Combined Inhibition of MAPK and mTOR Signaling Inhibits Growth, Induces Cell Death, and Abrogates Invasive Growth of Melanoma Cells

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The RAS–RAF–MEK–ERK and PI3K–AKT–mTOR signaling pathways are activated through multiple mechanisms and appear to play a major role in melanoma progression. Herein, we examined whether targeting the RAS–RAF–MEK–ERK pathway with the RAF inhibitor sorafenib and/or the PI3K–AKT–mTOR pathway with the mTOR inhibitor rapamycin has therapeutic effects against melanoma. A combination of sorafenib (4 μM) with rapamycin (10 nM) potentiated growth inhibition in all six metastatic melanoma cell lines tested. The absolute enhancement of growth inhibition rates ranged from 13.0–27.8% in different cell lines ($P<0.05$, combination treatment *vs* monotreatment). Similar results were obtained with combinations of the MEK inhibitors U0126 (30 μM) or PD98059 (50 μM) with rapamycin (10 nM). The combined treatment of melanoma cells with sorafenib and rapamycin led to an approximately twofold increase of cell death compared with sorafenib monotreatment ($P<0.05$) as assessed by propidium iodide staining and cell death detection ELISA. Moreover, sorafenib in combination with rapamycin completely suppressed invasive melanoma growth in organotypic culture mimicking the physiological context. These effects were associated with complete downregulation of the antiapoptotic proteins Bcl-2 and Mcl-1. Sorafenib combined with rapamycin appears to be a promising strategy for the effective treatment of melanoma and merits clinical investigation.

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INTRODUCTION

Cutaneous melanoma is one of the cancers with the greatest increase in incidence during the past three generations (Jemal *et al.*, 2001; Lasithiotakis *et al.*, 2006). The prognosis of patients with metastatic disease remains very poor with a 5-year survival probability of less than 5%, largely reflecting the failure of chemotherapy or immunotherapy regimens to impact the natural history of advanced disease (Flaherty, 2006). New therapies are urgently needed for melanoma and the increase in our understanding of the molecular biology of melanoma offers the first opportunity for a rational treatment strategy.

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Abbreviations: Bcl-1, B-cell lymphoma-2; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia-1; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; RAF, recombinant activated factor

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The RAS–RAF–MEK–ERK (MAPK) and the PI3K–AKT–mTOR (AKT) signaling pathways are constitutively activated through multiple mechanisms and subserve key functions in the progression of melanoma (Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003; Dai *et al.*, 2005; Meier *et al.*, 2005). The mitogen-activated protein kinase (MAPK) and acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (AKT) signal transduction pathways modulate the function of numerous substrates that regulate cell survival, proliferation, and invasion (Meier *et al.*, 2005). Interestingly, a number of molecules, for example, the adhesion molecules E-/N-cadherin, MelCAM, and αvβ3 integrin, whose essential role in the development and progression of melanoma is well known, activate these signaling pathways and/or are regulated by them (Meier *et al.*, 2005). Thus, the MAPK and AKT signal transduction pathways may be promising targets for the effective treatment of melanoma.

Sorafenib (BAY 43-9006), a potent recombinant activated factor (RAF) inhibitor, inhibits the MAPK signaling pathway both *in vitro* and *in vivo* (Karasarides *et al.*, 2004). However, a phase II clinical study revealed that BAY 43-9006 as a monotherapy is not effective in patients with metastatic melanoma (Eisen *et al.*, 2006). In a recently published phase I study where melanoma patients were treated with the MAPK/ERK kinase (MEK) inhibitor PD-0325901, only 2 out of 27 patients showed an objective response even though

near-complete inhibition of the MAPK signaling pathway was histologically confirmed (Flaherty, 2006). Similarly, the selective mTOR inhibitor CCI-779 inhibits its target *in vivo* but has been proved ineffective in melanoma patients (Margolin *et al.*, 2005). It has recently been demonstrated that aggressive melanoma cell lines are resistant to both MEK and PI3K inhibitors, whereas the combination of MEK- with PI3K-inhibitors suppresses the growth and invasion of metastatic melanoma cells (Smalley *et al.*, 2006; Meier *et al.*, 2007). These data support the hypothesis that in the treatment of melanoma it is not sufficient to inhibit only a single constitutively activated signaling pathway and that an effective treatment strategy must take into account more than one deregulated signaling pathway.

We investigated whether the combined targeting of MAPK and AKT signaling pathways has therapeutic effects in melanoma with particular interest in the combination of sorafenib with rapamycin, which are both available for clinical administration.

RESULTS

The MAPK inhibitors sorafenib, PD98059, and U0126 and the AKT/mTOR inhibitors LY294002, wortmannin, and rapamycin inhibit the MAPK and AKT/mTOR signaling pathways, respectively

The efficacy of the RAF inhibitor sorafenib, the MEK inhibitors PD98059 and U0126, the PI3K inhibitors LY294002 and wortmannin, and the mTOR inhibitor rapamycin in inhibiting their target pathways was verified by Western blot analyses for total and phosphorylated extracellular signal-regulated kinase (ERK), AKT, and p70S6K (ribosomal protein S6 kinase, which is phosphorylated by mTOR). The RAF inhibitor sorafenib (4 μM) and the mTOR inhibitor rapamycin (10 nM) effectively inhibited phosphorylation of ERK and p70S6K, respectively. The MEK inhibitors U0126 (30 μM) and PD98059 (50 μM), and the PI3K inhibitors LY294002 (30 μM) and wortmannin (4 μM) were effective in inhibition of phosphorylation of ERK and AKT/p70S6K, respectively. Combinations of sorafenib, MEK inhibitors, or PI3K inhibitors with rapamycin did not augment the pathway inhibition observed with the agents individually. In Figure 1, the data for SKMel28 metastatic melanoma cells are presented (Figure 1). Similar results were obtained for 451Lu and 1205Lu metastatic melanoma cells (data not shown).

