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Combination of MEK and SRC inhibition suppresses melanoma cell growth and invasion

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Abstract

The RAS-RAF-MEK-ERK pathway is deregulated in over 90% of malignant melanomas and targeting MEK as central kinase of this pathway is currently tested in clinical trials. However, dose-limiting side effects are observed, and MEK inhibitors that sufficiently reduce ERK activation in patients show a low clinical response. Apart from dose-limitations, a reason for the low response to MEK targeting drugs is thought to be the up-regulation of counteracting signalling cascades as a direct response to MEK inhibition. Therefore, understanding the biology of melanoma cells and the effects of MEK inhibition on these cells will help to identify new combinatorial approaches that are more potent and allow for lower concentrations of drug being used. We have discovered that in melanoma cells MEK inhibition by selumetinib (AZD6244, ARRY-142886) or PD184352 while efficiently suppressing proliferation stimulates increased invasiveness. Inhibition of MEK suppresses actin-cortex contraction and increases integrin-mediated adhesion. Most importantly, and surprisingly MEK inhibition results in a significant increase in MMP-2 and MT1-MMP expression. All together MEK inhibition in melanoma cells induces a 'mesenchymal' phenotype that is characterised by protease driven invasion. This mode of invasion is dependent on integrin-mediated adhesion, and because SRC kinases are main regulators of this process, the SRC kinase inhibitor saracatinib (AZD0530) completely abolished the MEK inhibitor induced invasion. Moreover, the combination of saracatinib and selumetinib effectively suppressed the growth and invasion of melanoma cells in a 3D environment, suggesting that combined inhibition of MEK and SRC is a promising approach to improve the efficacy of targeting the ERK/MAP kinase pathway in melanoma.

Keywords

melanoma; MEK; SRC; MMP-2; invasion; combination therapy

Introduction

The RAS-RAF-MEK-ERK pathway is deregulated in over 90% of malignant melanomas. In over 40% of cases this is due to activating mutations in the serine-threonine kinase BRAF, and in a further 20% this is caused by mutations in NRAS (1). Targeting mutant BRAF has proven a very promising therapy approach, but development of resistance and the complex molecular mechanisms of RAF-kinase regulation pose a major problem (2-4). An alternative approach that can circumvent these problems is directly targeting the common NRAS and BRAF downstream effector MEK.

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A potent orally active MEK inhibitor that blocks ERK activation in cells at a concentration of 10-100nM is selumetinib (AZD6244, ARRY-142886) (5). In a phase II clinical trial for melanoma selumetinib efficiently reduced ERK phosphorylation in tumours, however the clinical response of patients with BRAF mutations expected to be sensitive to MEK inhibition, was only 12% (6). One reason for this may be the induction of counteracting signalling events, such as up-regulation of FOXO3a and activation of PI3-kinase signalling as a direct response to MEK inhibition (7, 8). Thus, by using MEK inhibitors in monotherapies adaptive responses can create cells that acquire resistance to MEK inhibition or induce further signalling events that will interfere with the tumour suppressing function of the drug. In this respect it is important to mention that whereas it is well established that MEK inhibition suppresses proliferation and can induce cell death in melanoma cells, the impact of MEK inhibitors on melanoma cell invasion is not clear (5, 9).

Melanoma cells are highly motile and invasive cells, and under conditions resembling the three-dimensional extracellular matrix (ECM) they use two major modes of invasion: cells invade by modifying the ECM through protease activities, such as matrix-metalloproteases (MMPs), and they negotiate the 3D-architecture by undergoing cell-shape changes based on Rho/ROCK mediated actin-cytoskeleton dynamics (10, 11). Treating melanoma cells with inhibitors of either proteases or Rho/ROCK activities can force the cells to predominantly adopt one mode of invasion (10, 11), however it is important to mention that under steady state conditions a mixture of modes is observed (12, 13). At the cellular level the Rho/ROCK mediated mode of invasion is represented by a round cell-shape that shows reduced ECM adhesion, whereas the MMP/protease mediated mode of invasion is linked to a more elongated 'mesenchymal' morphology and is dependent on integrin-mediated ECM adhesion (10, 11). Importantly, because integrin-mediated cell adhesion is tightly regulated by SRC kinases (14, 15), the 'mesenchymal' mode of invasion is extremely sensitive to SRC kinase inhibitors (16).

