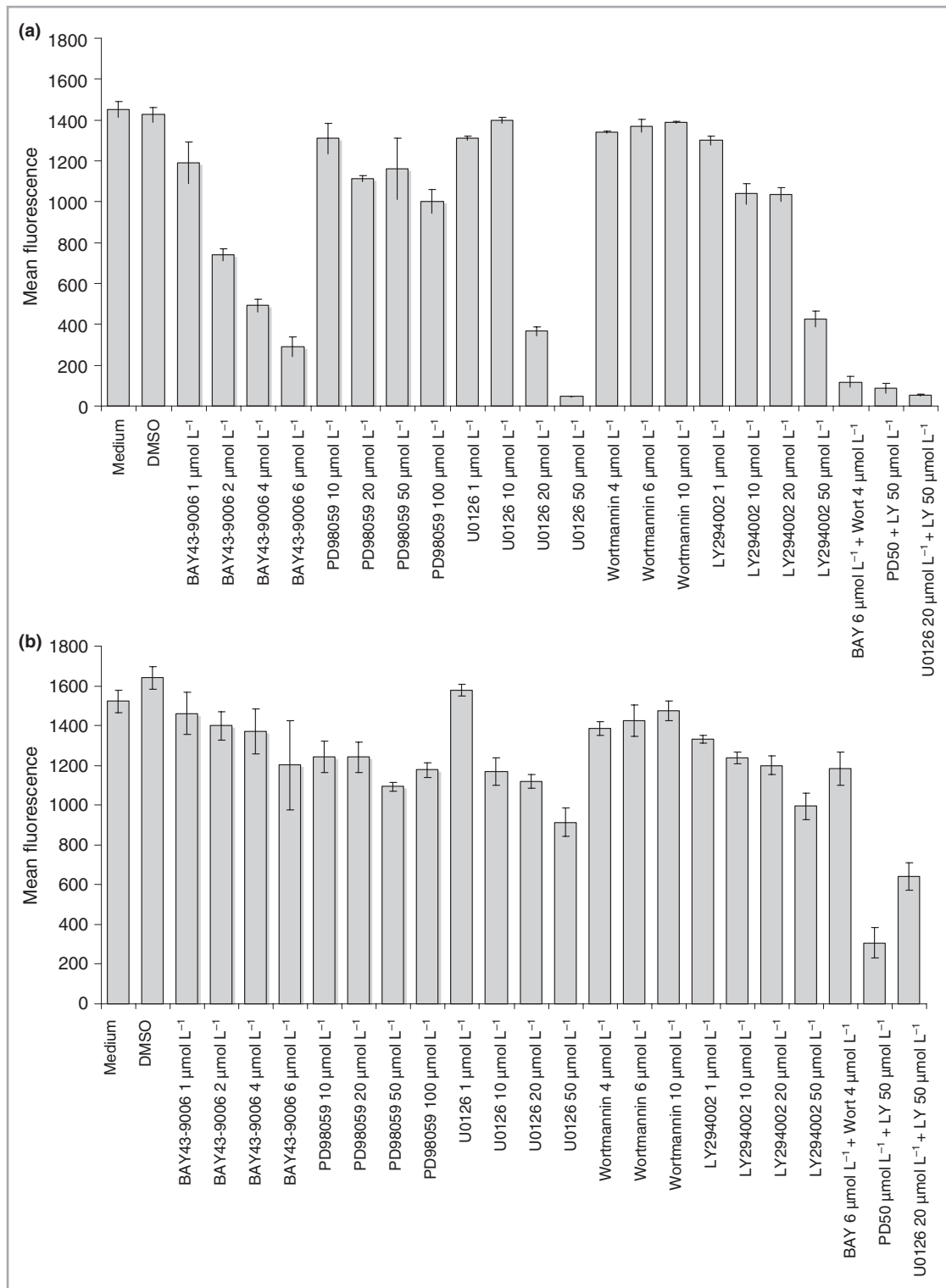
20  $\mu\text{mol L}^{-1}$  (Fig. 2a,b). At a dosage of 50  $\mu\text{mol L}^{-1}$  LY294002 reduced growth in 451Lu and SKMel28 cells, and was most pronounced in 451Lu cells. The proliferation rate of 451Lu cells was significantly reduced after treatment with the RAF kinase inhibitor BAY 43-9006 at 6  $\mu\text{mol L}^{-1}$  and the MEK inhibitor U0126 at 20  $\mu\text{mol L}^{-1}$  whereas the number of viable 451Lu cells after treatment with the MEK inhibitor PD98059 at dosages from 10  $\mu\text{mol L}^{-1}$  to 100  $\mu\text{mol L}^{-1}$  was not significantly reduced (Fig. 2a). Generally, 451Lu cells