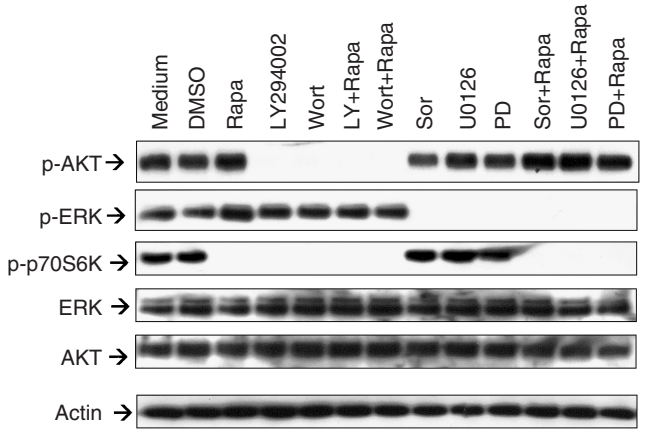

Figure 1. RAF/MEK and PI3K/mTOR inhibitors inhibit the MAPK and AKT/mTOR signaling pathways, respectively. Western blot analyses of cell lysates from metastatic melanoma cells (SKMel28) 6 hours after treatment with culture medium without or with DMSO as controls, the mTOR inhibitor rapamycin (Rapa 10 nM), the PI3K inhibitors LY294002 (LY 30 μM) or wortmannin (Wort 4 μM), the RAF inhibitor sorafenib (Sor 4 μM), the MEK inhibitors U0126 (30 μM) or PD98059 (PD 50 μM), alone or in combination with rapamycin for phosphorylated and total AKT, ERK, and p70S6K.

Combined MAPK and mTOR inhibition significantly inhibits melanoma cell growth

Using a panel of six human metastatic melanoma cell lines (451Lu, SKMel28, 1205Lu, WM852, SKMel19, Mewo), the effects of the RAF inhibitor sorafenib and/or the mTOR inhibitor rapamycin on melanoma cell growth in monolayer culture were determined by a fluorimetric assay using 4-methylumbelliferyl heptanoate (Zouboulis *et al.*, 1991). Treatment of metastatic melanoma cells with the RAF inhibitor sorafenib alone (2–6 μM) yielded variable growth inhibition of melanoma cells in monolayer culture (Figure 2a). 451Lu, WM852, and 1205Lu cells appeared to be more sensitive to sorafenib (4 μM) with growth inhibition rates ranging from 48% (451Lu) to 95% (1205Lu). SKMel28, SKMel19, and Mewo cells were relatively resistant to sorafenib (4 μM), yielding growth inhibition rates that ranged from 16.5% (SKMel28) to 21.1% (Mewo). Treatment of metastatic melanoma cells with the mTOR inhibitor alone (1–100 nM) did not significantly inhibit melanoma cell growth with corresponding growth inhibition rates not exceeding 30% in any of the six metastatic melanoma cell lines tested (Figure 2b). The combination of the RAF inhibitor sorafenib (4 μM) with the mTOR inhibitor rapamycin (10 nM) significantly potentiated growth inhibition compared with sorafenib alone in all metastatic melanoma cell lines tested (Figure 2c). The absolute enhancement of growth inhibition rates ranged from 13% (SKMel19) to 27.8% (WM852) ($P<0.05$, combination treatment *vs* monotreatment with sorafenib). Of note, the difference in growth inhibition between 4 μM sorafenib plus 10 nM rapamycin and 6 μM sorafenib alone was significant in four out of six metastatic melanoma cell lines tested ($P<0.05$: 451Lu, WM852, SKMel19, Mewo; $P>0.05$: 1205Lu, SKMel28).

The MEK inhibitor U0126 (30 μM) reduced the growth of most melanoma cell lines with inhibition rates ranging from 28% (Mewo) to 68.9% (451Lu). The MEK inhibitor PD98059 (50 μM) reduced the growth of melanoma cell lines to a lesser degree compared with U0126 with inhibition rates ranging from 16.9% (Mewo) to 50.5% (451Lu). When rapamycin (10 nM) was combined with the MEK inhibitor U0126 (30 μM), there was an absolute enhancement of growth inhibition rates ranging from 10.2% (SKMel28) to 27.4% (Mewo) ($P<0.05$, combination treatment *vs* monotreatment with U0126). PD98059 (50 μM) combined with rapamycin (10 nM) yielded an absolute enhancement of growth inhibition ranging from 16.4% (Mewo) to 46.3% (1205Lu) ($P<0.05$, combination treatment *vs* monotreatment with PD98059). Combinations of the PI3K inhibitors wortmannin (4 μM) or LY294002 (30 μM)