SRC kinases play a central role in cell migration and invasion, and since aberrant activation of SRC itself has been widely implicated in cancer, SRC kinase inhibitors have been developed and are currently tested in the clinic. Saracatinib (AZD0530) is a potent orally available SRC inhibitor with an in vitro IC50 for SRC of 2.7 nM (17). Its impact on proliferation varies (0.2->10 μ M) and is dependent on the cell type (18), which confirms the emerging evidence that in cancer cells SRC might not be a major regulator of proliferation, but predominantly involved in invasion and tumour progression (14, 19, 20). Accordingly, saracatinib efficiently suppressed metastases formation in several preclinical mouse models (18, 21). Saracatinib has been tested in various phase II trials against epithelial cancers such as breast, colon and prostate, and a melanoma trial finished in January 2011 with results pending.

We have analysed the impact of MEK inhibition on melanoma cells in a 3D-environment and observed an unexpected increase in invasiveness. However, SRC inhibition by saracatinib could overcome this effect and the combination of selumetinib with saracatinib resulted in sufficient suppression of cell growth and invasion in melanoma cell spheres, suggesting that combining MEK and SRC inhibitors represents a promising rationale for the design of improved therapies.

Results

MEK inhibition increases melanoma cell invasion into collagen

In melanoma cells expressing the oncogenic mutated BRAF MEK is constitutively active, but treatment with the MEK inhibitor selumetinib suppresses this activity and this can be detected in a loss of phosphorylation of ERK (Fig.1a). At selumetinib concentrations that

lead to efficient loss of ERK phosphorylation in WM266-4 cells, their proliferation is almost completely suppressed (Fig.1b). Following this confirmation of previous findings in melanoma cells (5, 8), we analysed the impact of MEK inhibition on cell growth in a 3D environment and monitored WM266-4 spheres embedded in fibrillar collagen. Selumetinib efficiently suppressed WM266-4 cell growth, and at a concentration of 1 μ M also induced cell death over a period of 72 hours. However, not all cells died and surprisingly we found surviving cells invading the collagen gel as individual cells in a significant distance to the original sphere (Fig.1c). Furthermore, whereas untreated cells invaded the collagen in a mixed, but predominantly rounded mode, selumetinib treated cells exhibited a very elongated shape (Fig.1c). We next wanted to quantify the invasiveness of selumetinib treated cells. To our surprise, treatment of WM266-4 or A375 cells with selumetinib for 24h resulted in a significant increase in their invasion into 3D fibrillar collagen (Fig1.d). This effect was also seen with another MEK inhibitor PD184352/CI-1040 (Fig.1d), clearly demonstrating that in melanoma cells MEK inhibition stimulates increased invasiveness.

MEK inhibition induces a ‘mesenchymal’ shape in melanoma cells

Under steady state conditions both A375 and WM266-4 cells exhibit a mixed mode of invasion, with the majority of cells displaying a round shape (Fig.1c and suppl. Fig.1a). This was also seen when A375 cells were cultured on top of a thick layer of collagen; the majority of cells showed a round phenotype and only 20% of A375 cells adopted a ‘mesenchymal’ shape (Fig.2a and b). The round shape is dependent on actin-myosin driven contraction and regulated by Rho/ROCK mediated myosin-light chain phosphorylation present in the actin cortex and membrane blebs of round cells (22). Importantly, within 24h of MEK inhibition 67% of cells took on an elongated ‘mesenchymal’ shape (Fig.2a and b). Because of this change in cell shape we analysed the level of myosin light chain (MLC) phosphorylation. Although we only observed a slight reduction in total phospho-MLC by Western blotting (Fig.2c), an immunofluorescence analysis of cells embedded in collagen revealed that the amount of phospho-MLC present in the actin cortex of round cells was strongly reduced (Fig.2d). Instead, in MEK inhibited cells phospho-MLC was restricted to the retracting end of the cell (Fig.2d), which is indicative for the ‘mesenchymal’ mode of invasion, where Rho/ROCK activities are high in the rear of the cells and Rac driven actin assembly occurs at the leading edge (23). In addition to actin-myosin regulated shape changes, a further characteristic of the ‘mesenchymal’ mode of invasion is increased adhesion to the ECM substrate. When we analysed melanoma cell adhesion to collagen, we found that in line with inducing a ‘mesenchymal’ shape, MEK inhibition induced a modest, but significant increase in the number of cells adhering to collagen (Fig.2e).