Fig 1. The MAPK inhibitors BAY 43-9006, PD98059 and U0126 and the PI3K/AKT inhibitors wortmannin and LY294002 inhibit the MAPK and AKT signalling pathways, respectively. (a) Western blot analysis of cell lysates from metastatic melanoma cells 1 h after treatment with culture medium or medium plus DMSO as controls and the PI3K/AKT inhibitors LY294002 at 10, 50 and 100  $\mu\text{mol L}^{-1}$  and wortmannin at 4 and 6  $\mu\text{mol L}^{-1}$ . The data indicate that at dosages of 50  $\mu\text{mol L}^{-1}$  LY294002 and 4  $\mu\text{mol L}^{-1}$  wortmannin the phosphorylation of AKT was affected. (b) Western blot analysis of cell lysates from metastatic melanoma cells 1 h after treatment with culture medium or medium plus DMSO as controls, the MEK inhibitor U0126 at 10 and 20  $\mu\text{mol L}^{-1}$  and the MEK inhibitor PD98059 at 20, 50 and 100  $\mu\text{mol L}^{-1}$ . The data indicate that at dosages of 20  $\mu\text{mol L}^{-1}$  U0126 and 50  $\mu\text{mol L}^{-1}$  PD98059 the phosphorylation of ERK was affected. (c) Western blot analysis of cell lysates from metastatic melanoma cells 48 h after treatment with culture medium or medium plus DMSO as controls, wortmannin (4  $\mu\text{mol L}^{-1}$ ), BAY 43-9006 (6  $\mu\text{mol L}^{-1}$ ) and wortmannin (4  $\mu\text{mol L}^{-1}$ ) plus BAY 43-9006 (6  $\mu\text{mol L}^{-1}$ ) for total ERK, the phosphorylated form of ERK (p-ERK), total AKT and the phosphorylated form of AKT (p-AKT). At the doses used wortmannin specifically inhibits the AKT pathway, BAY 43-9006 the MAPK pathway, and wortmannin plus BAY 43-9006 both signalling pathways.