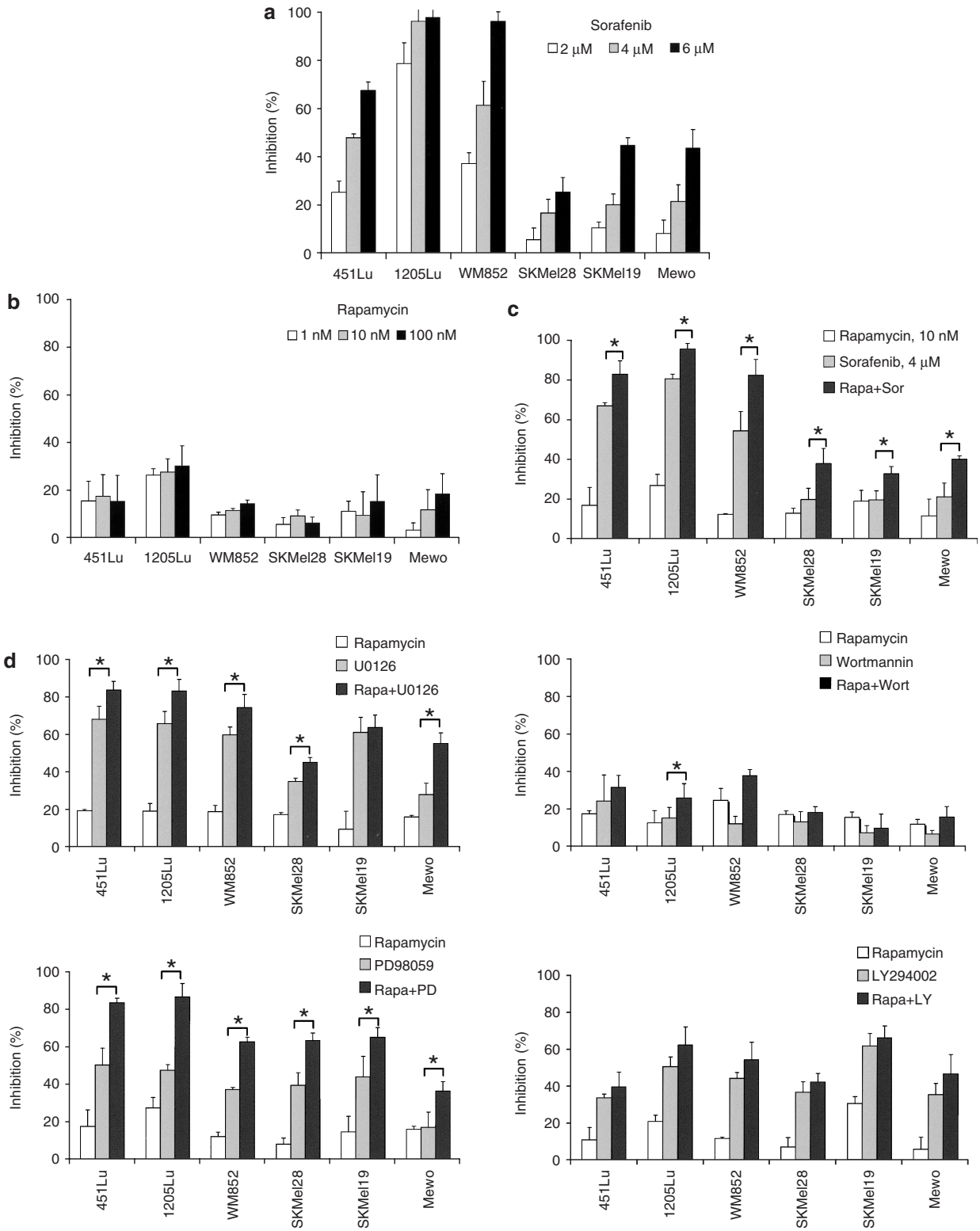

Figure 2. Combinations of MAPK inhibitors but not AKT inhibitors with rapamycin significantly inhibit melanoma cell growth. Metastatic melanoma cells (451Lu, 1205Lu, WM852, Mewo, SKMel28, SKMel19) were treated with the RAF inhibitor sorafenib (**a**), the mTOR inhibitor rapamycin (**b**), rapamycin in combination with sorafenib (**c**), and combinations of rapamycin with the MEK inhibitors U0126 or PD98059 or the PI3K inhibitors wortmannin or LY294002 (**d**). Melanoma cell growth was determined by a fluorimetric assay. $^*P<0.05$.

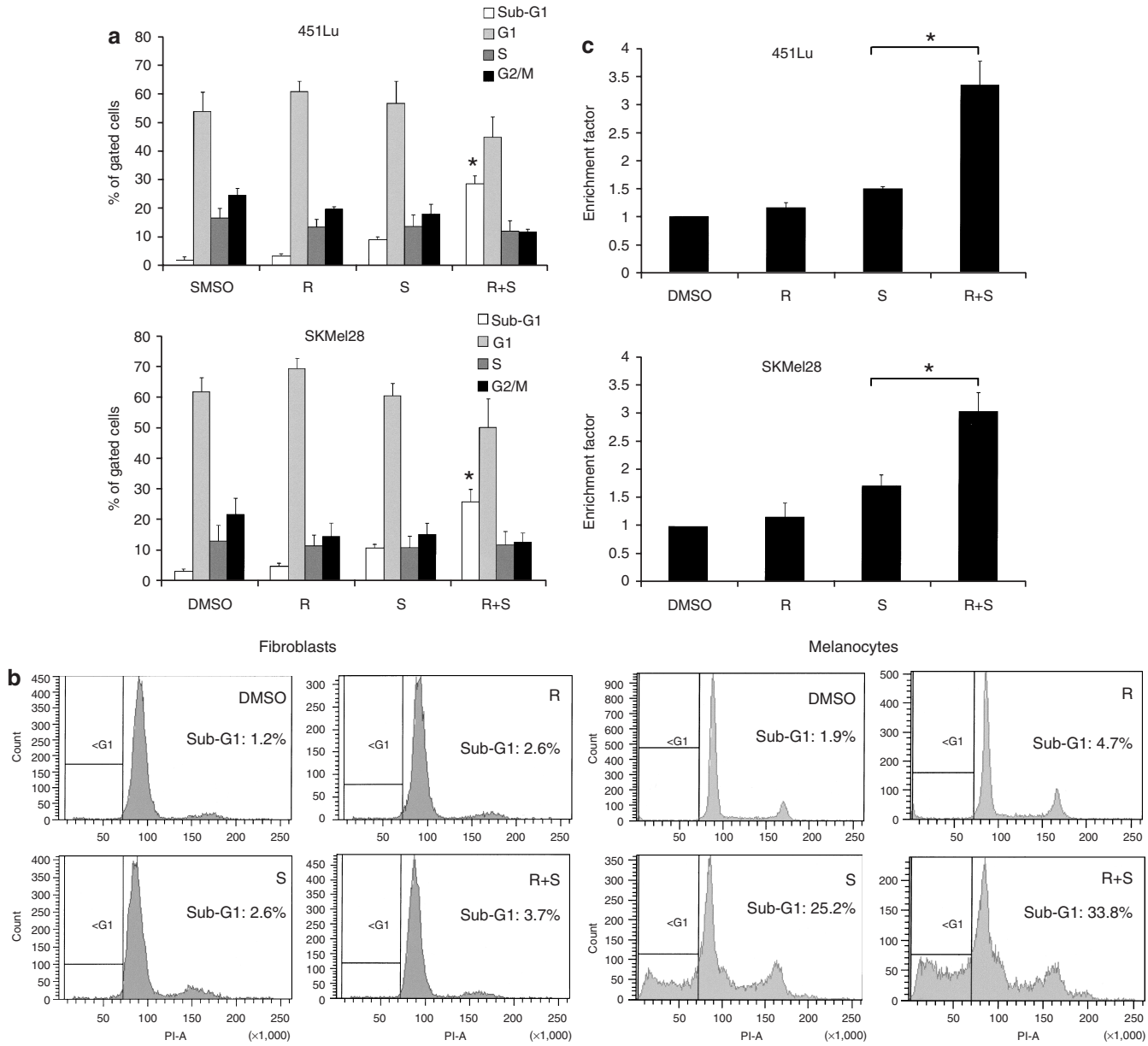

Figure 3. Sorafenib combined with rapamycin potently induces cell death of melanoma cells. (**a**) Cell cycle distribution of metastatic melanoma cells (451Lu, SKMel28) 30 hours after treatment with DMSO as control, rapamycin (R 10 nM), sorafenib (S 4 μM), or rapamycin combined with sorafenib (R + S). *$P<0.05$ compared with sorafenib alone. (**b**) Cell cycle analysis of human fibroblasts and melanocytes 48 hours after treatment with DMSO as control, rapamycin (R 10 nM) and/or sorafenib (S 4 μM). (**c**) Metastatic melanoma cells (451Lu, SKMel28) were treated with culture medium plus DMSO as control, rapamycin (R 10 nM) and/or sorafenib (S 4 μM), and subjected to a cell death detection ELISA. *$P<0.05$ compared with sorafenib alone.

with rapamycin (10 nM) did not yield significant additional growth inhibition in most melanoma cell lines tested (Figure 2d).