MEK inhibition promotes protease dependent melanoma cell invasion

Our data indicate that MEK inhibition induces a ‘mesenchymal’ shape in melanoma cells. For cells to invade with this shape they need to modulate the ECM through protease activities, such as matrix-metalloproteinases (MMPs) (10, 11). It is generally accepted that the ERK/MAP kinase pathway regulates MMP expression in cancer cells, although in melanoma only few reports document such a regulation, in particular of MMP-1 or MMP-13 (24, 25). Therefore we analysed the effect of MEK inhibition on MMP-production in melanoma cells. A zymographic analysis revealed that although low amounts of MMP-9 were present, MMP-2 was by far the most abundant collagenase present in the medium of A375 or WM266-4 cells when cultured on a collagen gel (Fig.3a). Furthermore, a significant amount of MMP-2 was present in its cleaved and hence active form (Fig.3a). Most importantly inhibition of MEK by either selumetinib or PD184352 did not reduce the amount of pro- and active MMP-2. For a better quantification of MMP activities, we examined the effect of MEK inhibition on the mRNA expression of MMP-2 and MT1-MMP, a membrane tethered MMP, which apart from being a collagenase itself is essential

for the cleavage of pro-MMP-2. This revealed that inhibition of MEK by either selumetinib or PD184352 results in a significant up-regulation of MMP-2 and MT1-MMP expression (Fig.3b, c), suggesting that this increases the ability of MEK inhibited cells to invade in an MMP-dependent fashion. To test this hypothesis, we determined the selumetinib-induced invasiveness of WM266-4 cells in the presence of protease inhibitors. The suppression of proteolytic activities completely abolished the MEK inhibitor induced invasiveness (Fig.3d), clearly demonstrating that MEK inhibition in melanoma cells promotes a protease dependent 'mesenchymal' mode of invasion.

Selumetinib enhances beta1-integrin mediated adhesion to collagen

As mentioned above the 'mesenchymal' mode of invasion is dependent on ECM adhesion, and integrin adhesion receptors are central to this process (26), and one of the main mediators of collagen binding is beta1-integrin. Importantly, a beta1-integrin blocking antibody (MAB13) completely abolished the increase in adhesion induced by MEK inhibition in A375 or WM266-4 cells, whereas no effect was seen when a control antibody was used (Fig.4a, b). This demonstrated that MEK inhibition increases the adhesion of melanoma cells to collagen through beta1-integrin.

Focal adhesion kinase (FAK) is essential for integrin-mediated adhesion, and selumetinib treatment resulted in loss of phosphorylation at S910 (Fig.4c). Phosphorylation of this site by ERK has been shown to regulate a decrease in FAK activity detectable as reduction in phosphorylation at Y397 (27). However, we could not detect any difference in Y397 phosphorylation in cells treated with selumetinib over 24h (Fig.4d), suggesting that S910 phosphorylation might have another function than directly regulation FAK activity. Further important regulators of integrin-mediated cell adhesion are SRC kinases, and melanoma cells and melanocytes predominantly express the members SRC, FYN and YES (suppl. Fig. 2). Using a PAN-SRC antibody we assessed the total SRC kinase activity in these cells. In melanocytes the basal activity was very low, but strongly increased in response to a growth-factor supplement containing bFGF, SCF and TPA (Fig.4e). In contrast basal SRC kinase activity was constitutively high in melanoma cells (Fig.4e). Importantly, although selumetinib increased integrin-mediated adhesion, it did not increase the amount of activated SRC kinase detectable as auto-phosphorylation activity at Y416 (Fig.4f).

Saracatinib suppresses melanoma cell collagen adhesion and invasion

The 'mesenchymal' mode of invasion is extremely sensitive to SRC kinase inhibition (16). Therefore, we hypothesised that inhibiting SRC kinase activities represents a potential strategy to overcome MEK-inhibitor effects on invasion.

Treatment of melanoma cells with the SRC kinase inhibitor saracatinib resulted in efficient suppression of SRC auto-phosphorylation activity at Y416 (Fig.5a) and led to a strong decrease in FAK Y861 phosphorylation (Fig.5b), which is required for efficient integrin-mediated focal adhesion formation. In line with this, treatment with saracatinib resulted in the reduction of vinculin containing focal adhesions (Fig.5c) and eventually in the complete detachment of cells from the collagen substrate (Fig.5d), which was even more noticeable in cells cultured on a thick collagen gel (Fig.5d, right panel). Most importantly, SRC inhibition by saracatinib prevented the increase in adhesion to collagen observed in selumetinib treated cells (Fig.5e and f).