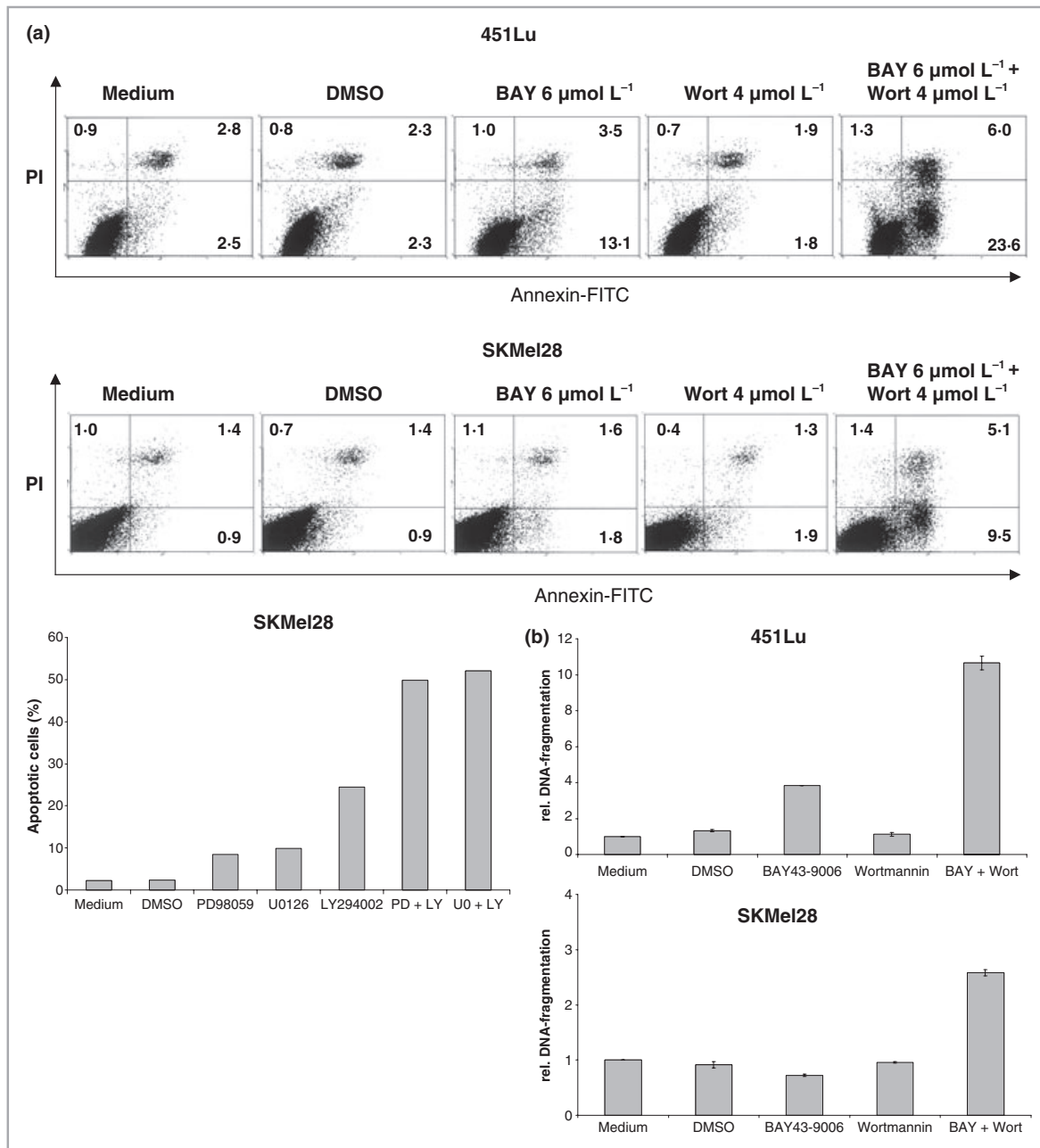
appeared to be more sensitive to MAPK and AKT inhibitors compared with SKMel28 cells. Of interest, combinations of MAPK and AKT inhibitors resulted in a dramatic reduction of the number of viable 451Lu and SKMel28 melanoma cells in most cases (Fig. 2a,b).

### Combined inhibition of MAPK and AKT signalling pathways significantly enhances apoptosis in melanoma cells

To investigate whether blockade of the MAPK and AKT signalling pathways induces apoptosis, monolayer cultures of 451Lu and SKMel28 metastatic melanoma cells were treated with DMSO as control, BAY 43-9006 (6  $\mu\text{mol L}^{-1}$ ), wortmannin (4  $\mu\text{mol L}^{-1}$ ) or a combination of both inhibitors. Apoptotic cells were identified by Annexin V-FITC staining (Fig. 3a) 48 h after beginning of treatment. The data show that at the dosages used the RAF kinase inhibitor BAY 43-9006 induced apoptosis in 451Lu but not in SKMel28 melanoma cells, whereas the PI3K inhibitor wortmannin did not increase the number of apoptotic cells in both cell lines. However, wortmannin significantly enhanced the proapoptotic effect of BAY 43-9006 in 451Lu and SKMel28 melanoma cells (Fig. 3a). These data were confirmed by measuring DNA fragmentation (Fig. 3b). Furthermore, simultaneous blockade of the MAPK and AKT signalling pathways using the MEK inhibitors PD98059 or U0126 and the PI3K inhibitor LY294002 dramatically increased the number of apoptotic cells (Fig. 3a). Taken together, the simultaneous blockade of both signalling pathways resulted in a highly augmented induction of apoptosis compared with the blockade of only one signalling pathway.



**Fig 2.** Combined inhibition of MAPK and AKT signalling pathways significantly inhibits melanoma cell growth. Growth assay of 451Lu (a) and SKMel28 (b) metastatic melanoma cells in monolayer culture using a fluorimetric assay with 4-methylumbelliferyl heptanoate (MUH). The intensity of fluorescence, given as mean values, indicates the number of viable cells in the wells. The MAPK signalling pathway inhibitors BAY 43-9006, PD98059 and U0126 and the AKT signalling pathway inhibitors wortmannin and LY294002 differently affected cell growth of 451Lu and SKMel28 melanoma cells. 451Lu melanoma cells were more sensitive to inhibitor-induced growth inhibition compared with SKMel28 melanoma cells. Combinations of MAPK and AKT inhibitors significantly decreased growth of both 451Lu and SKMel28 melanoma cells in most cases (451Lu: BAY 43-9006 + wortmannin,  $P = 0.005$ ; PD98059 + LY294002,  $P = 0.0002$ ; U0126 + LY294002,  $P = 0.00002$ . SKMel28: BAY 43-9006 + wortmannin,  $P = 0.903$ ; PD98059 + LY294002,  $P = 0.0003$ ; U0126 + LY294002,  $P = 0.0004$ ).