Sorafenib combined with rapamycin potently induces melanoma cell death

To investigate whether the RAF inhibitor sorafenib and the mTOR inhibitor rapamycin alone or in combination affect the cell cycle, two metastatic melanoma cell lines (the sensitive 451Lu and the less sensitive SKMel28 cell lines) were treated with DMSO as control, sorafenib (4 μM), rapamycin (10 nM), or a combination of both inhibitors for 30 hours, and fixed and stained with propidium iodide before cell cycle distribution was analyzed by flow cytometry (Figure 3a). In rapamycin-exposed cells, there was only a minor delay at the G1 phase of the cell cycle, which accounted for fewer than 10% of cells. Monotherapy with sorafenib did not result in an appreciable cell cycle arrest but increased the percentage of cells in the sub-G1 cell fraction to 10.7 and 9.3% in 451Lu and SKMel28 cells, respectively. Strikingly, coexposure of cells to rapamycin and sorafenib significantly increased the sub-G1 fraction to 29.0 and 25.7% in 451Lu and SKMel28 cells, respectively ($P<0.05$ compared with sorafenib alone).

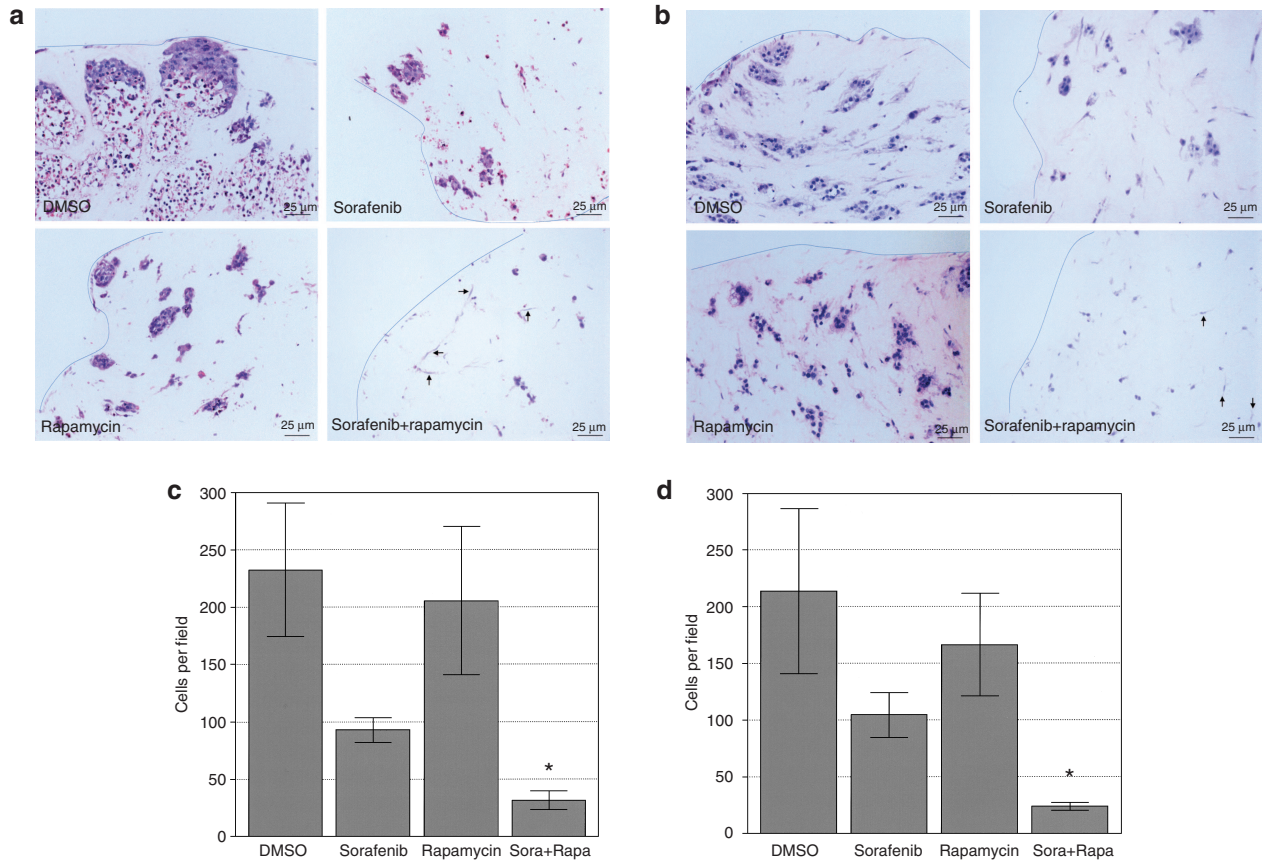

Figure 4. Sorafenib combined with rapamycin abrogates invasive tumor growth of melanoma cells in organotypic skin culture. Metastatic melanoma cells (**a**: 451Lu; **b**: SKMel28) grown in human dermal reconstructs were treated with culture medium plus DMSO as control, the RAF inhibitor sorafenib (4 μM; 6 μM), the mTOR inhibitor rapamycin (20 nM), or a combination of both inhibitors and were stained with hematoxylin. Line indicates the upper margin of the dermis. Arrows indicate dermal fibroblasts. Metastatic melanoma cells (**c**: 451Lu; **d**: SKMel28) in dermal reconstructs after control treatment (DMSO) or inhibitor treatment (sorafenib, rapamycin, sorafenib + rapamycin) were counted in six high-powered fields, and means + SD were depicted. *$P<0.05$ compared with sorafenib alone.

Of interest, treatment of human fibroblasts with rapamycin (10 nM), sorafenib (4 μM), or a combination of both inhibitors only marginally increased the sub-G1 fraction (Figure 3b). In contrast, sorafenib alone and, in particular, its combination with rapamycin significantly increased the percentage of human melanocytes in the sub-G1 fraction. Altogether, these results suggest that the induction of cell death is the primary mechanism of action of sorafenib combined with rapamycin on melanoma cells, with cell cycle inhibition playing a lesser role.

To confirm these results, 451Lu and SKMel28 metastatic melanoma cells were treated with DMSO as control, sorafenib (4 μM), rapamycin (10 nM), or sorafenib (4 μM) plus rapamycin (10 nM). After 48 hours, control-treated and inhibitor-treated melanoma cells were subjected to a cell death detection ELISA measuring the enrichment of histone-complexed DNA fragments in the cytoplasm of cells (Figure 3c). In agreement with the cell cycle data, coadministration of sorafenib with rapamycin yielded a 2.2-fold and 1.8-fold increase of cell death rates in 451Lu and SKMel28 cells, respectively ($P<0.05$ compared with sorafenib alone).