Because SRC inhibition resulted in reduced substrate adhesion of melanoma cells, we next wanted to assess its effect on the invasion of melanoma cells into 3D-collagen. We found that saracatinib at a concentration as low as 0.5 μ M almost completely suppressed the invasion of either WM266-4 or A375 cells into 3D-collagen (Fig.6a and b). Because under

steady state conditions the majority of the cells do not invade in a 'mesenchymal' mode, this suggested that also the round, membrane-blebb driven invasion ('amoeboid' mode) requires the cells to at least partially adhere to the matrix. This is in line with previous findings that - although to a lesser extent than the 'mesenchymal' mode- also the 'amoeboid' mode of invasion is suppressed in the presence of integrin blocking antibodies (16). However, despite this potent effect on invasion, saracatinib had only a minor effect on the proliferation of either WM266-4 or A375 cells at concentrations when invasion was suppressed to over 90% (Fig.6c and d). This suggests that SRC kinases do not play a major role in melanoma cell proliferation and is in line with the observation that the SRC inhibitor dasatinib also does not significantly affect melanoma cell proliferation at concentrations when invasion is blocked (19, 20).

Because saracatinib suppressed adhesion in the presence of MEK inhibitor (see Fig.5e and f), we assessed its effect on invasion under these conditions. When we co-treated cells with selumetinib and saracatinib, the saracatinib produced block in invasion dominated and hence completely abolished the MEK inhibitor induced increase invasion (Fig.6e and f).

Combination of selumetinib and saracatinib suppresses melanoma cell growth and invasion within dermal collagen

In order to fully assess the impact of combinatorial treatment of melanoma cells with selumetinib and saracatinib, we analysed melanoma spheres embedded into 3D fibrillar collagen for cell growth, survival and invasion. 16h after embedding the spheres, the cells had started leaving the sphere and invading the collagen. At this point selumetinib was added to the spheres at different concentrations in the presence or absence of saracatinib. Already within 6h after the addition of selumetinib an increase in elongated cells could be observed and this effect was even more apparent at 24h (suppl. Fig.1a and b). After 72h DMSO treated cells had invaded the collagen covering an area ~4 times the size of the original sphere, but this was significantly suppressed by saracatinib (Fig.7a and b). Although the majority of cells cultured in the presence of 1 μ M selumetinib for 72h died and stained positive for EtBr (Fig.7c and d), surviving cells still invaded the collagen gel at the same distance and thus covered an area similar to DMSO treated cells (Fig.7a and b). Strikingly, although 0.1 μ M selumetinib still had a toxic effect (Fig.7d), surviving cells invaded the collagen gel with an even longer distance than DMSO treated cells (Fig.7a and b). However, the addition of saracatinib effectively suppressed this increase in invasion (Fig.7a and b). Together this demonstrated that even at low concentrations of selumetinib the additional inhibition of SRC kinases produces a potent inhibitory effect on melanoma cell growth and spread.

Discussion

We have discovered that although MEK inhibition in BRAF mutant melanoma cells produces a very potent block in proliferation, it also increases the invasive behaviour of these cells into 3D fibrillar collagen. This finding was surprising, because it is generally accepted that ERK/MAP-kinase signalling is required for growth factor receptor induced 2D cell migration (28). Furthermore, pre-treatment of melanoma cells for 24h on plastic, thus affecting ERK mediated transcriptional events before exposure to a 3D matrix, results in suppression of invasion (29). Crucially however, when MEK is inhibited in melanoma cells while already grown in a 3D matrix, this does not reduce their invasion (9). Together this suggests not only that effects of MEK inhibition on invasive activities differ at early and later time points, but also that in 3D MEK regulates invasive properties that are not detectable in 2D.

We found that inhibition of MEK in the context of a 3D matrix resulted in the induction of an elongated cell shape and increased integrin-mediated adhesion. The cell shape of this so called 'mesenchymal' phenotype is based on the loss of actin-myosin contraction within the cell cortex, which is due to reduced MLC phosphorylation downstream of Rho/ROCK signalling at the membrane (10, 13). We found that inhibition of MEK led to a decrease in MLC phosphorylation, and this is in line with previous reports showing that ERK can regulate Rho/ROCK signalling by either directly phosphorylating and activating MLC kinase (28) or phosphorylating and inhibiting RhoGAP (30). Importantly, reduced Rho activity at the membrane regulates the early stages of cell spreading, where it leads to increased substrate adhesion (31). Thus the reduced Rho activity in MEK inhibited cells might contribute to the increased adhesion of melanoma cells to collagen that we observed. On the other hand, adhesion also depends on integrin interactions, and beta1 integrin has been found to cluster at sites of interaction with collagen fibres (26). Accordingly, we identified beta1 integrin to be crucial for collagen adhesion of melanoma cells, but we did not observe a change in beta-1 integrin surface expression or activity in response to MEK inhibition (not shown) that would explain the increased adhesion. Nevertheless, MEK/ERK signalling also contributes to the regulation of focal adhesion dynamics. For instance, ERK can directly phosphorylate and activate the m-isoform of the calcium-dependent protease calpain (calpain2), and this is crucial for the proteolysis and turnover of several adhesion components including focal adhesion kinase (FAK) (32-34). However, we failed to detect any increase in FAK protein after MEK inhibition (Fig.4d), which suggests that under our experimental conditions compensatory mechanisms that are independent of ERK, such as activation by calcium (34) can regulate calpain2 activity. This is important, because it has been shown that calpain2 is essential for 'mesenchymal' invasion (16), the mode that we find to be increased when MEK is inhibited.