**Fig 3.** Combined inhibition of MAPK and AKT signalling pathways significantly enhances apoptosis in melanoma cells. (a) Monolayer cultures of metastatic melanoma cells (451Lu, SKMel28) were left untreated or were treated with DMSO as control,  $6 \mu\text{mol L}^{-1}$  BAY 43-9006,  $4 \mu\text{mol L}^{-1}$  wortmannin or  $6 \mu\text{mol L}^{-1}$  BAY 43-9006 combined with  $4 \mu\text{mol L}^{-1}$  wortmannin for 48 h before staining with Annexin V-FITC and propidium iodide. In 451Lu but not SKMel28 cells the RAF kinase inhibitor BAY 43-9006 induced apoptosis to some degree, whereas the PI3K inhibitor wortmannin did not affect tumour cell survival in either cell line. Interestingly, wortmannin significantly enhanced the proapoptotic effect of BAY 43-9006 in 451Lu and SKMel28 melanoma cells. In addition, metastatic melanoma cells (SKMel28) were treated with medium or medium plus DMSO as controls, the MEK inhibitors PD98059 ( $50 \mu\text{mol L}^{-1}$ ) or U0126 ( $20 \mu\text{mol L}^{-1}$ ), the PI3K inhibitor LY294002 ( $50 \mu\text{mol L}^{-1}$ ) and inhibitor combinations (PD98059 + LY294002, U0126 + LY294002), respectively. Forty-eight hours after treatment the percentage of apoptotic melanoma cells was assessed by Annexin V-FITC and propidium iodide staining. Inhibitors alone (PD98059, U0126, LY294002) enhanced and inhibitor combinations (PD98059 + LY294002, U0126 + LY294002) dramatically increased the number of apoptotic melanoma cells. (b) After treatment of metastatic melanoma cells (451Lu, SKMel28) with culture medium, or medium plus DMSO as controls, BAY 43-9006 ( $6 \mu\text{mol L}^{-1}$ ), wortmannin ( $4 \mu\text{mol L}^{-1}$ ) or BAY 43-9006 ( $6 \mu\text{mol L}^{-1}$ ) plus wortmannin ( $4 \mu\text{mol L}^{-1}$ ) for 48 h, apoptosis was determined using a cell death detection enzyme-linked immunosorbent assay. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the y-axis (mean  $\pm$  SD of duplicate samples). BAY 43-9006 increased apoptosis in 451Lu cells but not in SKMel28 cells. Wortmannin did not alter the apoptotic rate in both cell lines. BAY 43-9006 combined with wortmannin caused a marked increase of apoptosis in both metastatic melanoma cell lines (451Lu: BAY 43-9006 + wortmannin,  $P = 0.005$ . SKMel28: BAY 43-9006 + wortmannin,  $P = 0.004$ ).

### Combined inhibition of MAPK and AKT signalling pathways abrogates migration, invasion and invasive tumour growth of melanoma cells in monolayer and organotypic skin culture