Sorafenib combined with rapamycin abrogates invasive melanoma growth in organotypic skin culture

To investigate whether the RAF inhibitor sorafenib and the mTOR inhibitor rapamycin are able to suppress invasive melanoma growth in a physiological context, metastatic melanoma cells (451Lu, SKMel28) were incorporated into human dermal reconstructs and treated with DMSO as control, the RAF inhibitor sorafenib (1–6 μM), and/or the mTOR inhibitor rapamycin (5–20 nM) (Figure 4). Control-treated metastatic melanoma cells exhibited rapid growth of multiple tumor cell clusters and nests in the dermis. The RAF inhibitor sorafenib (4–6 μM), and to a lesser degree, the mTOR inhibitor rapamycin (10–20 nM) decreased the number and size of melanoma cell nests with small melanoma cell nests and single melanoma cells scattered throughout the dermis. Intriguingly, coadministration of sorafenib with rapamycin completely suppressed invasive tumor growth of both melanoma cell lines with very few rounded melanoma cells left in the dermis. Of interest, nontumor-derived cells of human skin such as fibroblasts did not appear to be affected by inhibitor treatment (Figure 4a and b).

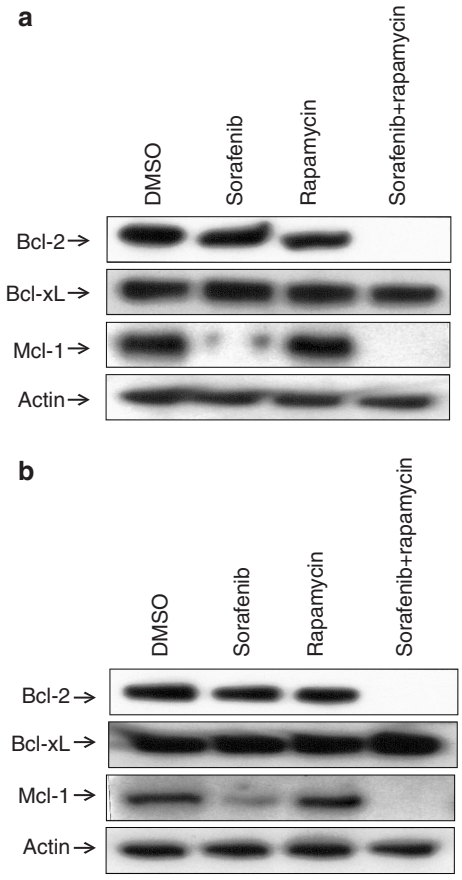

Figure 5. Sorafenib combined with rapamycin downregulates Bcl-2 and Mcl-1. Metastatic melanoma cells (**a**: 451Lu; **b**: SKMel28) were treated with culture medium plus DMSO as control, the RAF inhibitor sorafenib (4 μM), the mTOR inhibitor rapamycin (10 nM), or the inhibitor combination for 48 hours. Expression of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 was determined by Western blot analysis.

Sorafenib combined with rapamycin downregulates the antiapoptotic proteins Bcl-2 and Mcl-1

Recent studies suggest that cell death induced by sorafenib or rapamycin involves downregulation of antiapoptotic Bcl-2 (B-cell lymphoma-2) family proteins such as Bcl-2, Bcl-xL, and Mcl-1 (myeloid cell leukemia-1) (Rahmani *et al.*, 2005; Tirado *et al.*, 2005; Yu *et al.*, 2005; Panka *et al*, 2006; Vega *et al.*, 2006). Thus, the striking effects of coexposure of melanoma cells to sorafenib and rapamycin were examined in relation to expression of Bcl-2, Bcl-xL, and Mcl-1 by Western blot analysis (Figure 5a and b). The exposure of metastatic melanoma cells (451Lu, SKMel28) to sorafenib (4 μM) or rapamycin (10 nM) individually did not significantly alter expression of Bcl-2 or Bcl-xL. As recently described in human leukemia cells and other human cancer cell lines (Rahmani *et al.* 2005; Yu *et al.*, 2005), the treatment of metastatic melanoma cells (451Lu, SKMel28) with sorafenib resulted in significant downregulation of Mcl-1, whereas rapamycin did not appear to affect Mcl-1. Strikingly, coadministration of sorafenib and rapamycin completely abolished Bcl-2 and Mcl-1 without affecting Bcl-xL.

DISCUSSION

We examined whether targeting the MAPK pathway at the RAF level and the AKT pathway at the mTOR level would have therapeutic effects against melanoma. In particular, we investigated the effects of the RAF inhibitor sorafenib and/or the mTOR inhibitor rapamycin on growth, survival, and invasive tumor growth of metastatic melanoma cells in monolayer and organotypic skin culture. In contrast to monotherapy with sorafenib or rapamycin, combination therapy with sorafenib and rapamycin potently inhibited growth and survival of all melanoma cell lines tested in monolayer culture and completely suppressed invasive melanoma growth in organotypic skin culture. These effects were associated with complete downregulation of the antiapoptotic proteins Bcl-2 and Mcl-1.

In our study, the effects of the RAF inhibitor sorafenib on melanoma cell growth varied significantly among the different cell lines tested. 451Lu, 1205Lu, and WM852 cells appeared to be more sensitive to sorafenib compared to SKMel28, SKMel19, and Mewo cells. Notably, the BRAF mutation status did not reliably predict sensitivity of cell lines to RAF kinase inhibition. For example, the analysis of our melanoma cell lines for BRAF and NRAS mutations revealed that the sensitive WM852 melanoma cell line lacks the activating BRAF mutation and harbors the activating NRAS mutation, whereas the more resistant SKMel19 melanoma cell line harbors the BRAF mutation and lacks the NRAS mutation (unpublished data). This observation is in contrast to a previous study showing that the mutation of BRAF is associated with enhanced sensitivity to MEK inhibition compared to either wild-type cells or cells harboring an RAS mutation (Solit *et al.*, 2006). However, a recent study showed that there is no correlation between the concentrations of the MEK inhibitor U0126 required to block phospho-ERK activity and to inhibit melanoma cell growth (Smalley *et al.*, 2006). These and our data suggest that the MEK inhibitor U0126 and the RAF inhibitor sorafenib may also affect melanoma cell growth through their impacts on targets other than MAPK signaling. Indeed, sorafenib is not only an inhibitor of RAF kinases (BRAF, CRAF) but also a potent inhibitor of vascular endothelial growth factor receptor-2 and -3, platelet-derived growth factor receptor β, Flt-3, and c-KIT (Wilhelm *et al.*, 2004). Thus, one may speculate that some of the antitumor effects of sorafenib may be due to the inhibition of non-RAF targets (Panka *et al.*, 2006).