It is commonly accepted that ERK/MAP kinase signalling activates the expression of various MMP genes such as *MMP-9*, *MMP-1* or *MMP-13*. Also in melanoma MEK/ERK signalling regulates the *MMP-1* promoter (25) and *MMP-13* expression (24), but the role of MEK in *MMP-9* expression is less clear. Although we detected MMP-9 activity in melanoma cell conditioned medium, we found MMP-2 to be the major collagenase activity secreted by these cells. Most importantly, MEK inhibition resulted in an increase in *MMP-2* expression, indicating that in melanoma cells MEK/ERK signalling suppresses the *MMP-2* promoter. In line with this, an inhibitory function of ERK on the *MMP-2* promoter has been described previously in the context of IGF-I signalling (35). Furthermore the ATF/CREB transcription factor ATF3 can suppress the *MMP-2* promoter, and the expression by ATF3 is regulated by ERK (36, 37). Besides *MMP-2* we found that MEK also suppressed MT1-MMP expression. This is an important finding, because MT1-MMP is required for *MMP-2* processing (38), and consequently MEK inhibition results in the production of a fully active *MMP-2* enzyme. In addition MT1-MMP is a collagenase itself and as such essential for cancer cell invasion (39, 40). Thus, although the MAP kinase pathway often activates *MMP* genes, we have shown that it also can suppress *MMP* expression most probably depending on the cell type and the signalling context.

In summary, MEK inhibition of melanoma cells in fibrillar collagen produces all characteristics of a 'mesenchymal' invasion phenotype with an elongated morphology based on reduced Rho mediated MLC phosphorylation, enhanced integrin-mediated adhesion and increased expression of MMPs. Importantly, because this mode of invasion is more dependent on integrin-mediated adhesion, it is more sensitive to inhibitors of adhesion, such as inhibitors of SRC kinases (16), the crucial regulators of cell migration and invasion.

Elevated SRC kinase expression and auto-phosphorylation has been reported in melanoma and SRC itself is involved in melanoma cell migration and metastasis (41-43). Furthermore,

increased FYN activity induces melanocyte transformation, regulates melanoma cell migration and invasion, and its activity is up-regulated during tumour progression *in vivo* in a fish model for melanoma (44-46). Dasatinib (BMS-354825), a dual specific SRC/BCR-ABL inhibitor that is currently tested in clinical trials, has been shown to significantly reduce migration and invasion of melanoma cells *in vitro* at concentrations when no major effect on melanoma cell proliferation or survival was observed (19, 20). This emphasizes the fact that in melanoma cells SRC kinases are not important regulators of cell growth, and might explain the rather disappointing result of the first published dasatinib phase II trial in melanoma that used reduction of tumour volume as endpoint and achieved only a response rate of 5% (47). It seems that if tumour reduction is the aim in SRC inhibitor therapies, higher concentrations need to be achieved and this might be difficult due to toxicity limitations. On the other hand with the potent suppression of invasion and metastasis by SRC inhibitors in preclinical settings, a more meaningful assessment in clinical studies would be to measure effects on the reduction of motility and invasion.

Overall it appears that SRC inhibitors in monotherapies are not sufficient to affect tumour size, and therefore combinations with other anti-proliferative or cytotoxic drugs have been considered and various trials combining e.g. dasatinib or saracatinib with cytotoxic agents such as gemcitabine, paclitaxel or EGFR inhibitors have been carried out (48-50). We discovered that the combination of the MEK inhibitor selumetinib with saracatinib has potent anti-proliferative, cytotoxic and anti-invasive effects on melanoma cells. Most importantly, we found that SRC kinase inhibition counteracts the pro-invasive activities produced by MEK inhibition. The central role of the ERK/MAP kinase pathway in melanoma makes it an obvious target for therapeutic intervention, but we have demonstrated that additional inhibition of SRC kinases is a relevant factor that should be considered in future designs for rationale approaches in melanoma therapy.