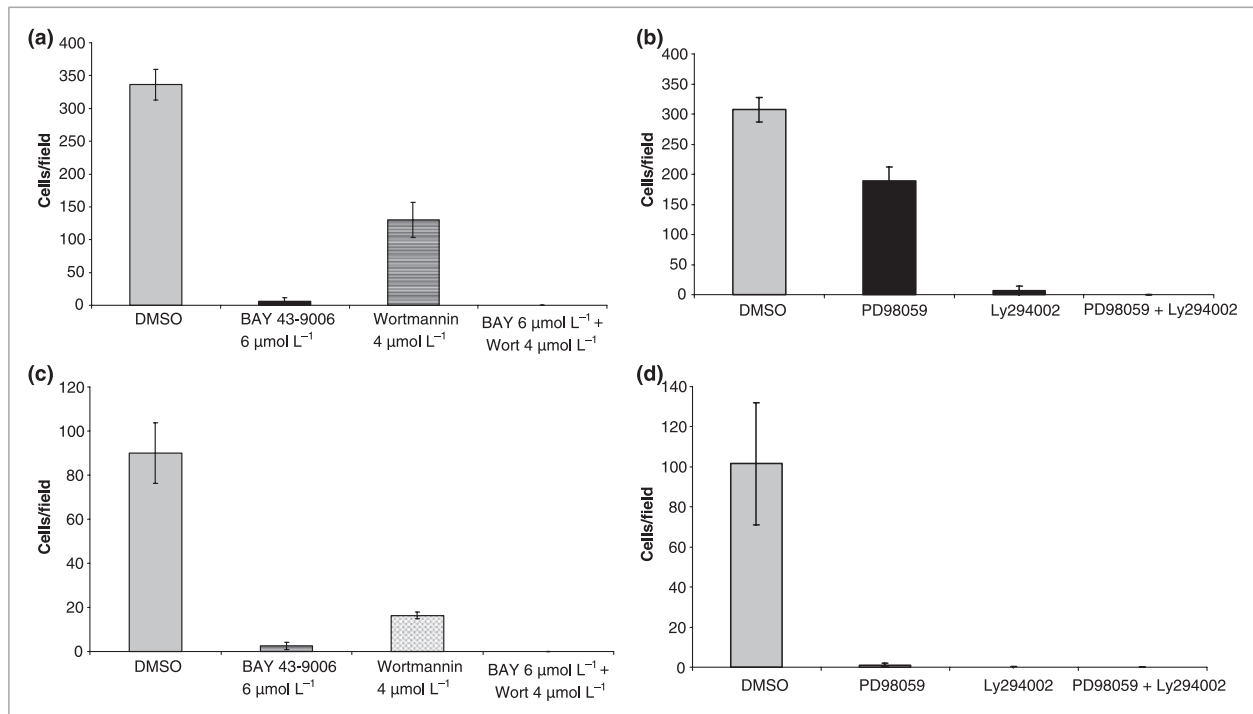
We analysed the effects of MAPK and AKT signalling pathway inhibition on motility and invasive competence of metastatic melanoma cells in a Boyden chamber assay with or without Matrigel (Fig. 4). Both the RAF kinase inhibitor BAY 43-9006 ( $6 \mu\text{mol L}^{-1}$ ) and the PI3K inhibitor wortmannin ( $4 \mu\text{mol L}^{-1}$ ) significantly decreased migration (Fig. 4a) and invasion (Fig. 4c) of SKMel28 melanoma cells. The combination of both signalling pathway inhibitors completely abrogated melanoma cell motility and invasive competence (Fig. 4a,c). Similar results were obtained with the MEK inhibitor PD98059 and the PI3K inhibitor LY294002 (Fig. 4b,d).

Finally, we determined whether MAPK and AKT signalling pathway inhibition is able to suppress invasive melanoma growth in a more physiological context. Metastatic melanoma cells (451Lu, SKMel28) were incorporated into human dermal reconstructs. The reconstructed melanomas were treated with BAY 43-9006, wortmannin or BAY 43-9006 combined with wortmannin (Fig. 5). Nontumour-derived cells of human skin such as fibroblasts in the organotypic skin reconstruction were