We demonstrated that the mTOR inhibitor rapamycin alone did not significantly inhibit the growth of any of the melanoma cell lines tested. Our *in vitro* data are in line with recent data of a phase II clinical trial demonstrating that the mTOR inhibitor CCI-779, an analog of rapamycin, is not active in patients with metastatic melanoma (Margolin *et al.*, 2005). Loss of PTEN, a major negative regulator of the AKT pathway, and activation of AKT and mutant or defective p53 are thought to render tumor cells sensitive to the mTOR inhibitor rapamycin (Majumder *et al.*, 2004; Majumder and Sellers, 2005; Kurmasheva *et al.*, 2006). It is likely that the mechanisms for resistance to rapamycin are even more complex (Corradetti and Guan, 2006; Kurmasheva *et al.*,

2006; Vega *et al.*, 2006; Wullschleger *et al.*, 2006). For example, phosphorylation of p70S6K, which is potently inhibited by rapamycin and thought to be essential for the antitumor effects of rapamycin, has also been shown to initiate a negative feedback loop leading to inhibition of insulin receptor substrate-1 and reduced activation of PI3K (Averous and Proud, 2006; Corradetti and Guan, 2006). Thus, the inhibitory effect of rapamycin on p70S6K phosphorylation could lead to increased activation of PI3K and improve the survival of cancer cells. In our study, however, rapamycin did not enhance AKT phosphorylation. Moreover, combinations of rapamycin with the PI3K inhibitors wortmannin or LY294002 efficiently inhibited AKT phosphorylation but did not significantly potentiate growth inhibition of melanoma cells. Additionally, it has been shown that rapamycin can augment the stability of the antiapoptotic protein Bcl-2 by inhibiting its phosphorylation and degradation (Calastretti *et al.*, 2001). However, in our study, 451Lu and SKMel28 metastatic melanoma cells expressed high levels of Bcl-2, and rapamycin did not further increase Bcl-2 levels.

Strikingly, the treatment of metastatic melanoma cells with the RAF inhibitor sorafenib in combination with the mTOR inhibitor rapamycin significantly inhibited growth and induced marked cell death in monolayer culture and completely suppressed invasive tumor growth in organotypic culture. Similarly, combinations of other MAPK pathway inhibitors (U0126, PD98059) with rapamycin efficiently inhibited melanoma cell growth. Furthermore, in our previous study, we inhibited the MAPK and AKT signaling pathways in two metastatic melanoma cell lines at different levels using a panel of pharmacological inhibitors. Whereas the different MAPK and AKT inhibitors differentially affected melanoma cell growth, survival, and invasion, combinations of MAPK and AKT inhibitors significantly inhibited growth, induced apoptosis, and suppressed invasion of both melanoma cell lines (Meier *et al.*, 2007). These data are in line with results of a recent *in vitro* study (Smalley *et al.*, 2006) investigating the antitumor activity of a panel of MAPK and AKT inhibitors in a series of melanoma cell lines in a three-dimensional spheroid model. Metastatic melanoma cell lines were resistant to MAPK or AKT inhibitors alone, whereas MAPK and AKT inhibitors in combination efficiently blocked growth and invasion of metastatic melanoma three-dimensional spheroids. Moreover, a recent *in vivo* study demonstrated that inhibition of MAPK and AKT pathways by topical application of the MEK inhibitor U0126 and the PI3K inhibitor LY294002 results in complete remission in 33% and partial remission in 46% of 7,12-dimethylbenzanthracene-treated TPRas mice (Bedogni *et al.*, 2006). These effects were associated with reduced proliferation and increased apoptosis. Taken altogether, these findings provide further support for the evolving concept that the simultaneous interruption of two cytoprotective signaling pathways represents a particularly potent strategy to inhibit growth and stimulate cell death in neoplastic cells (Hahn *et al.*, 2005).

In monolayer culture, the difference in growth inhibition between 6 μM sorafenib alone and 4 μM sorafenib plus 10 nM rapamycin was statistically significant in most but not all metastatic melanoma cell lines tested. These data are supported by a previous experimental study showing the synergistic inhibition of proliferation of different melanoma cell lines in monolayer culture by the combination of sorafenib and rapamycin (Molhoek *et al.*, 2005). Moreover, in organotypic culture, monotherapy with sorafenib reduced invasive melanoma growth, whereas combination therapy with sorafenib and rapamycin completely suppressed tumor growth of metastatic melanoma cells, suggesting that this drug combination may be even more effective in a more physiological context. However, this speculative hypothesis needs to be confirmed by *in vivo* and clinical studies.

Our experiments with monolayer cultures of melanoma cells demonstrated a sub-G1 increase and intranucleosomal DNA fragmentation after combination treatment with sorafenib and rapamycin, suggesting that sorafenib combined with rapamycin triggers cell death, thus contributing to abrogation of invasive melanoma growth in organotypic culture. Interestingly, a recent experimental study demonstrated that in human melanoma cell lines, sorafenib-induced cell death is independent of caspase activation and largely mediated through the apoptosis-inducing factor, which has the ability to translocate from the mitochondria to the nucleus, inducing DNA fragmentation independently of caspase activity (Panka *et al.*, 2006). Moreover, release of apoptosis-inducing factor rather than the activation of caspases was reported to be the mediator of melanoma cell death induced by MEK inhibition through U0126 and siRNA (Wang *et al.*, 2007). Altogether, these data strongly emphasize the importance of further studies to determine the mechanisms by which these drugs induce cell death in melanoma cells.

Rapamycin and/or sorafenib had no effect on cell cycle distribution of fibroblasts in monolayer or organotypic culture, suggesting that nontumor-derived cells are not significantly affected by inhibitor treatment. In contrast, sorafenib alone, and in particular, its combination with rapamycin significantly increased the percentage of human melanocytes in the sub-G1 fraction. However, melanocytes in monolayer culture may be more sensitive to inhibitor treatment than melanocytes in a physiological context, as loss of melanocytes (vitiligo) has not been reported in patients treated with sorafenib.