Materials and Methods

Cell culture and reagents

A375 and WM266-4 cells were grown in DMEM/10% FCS (Invitrogen, Carlsbad, CA, USA). Selumetinib and saracatinib were obtained under a material transfer agreement with Astra Zeneca (Alderly Park, Macclesfield, UK). PD184352 was from Axon Medchem, Groningen, The Netherlands). To culture cells on thick collagen layers, cells were seeded on a 12 well containing 0.6 ml fibrillar bovine dermal collagen (2.3mg/ml, Nutacon, Leimuiden, The Netherlands) in DMEM/10% FCS.

EdU incorporation analysis

20h after inhibitor treatment, cells were labeled with 10 μ M EdU (Invitrogen) for 4h before they were formalin fixed and processed following the manufacturer's instructions. Stained cells were analysed using a BDpathway 855 Bioimager.

Immunoblots and antibodies

Cell lysates were prepared and analysed as described (51). The primary antibodies used were: phospho-ERK (MAPK-YT) from Sigma, St Louis, MO, USA, ERK2 (C-14) and SRC (SRC-2) from SCBT, Santa Cruz, CA, USA, phospho-SRC (Y416) and phospho-MLC2 (Ser19) from Cell Signaling, Boston, MA, USA, SRC (GD-11) from Millipore, Watford, UK, phospho-FAK (Y861), (Y397), (S910) from Invitrogen.

Melanoma spheres

Cells were taken up in DMEM/5%FCS containing 1.5% methylcellulose and spheres were allowed to form in a 96 well U-bottom plate over a period of 48-72h. Spheres were then

transferred into 0.5 ml fibrillar bovine dermal collagen (2.3mg/ml) per 24 well. Once the collagen was set, DMEM/10%FCS was added and after ~16h drugs were added as indicated. Dead cells were identified by staining of non-fixed cells with EtBr (100µg/ml) for 10 min, followed by 3 washes with PBS.

In vitro invasion assay

Invasion assays analysing melanoma cell invasion into collagen were performed as described (29). Briefly, a total of 5×10^3 cells in 100 µl serum-free dermal collagen (2.3mg/ml, Nutacon) was dispensed into 96-well ViewPlates (Perkin-Elmer, UK) coated with BSA. The cells were sedimented at $300 \times g$ and incubated at 37°C/10% CO₂ for 30 min to coagulate the collagen, then overlaid with DMEM/10% FCS and inhibitors as indicated. After 24 hr, cells were fixed (4% formaldehyde) and stained with Hoechst 33258 (Invitrogen). Confocal Z sections were collected at the bottom of the wells and at 50 µm using a BD pathway Bioimager 855 high-content microscope. Nuclear staining was quantified using BD Bioimager software. Relative invasion relates to: [cells at 50 µm]/[cells at 1 µm]. Means of quadruplicate samples are presented as fold compared to controls. The cocktail of protease inhibitors was prepared as described (10): 20µM GM6001 (Calbiochem), 10µM calpeptin (Calbiochem) and 10µg/ml aprotinin and leupeptin (Sigma). Data are from at least three independent measurements and are shown as mean +/- SEM.

Adhesion assay

The adhesion assay was performed as previously described (51). A 96-well ELISA plate was coated with collagen (0.2 mg/ml) or BSA (1%/PBS) o/n at 4°C and blocked with 1% BSA/PBS. Melanoma cells were preincubated with either anti-beta1 integrin (MAB13, (52)) an unspecific control antibody or without antibody in DMEM/0.1% BSA for 15 min at RT, cells were allowed to adhere to the substrate at 37°C for 30 to 60 min. Non-adhering cells were washed off with PBS, and adhering cells were formalin fixed, stained with 0.5% toluidine blue, and the solubilized colour (in 1% SDS) was measured at 595 nm. Data are from at least three independent measurements and are shown as mean +/- SEM.

Gelatin zymography

An equal number of A375 or WM266-4 cells were seeded on top of collagen gels and after the indicated times aliquots of conditioned media were mixed with non-reducing sample buffer and analysed on gelatin containing (1.2mg/ml) acrylamide gels. Gels were stained with 0.025% Coomassie and then destained until bands were visualized.

In vitro kinase assay

SRC kinases were immuno-precipitated using the SRC-2 antibody (2 µg) and the precipitated proteins were analysed as described previously (45).

Quantitative Real-Time PCR

RNA was isolated with TRIZOL® and selected genes were amplified by quantitative real time PCR using SYBR green (Qiagen, Valencia, CA, USA). Primers sequences were *MMP-2*: CTGGCTCATGCCTTCGCCCC, ACTCCCCATCGGCGTTCCCA; *MT1-MMP*: CCGAGGGCTTCCATGGCGAC, ATTCTGACAGTCCAAGGCTCGGC; *GAPDH*: CAATGACCCCTTCATTGACC, GACAAGCTTCCCGTTCTCAG.