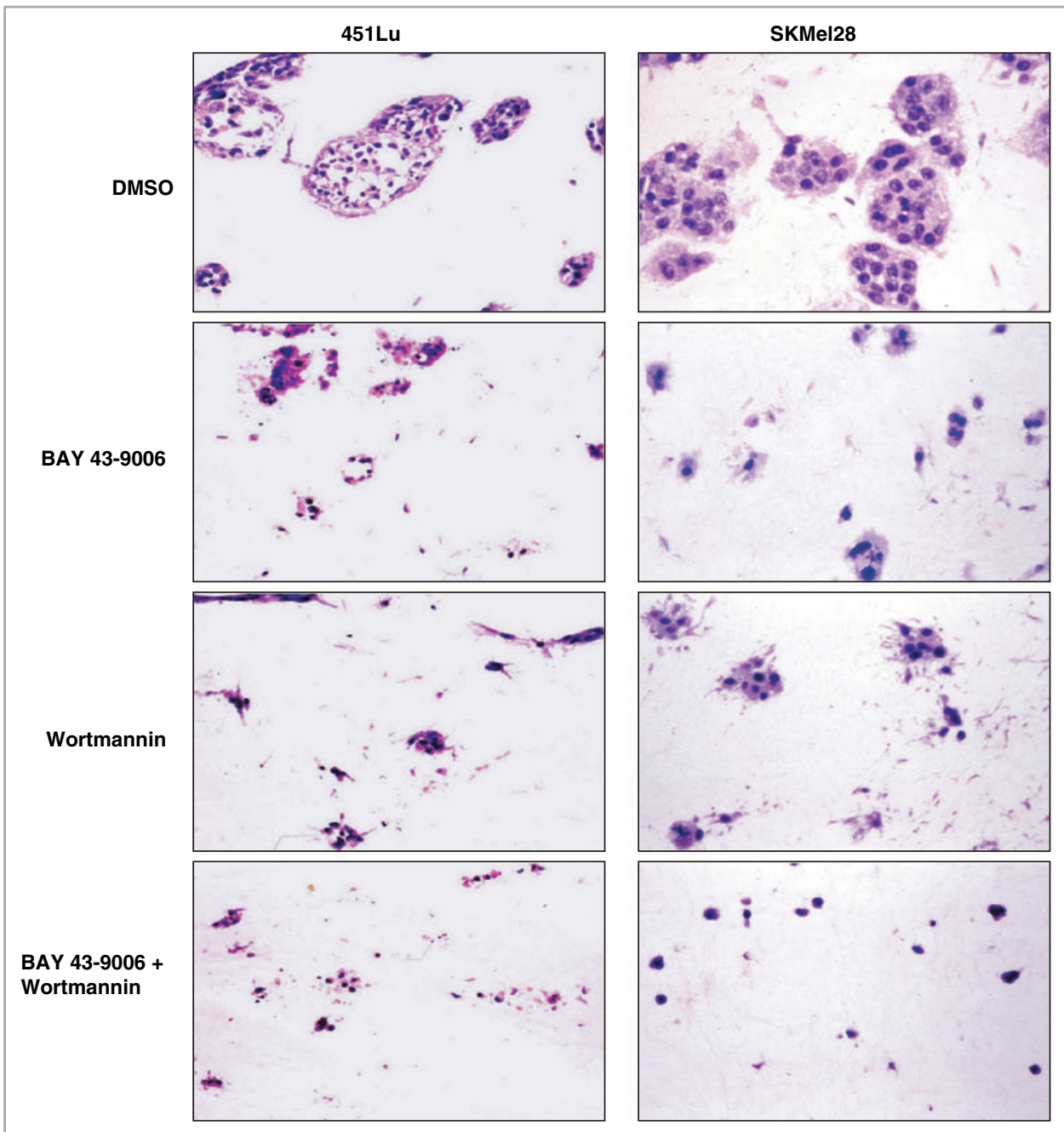
not affected by the treatment. Control-treated metastatic melanoma cells exhibited rapid growth of multiple tumour cell nests throughout the entire dermis. Either the inhibition of the MAPK signalling pathway by BAY 43-9006 or the inhibition of the AKT signalling pathway by wortmannin resulted in reduced invasive tumour growth of metastatic melanoma cells in reconstructed dermis. The application of the RAF kinase inhibitor BAY 43-9006 reduced the number and the size of melanoma cell nests. Small melanoma cell nests and single melanoma cells were scattered throughout the dermis. After treatment with the PI3K inhibitor wortmannin, melanoma cell nests were also reduced in number and size and appeared to be loosened suggesting a decrease in melanoma cell cohesion. Furthermore, melanoma cells displayed a multidendritic morphology. Interestingly, simultaneous blockade of the MAPK and AKT signalling pathways by BAY 43-9006 combined with wortmannin completely suppressed invasive melanoma growth with very few rounded melanoma cells left in the dermis.