In melanoma, the antiapoptotic Bcl-2 family proteins Bcl-2, Bcl-xL, and Mcl-1 appear to increase with progression and may be involved in resistance to conventional therapies (Bush and Li, 2003; Zhang and Rosdahl, 2006). Intriguingly, in our study, the combination of the RAF inhibitor sorafenib and the mTOR inhibitor rapamycin completely downregulated Bcl-2 and Mcl-1 in melanoma cells. The literature suggests that both the RAS–RAF–MEK–ERK and the PI3-K–AKT–mTOR signaling pathways, which are constitutively activated in melanoma, modulate the expression of Bcl-2, Bcl-xL, and Mcl-1 (Wang *et al.*, 1999, 2006; Mori *et al.*, 2003; Asnaghi *et al.*, 2004; Rahmani *et al.*, 2005; Tirado *et al.*, 2005; Vega *et al.*, 2006). Indeed, inhibition of mTOR with rapamycin or mTOR-specific small interfering RNA downregulated antiapoptotic proteins including Bcl-2 and

Mcl-1 in ALCL cells (anaplastic large-cell lymphoma cells) (Vega *et al.*, 2006). However, several mTOR-independent activities have been reported for rapamycin (Zhou *et al.*, 2003; Hleb *et al.*, 2004; Schoffstall *et al.*, 2005). Likewise, sorafenib was shown to downregulate Bcl-2 and Mcl-1 through processes independent of MAPK inhibition (Rahmani *et al.*, 2005; Yu *et al.*, 2005; Panka *et al.*, 2006). Altogether, the RAF inhibitor sorafenib together with the mTOR inhibitor rapamycin may downmodulate the antiapoptotic proteins Bcl-2 and Mcl-1 through mechanisms dependent and independent of their RAF and mTOR inhibitory activity, respectively. Notably, the inactivation of Bcl-2, Bcl-xL, or Mcl-1 by RNA interference or blockade of Bcl-2, Bcl-xL, and Mcl-1 by the BH3 mimetic TW-37 failed to induce significant apoptosis in melanoma cells (Verhaegen *et al.*, 2006). These data suggest that in melanoma, the removal of antiapoptotic proteins is not sufficient to promote apoptosis and that additional proapoptotic inducers are required. Indeed, the death response of melanoma cells to the MEK inhibitor U0126 was significantly enhanced by the BH3 mimetic TW-37 or shRNA against Bcl-2, Bcl-xL, or Mcl-1 (Verhaegen *et al.*, 2006). Our data suggest that sorafenib combined with rapamycin may have similar effects on melanoma cell survival and expression of Bcl-2 and Mcl-1.

In the melanoma cell lines tested, sorafenib together with rapamycin completely downregulated Mcl-1. Mcl-1 is thought to protect cells from a variety of proapoptotic stimuli that activate the mitochondrial apoptotic pathway and has been implicated in resistance to anticancer drugs (Craig, 2002). Recent work underscores the critical role of Mcl-1 in melanoma drug resistance (Verhaegen *et al.*, 2006). As mentioned above, the death response of melanoma cells to the MEK inhibitor U0126 was significantly enhanced by shRNA against Bcl-2, Bcl-xL, or Mcl-1. Intriguingly, the most cytotoxic effect was seen after inactivating Mcl-1. These results indicate that the resistance of melanoma cells to the MEK inhibitor U0126 relies on expression of Mcl-1 and, to a lesser extent, Bcl-xL and Bcl-2. Furthermore, in view of recent experimental data, it has been suggested that downregulation of Mcl-1 may be more effective in sensitizing melanoma to chemotherapy than targeting Bcl-2 (Hersey, 2006). Indeed, Mcl-1 antisense therapy was shown to chemosensitize human melanoma in an SCID mouse xenotransplantation model (Thallinger *et al.*, 2003). Both the RAF inhibitor sorafenib and the mTOR inhibitor rapamycin have been reported to affect Mcl-1 protein levels (Rahmani *et al.*, 2005; Yu *et al.*, 2005; Vega *et al.*, 2006). In particular, sorafenib was shown to downregulate Mcl-1 in leukemia and lung cancer cells by MAPK-independent inhibition of Mcl-1 translation levels (Rahmani *et al.*, 2005) and enhancement of proteasome-mediated Mcl-1 degradation (Yu *et al.*, 2005), respectively. The mechanisms by which sorafenib and rapamycin cooperate to achieve complete suppression of antiapoptotic Mcl-1 protein deserve further study.

In summary, our data indicate that the combination of the RAF inhibitor sorafenib with the mTOR inhibitor rapamycin potently inhibits growth, induces cell death, and abrogates invasive tumor growth of melanoma cells. Sorafenib and rapamycin may exert their antitumor activity, at least in part, through downmodulation of antiapoptotic Bcl-2 family proteins such as Bcl-2 and Mcl-1. As the RAF inhibitor sorafenib and the mTOR inhibitor rapamycin have reached clinical application, sorafenib combined with rapamycin appears to be a promising strategy for the effective treatment of melanoma in the near future and merits in-depth investigation.

MATERIALS AND METHODS

The use of human skin tissues in this study was approved by the medical ethical committee of the University of Tuebingen and was performed in accordance with the Declaration of Helsinki Principles.

Isolation and culture of human cells

Human metastatic melanoma cells (451Lu, SKMel28, 1205Lu, WM852, SKMel19, Mewo) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. After obtaining informed consent, fibroblasts were isolated from human foreskin after routine circumcision. Samples were stored at 4°C in Hank's balanced salt solution without Ca^{2+} or Mg^{2+} and containing penicillin, gentamicin, and amphotericin. The subcutaneous fat was trimmed off and the cutis cut and digested in solution B containing 0.25% Trypsin (Pittelkow and Scott, 1986) at 4°C for approximately 19 hours. The action of Trypsin was stopped with solution A (Pittelkow and Scott, 1986) and the epidermis was separated from the dermis using forceps. Fibroblasts were obtained from dermal explants of human foreskin and cultured in DMEM with 10% fetal bovine serum. Fibroblasts up to passage 7 were used for organotypic cultures. Melanocytes were isolated as described previously (Meier *et al.*, 2000) and cultured in Melanocyte medium (Cell Systems, St Katharinen, Germany).

Treatment of melanoma cells with signaling pathway inhibitors

For inhibition of the MAPK signaling pathway, the RAF inhibitor sorafenib (Bayer Corporation, West Haven, CT) and the MEK inhibitors PD98059 and U0126 (Cell Signaling Technology, Beverly, MA) were used. For blockade of the AKT/mTOR signaling pathway, the mTOR inhibitor rapamycin (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and the PI3K inhibitors LY294002 (Cell Signaling Technology) and wortmannin (Sigma Aldrich Chemie GmbH) were used. The inhibitors were dissolved in DMSO and were added directly to the culture medium of melanoma cells in monolayer or organotypic skin culture at the combinations and concentrations to be tested. On the basis of our own previous studies and those of others (Smalley *et al.*, 2006) the following doses of inhibitors were tested for monotherapy and combination therapy: sorafenib 2–6 μM, PD98059 10–50 μM, U0126 10–50 μM, LY294002 10–50 μM, wortmannin 4–10 μM, and rapamycin 1–100 nM. Melanoma cells incubated with culture medium or culture medium with DMSO served as controls.