Immunofluorescence and microscopy

Cells on cover slips or collagen were stained as described (13). For fluorescence microscopy a Zeiss Axiovert 200 equipped with epifluorescence was used; images were taken by a Photometrics Cool Snap HQ CCD camera driven by Metamorph software (Universal

Imaging). GFP expressing cells were monitored using a Leica DM IL HC inverted microscope and a FC340 Cooled Monochrome camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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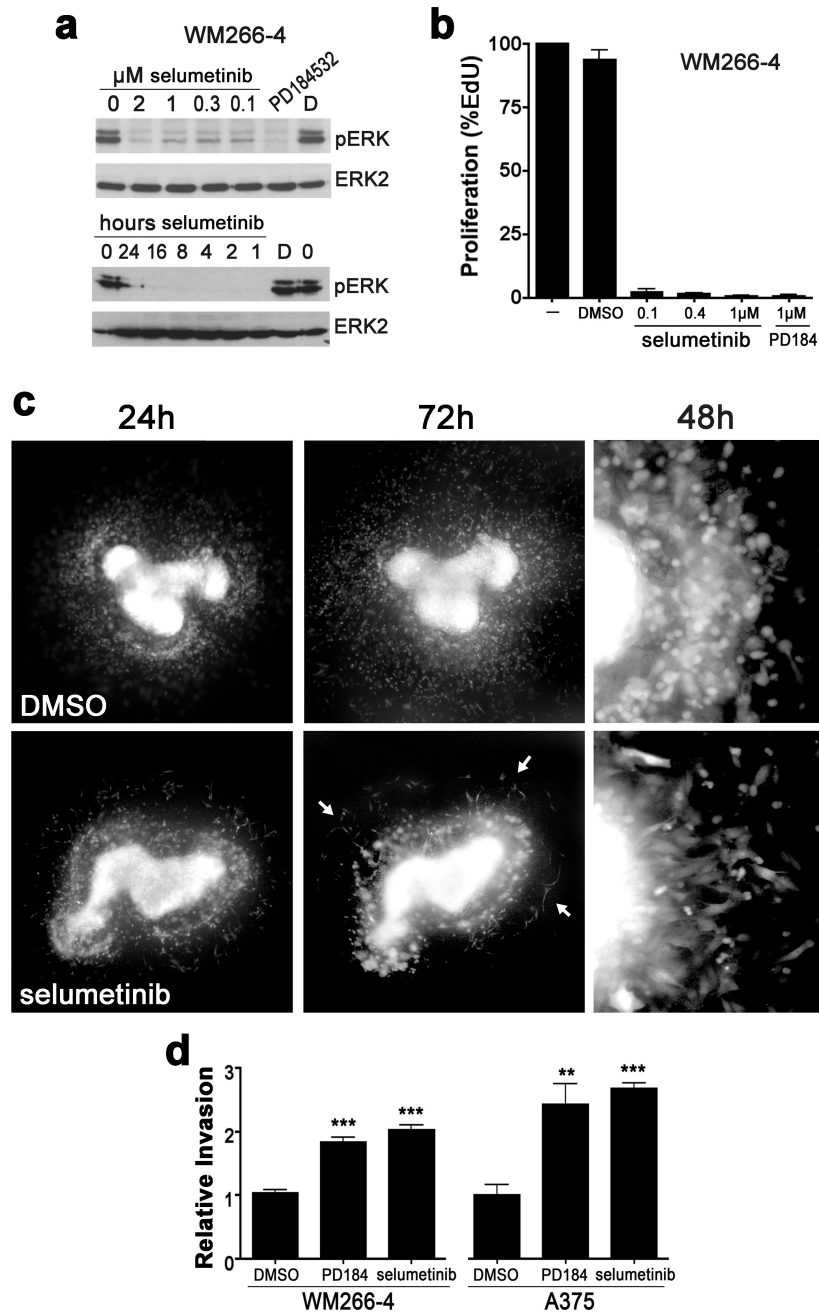


Figure 1. MEK inhibition increases melanoma cell invasion into collagen. (a) Phospho-ERK (pERK) Western blots for WM266-4 cells treated with selumetinib at different concentrations for 24h (left panel) and for different times at 1 μM (right panel); 1 μM PD184352 was used as control. ERK2 was used as loading control. (b) Proliferation assay. WM266-4 cells were treated with the indicated drugs and EdU incorporation was measured 24h after drug addition; untreated cells were set 100%. (c) GFP-expressing WM266-4 cells were cultured as spheres for 3 days before they were embedded in 3D collagen. After 16h, a front-wave of cells had started to invade the collagen, at which time the spheres were treated with DMSO or with 1 μM selumetinib. Images shown are from 24h and 72h after addition of the drug.