### Discussion

We examined whether targeting the MAPK and AKT signalling pathways would have therapeutic effects against melanoma. In particular, we investigated the effects of the MAPK signal-



**Fig 4.** Combined inhibition of MAPK and AKT signalling pathways abrogates migration and invasion of melanoma cells in monolayer culture. Rate of migrating (a, b) or invading (c, d) metastatic melanoma cells (SKMel28) assessed by a Boyden chamber assay with or without Matrigel. (a, c) Treatment of melanoma cells with the RAF kinase inhibitor BAY 43-9006 ( $6 \mu\text{mol L}^{-1}$ ) or the PI3K inhibitor wortmannin ( $4 \mu\text{mol L}^{-1}$ ) resulted in a significant decrease of melanoma cell migration (a) and invasion (c) through Matrigel, most pronounced after treatment with BAY 43-9006. The combination of both signalling pathway inhibitors completely abrogated melanoma cell motility ( $P = 0.0008$ ) and invasive competence ( $P = 0.002$ ) (b, d). Both the MEK inhibitor PD98059 ( $50 \mu\text{mol L}^{-1}$ ) and the PI3K inhibitor LY294002 ( $50 \mu\text{mol L}^{-1}$ ) inhibited melanoma cell migration (b) and strongly suppressed melanoma cell invasion (d). PD98059 combined with LY294002 completely abrogated migration ( $P = 0.001$ ) and invasion ( $P = 0.154$ ) of melanoma cells.



**Fig 5.** Combined inhibition of MAPK and AKT signalling pathways abolishes invasive tumour growth of melanoma cells in organotypic skin culture. Metastatic melanoma cells (451Lu, SKMel28) were incorporated into human dermal reconstructs, treated with culture medium plus DMSO as control, the RAF kinase inhibitor BAY 43-9006 (451Lu:  $2 \mu\text{mol L}^{-1}$ , SKMel28:  $6 \mu\text{mol L}^{-1}$ ), the PI3K inhibitor wortmannin (451Lu:  $10 \mu\text{mol L}^{-1}$ , SKMel28:  $4 \mu\text{mol L}^{-1}$ ) or a combination of both inhibitors and were stained with haematoxylin and eosin (original magnification  $\times 100$ ). Control-treated metastatic melanoma cells exhibited aggressive growth of numerous tumour cell nests and tumour cell clusters throughout the entire dermis. The RAF kinase inhibitor BAY 43-9006 reduced number and size of melanoma cell nests with small melanoma cell nests and single melanoma cells scattered throughout the dermis. Treatment with wortmannin also reduced the number and size of melanoma cell nests, decreased cohesion of melanoma cells, and changed morphology of melanoma cells with melanoma cells displaying a multidendritic phenotype. BAY 43-9006 in combination with wortmannin completely suppressed invasive melanoma growth with very few rounded melanoma cells left in the dermis.

ling pathway inhibitors BAY 43-9006, PD98059 and U0126 and the AKT signalling pathway inhibitors wortmannin and LY294002 on growth, survival and invasion of metastatic

melanoma cells in monolayer and organotypic skin culture. Antiproliferative and proapoptotic effects of inhibitors alone in monolayer culture were disappointing and significantly varied

among the different cell lines. However, combined inhibition of both signalling pathways potently inhibited growth and survival and completely suppressed migration and invasion of melanoma cells in monolayer and organotypic skin culture.

Our observations and those of other groups,<sup>2,3,6,11</sup> suggest that the RAF kinase inhibitor BAY 43-9006, the MEK inhibitors PD98059 and U0126 and the PI3K inhibitors wortmannin and LY294002 specifically block the MAPK and AKT signalling pathways, respectively, suggesting that the described findings are due to the activity of the selected signalling pathway inhibitors against their intended targets. However, we have to take into account that the agents BAY 43-9006, PD98059, U0126, wortmannin and LY294002 may additionally contribute to the described findings through their impact on targets other than RAF, MEK and PI3K. For example, BAY 43-9006 demonstrated significant activity against several receptor tyrosine kinases involved in tumour progression and neovascularization, including vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor  $\beta$ , Flt-3 and c-KIT.<sup>12</sup> However, it has been shown convincingly that the RAF kinase inhibitor BAY 43-9006 targets BRAF *in vitro* and *in vivo*, induces apoptosis in melanoma cell lines and elicits a significant retardation in the growth of human melanoma tumour xenografts.<sup>6</sup> Furthermore, BRAF depletion by siRNA elicited similar effects.<sup>6</sup> Because in our study we observed similar effects on growth, survival and invasion of melanoma cells using different combinations of MAPK and AKT inhibitors, we think that these effects are due mainly to the combined inhibition of MAPK and AKT pathways.