Western blot analyses

Cells were cultured in culture medium to 60–70% confluency. Inhibitors were added at the given concentrations, and after 6–48 hours the adherent cells were lysed directly in the dish for 30 minutes on ice with buffer containing 10 mM Tris pH 7.5, 0.5%

Triton X-100, 5 mM EDTA, 0.1 μM Phenylmethanesulfonylfluoride, 10 μM pepstatin A, 10 μM leupeptin, 25 μM aprotinin, 20 mM NaF, 1 mM pyrophosphate, and 1 mM orthovanadate. For the detection of phosphorylated proteins, the cells were serum starved for 14–16 hours and then stimulated for 1 hour with human recombinant IGF-1 (50 ng ml^{-1}) prior to cell lysis. Lysates were cleared by centrifugation at 13,000 *g* for 30 minutes and boiled at 100°C for 3 minutes, and 30 μg protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with primary antibodies in phosphate-buffered saline (PBS)/0.1% Tween-20/5% dry milk, washed with PBS for 3 × 10 minutes, and incubated with secondary biotin-conjugated antibody. After washing in phosphate buffer, the Streptavidin-AP-conjugate (Roche, Mannheim, Germany) was used for the detection of biotin-labeled secondary antibody. The membrane was immersed in CDP-Star solution (Roche) for 10 minutes and then exposed to X-ray film (Eastman Kodak, Rochester, NY). The following primary antibodies were used: anti-ERK, anti-phospho-ERK (Thr202/Tyr204), anti-AKT, anti-phospho-AKT (Thr308), anti-phospho-p70S6K, anti-β-actin (Cell Signaling Technology Inc.), anti-Bcl-2, anti-Bcl-xL, and anti-Mcl-1 (BD Biosciences Pharmingen, San Diego, CA).

Growth assay

Cells were seeded as triplicates in 96-well plates at a density of 5,000 cells per well in 150 μl medium (3.3 × 10^4 cells per ml). After 24 hours, the medium was replaced by a medium containing signaling pathway inhibitors alone or in combination at the concentrations to be tested. Cells incubated with culture medium with or without DMSO served as controls. Cells were incubated for 72 hours, washed two times with PBS, and 100 μl of a solution containing 100 μg 4-methylumbelliferyl-heptanoate per ml PBS was added. Plates were incubated at 37°C for 1 hour and measured in a Fluoroskan II (Labsystems, Helsinki, Finland), with an λ_{em} of 355 nm and an λ_{ex} of 460 nm. The intensity of fluorescence indicates the number of viable cells in the wells (Zouboulis *et al.*, 1991). Results were expressed as mean ± SE values of triplicates derived from at least two independent experiments.

Cell cycle analysis

Cells were seeded at a density of 2.5 × 10^5 cells per ml into 6-well plates. After 48 hours, the culture medium was replaced by a medium containing the inhibitors at the concentrations to be tested for 30–48 hours. Cells were harvested with Trypsin and centrifuged at 400 *g* for 5 minutes. The cell pellet was resuspended in PBS and fixed in ice-cold 70% ethanol for at least 60 minutes. Cells were then centrifuged and washed twice in cold PBS. The resulting cell pellet was stained in 500 μl propidium iodide solution (propidium iodide 40 μg ml^{-1} and RNase 100 μg ml^{-1} in PBS) for 20 minutes at 4°C. The cell cycle was analyzed using flow cytometry and FACSDiva software (BD Biosciences, Heidelberg, Germany). Results were expressed as mean ± SE values of three independent experiments.

Cell death assessment by DNA fragmentation assay

Intranucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes (Cell Death Detection ELISA plus kit; Roche) that accumulated in dying melanoma cells with intact membrane. Briefly, cells (5,000 cells in 150 μl medium in 96-well plates) were treated for 48 hours with culture medium containing the inhibitors at the concentrations to be tested or culture medium plus DMSO as controls. Cell culture supernatants were washed away to remove fragmented DNA from necrotic cells, and cells were lysed directly in the well with 200 μl buffer supplied by the manufacturer for 30 minutes at room temperature. After pelleting nuclei (200 *g*, 10 minutes), 20 μl of the supernatant (cytoplasmic fraction) was used in the ELISA following the manufacturer's standard protocol. Finally, absorbance at 405 and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 15 minutes, was determined with a microplate reader (SLT, Spectra LabInstruments Deutschland GmbH, Crailsheim, Germany). The rate of apoptosis is reflected by the enrichment (fold increase) of mono- and oligonucleosomes accumulated in the cytoplasm and was calculated according to the formula: absorbance of sample cells/absorbance of control cells. Results were expressed as mean ± SE values of triplicates of two independent experiments.

Organotypic culture of human skin and melanoma

A buffered collagen solution was prepared that consisted of rat tail collagen type I (BD Biosciences, Bedford, MA, USA) at a final concentration of 1.35 mg ml^{-1} in DMEM with 10% fetal bovine serum (Meier *et al.*, 2000). One millilitre of the collagen solution was added to tissue culture inserts (Millicell PC, Millipore, Bedford, MA) placed in six-well tissue culture plates. While the 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 × 10^5 ml^{-1} and a fibroblast to melanoma cell ratio of 1:1. Three millilitre of the cellular collagen solution was 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. For submerged culture conditions, 3 ml of melanoma cell culture medium supplemented with 10% fetal bovine serum was added beneath the insert and 2 ml inside the insert. The culture medium was changed every 2 days. After 14 days of submerged culture, the melanoma reconstructs were harvested. Melanoma reconstructs were fixed with 4% formaldehyde for 8–9 hours, dehydrated, and embedded in paraffin. For routine light microscopy, paraffin sections were stained with hematoxylin.

To test the efficacy of the inhibitors alone or in combination against invasive melanoma growth, 451Lu and SKmel28 metastatic melanoma cells were incorporated into human dermal reconstructs and treated with sorafenib (1–6 μM) and/or rapamycin (5–20 nM). 451Lu and SKMel28 cells treated with culture medium or culture medium with the addition of DMSO served as controls.

Statistical analysis

Statistical analyses were performed with a two-tailed unpaired *t*-test. *P*-values <0.05 were considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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