In our study, the RAF kinase inhibitor BAY 43-9006 and the MEK inhibitor U0126 significantly inhibited growth of 451Lu metastatic melanoma cells. These observations are in agreement with recent data demonstrating that the RAF kinase inhibitor BAY 43-9006 and MEK inhibitors inhibit growth of human melanoma cells.<sup>2,6</sup> In contrast, growth of SKMel28 metastatic melanoma cells was not significantly affected by the RAF kinase inhibitor BAY 43-9006 or the MEK inhibitor U0126. Recent data suggest that the BRAF mutational status predicts sensitivity to MAPK inhibition.<sup>13</sup> However, analysis of BRAF genotypes of our melanoma cell lines revealed that both melanoma cell lines harbour the BRAF (V600E) mutation.<sup>14,15</sup> Furthermore, the MEK inhibitor PD98059 did not significantly inhibit growth of both cell lines suggesting that the MAPK inhibitors BAY 43-9006 and U0126 inhibit growth of 451Lu cells through their impacts on targets other than MAPK signalling.

The PI3K inhibitor wortmannin did not affect growth and survival of both cell lines tested, but significantly enhanced the proapoptotic effect of the MAPK inhibitor BAY 43-9006. These findings are in line with recent studies indicating that the AKT signalling pathway promotes tumour cell survival and resistance to chemotherapy and that inhibition of the AKT signalling pathway sensitizes tumour cells to chemotherapy or targeted agents such as the c-Kit kinase inhibitor imatinib.<sup>16-18</sup> In contrast, the PI3K inhibitor LY294002 significantly

inhibited growth of 451Lu cells at 50  $\mu\text{mol L}^{-1}$  but only slightly affected growth of SKMel28 cells. This observation also suggests that the PI3K inhibitor LY294002 affects growth of 451Lu cells through hitting targets other than PI3K. Indeed, LY294002, widely used as a specific inhibitor of PI3K, is known to induce effects independently of its action on PI3K.<sup>19,20</sup>

Both the MAPK and AKT signalling pathways are known regulators of tumour invasion.<sup>2,21-24</sup> In our study, migration and invasion of melanoma cells were decreased with both the MAPK inhibitors BAY 43-9006 and PD98059 and the AKT inhibitors wortmannin and LY294002 and completely abrogated when applied in combination. Our results are in accordance with a recent report suggesting that treatment with the MEK inhibitor U0126 and the PI3K inhibitor LY294002, alone or in combination, decreased the ability of 1984-1 melanoma cells to penetrate Matrigel.<sup>11</sup> Moreover, expression and activity of matrix metalloproteinase (MMP)-2, which has been associated with highly invasive potential and melanoma progression,<sup>25,26</sup> were significantly decreased with either of the inhibitors and also with the combination.<sup>11</sup> These results support previous observations suggesting a critical role for both the MAPK and AKT pathways in the regulation of proteases such as MMP-2.<sup>2,27,28</sup>

It has recently been proposed that the environment determines the susceptibility of melanoma cells to the inhibition of signalling pathways.<sup>29</sup> It has been shown that metastatic melanoma cells grown in monolayer are susceptible to inhibition of either the MAPK or the AKT signalling pathway. However, when they were grown in three-dimensional spheroids, most metastatic melanoma cells were resistant to the inhibition of only one signalling pathway and could be killed only by the inhibition of both pathways simultaneously. In our study we found similar sensitivities of the metastatic melanoma cell lines 451Lu and SKMel28 to inhibition of one or both pathways, irrespective of whether the cells were grown in monolayer or in an organotypic skin reconstruct. The reason for this discrepancy might be that the spheroids differ substantially from the organotypic skin model in that in the spheroids melanoma cells are not in contact with other skin-derived cells. The organotypic skin model used in our studies represents a physiological skin environment as melanoma cells are in close contact with fibroblasts, which are the natural environment of invasive melanoma cells. Further studies are necessary to elucidate the mechanisms which confer melanoma cell resistance to the inhibition of signalling pathways.

In summary, in recent years progress has been made in understanding what the critical signalling pathways are in melanoma. The increased understanding of signalling networks may be the key to curative treatment of advanced melanoma. The biological properties of malignant tumour cells are thought to be the sum of many activated genes. Targeting the most critical molecules at the same time may be feasible. The data of this study suggest that combined targeting of the MAPK and AKT signalling pathways may be a promising

strategy for the medical treatment of melanoma and should encourage further in-depth investigations.

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