Magnifications are from 48h treatment. Arrows indicate individual invading cells. (d) Relative invasion assay. The invasion of WM266-4 and A375 into a 3D collagen gel was analysed either in the absence or in the presence of DMSO, 1 μ M PD184352 or 1 μ M selumetinib. Drugs were added at the beginning of the experiment and invasion was quantified after 24h.

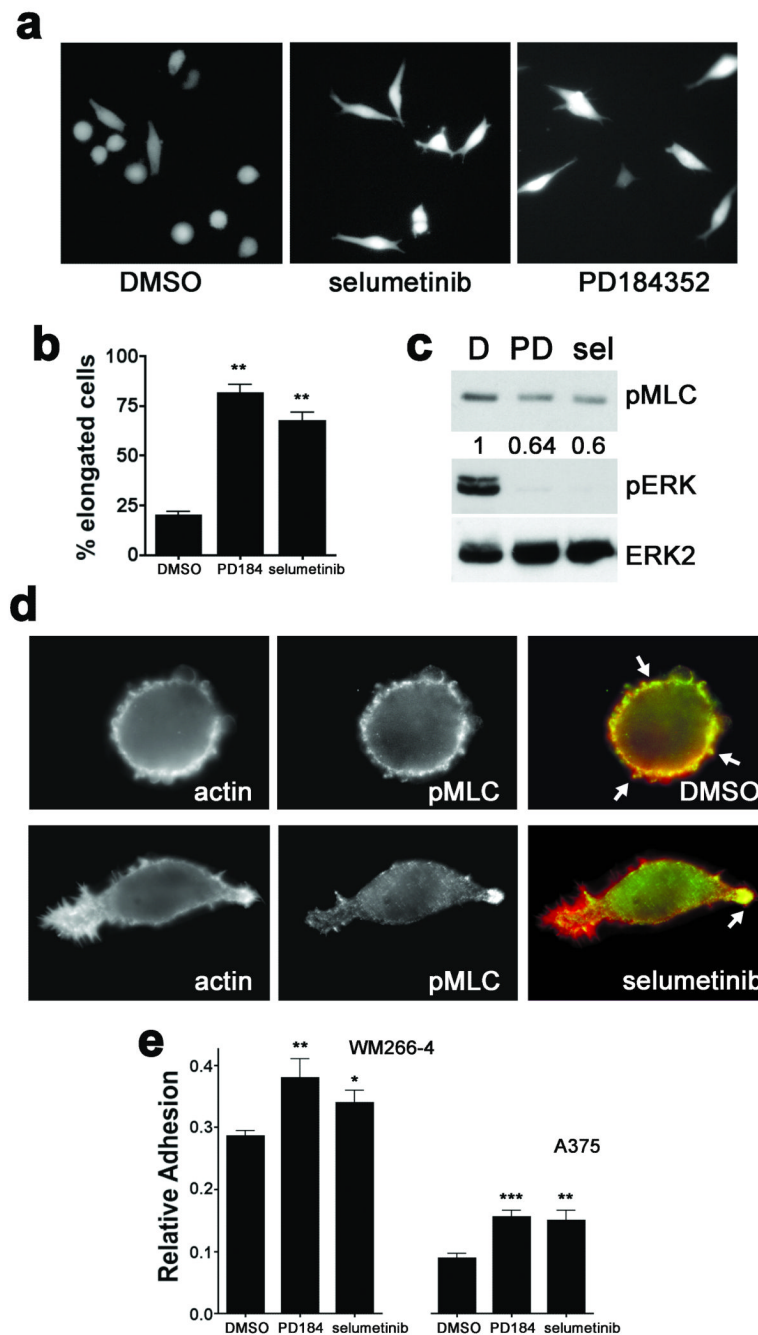


Figure 2. MEK inhibition induces a ‘mesenchymal’ shape in melanoma cells. (a) GFP-expressing A375 cells were cultured on a thick collagen gel and treated with DMSO or selumetinib or PD184352 for 24h before images were taken. (b) Quantification of GFP-A375 cells cultured on a thick collagen gel and treated for 24h with DMSO, selumetinib or PD184352. (c) Western blot analysis of A375 cells cultured on a thick collagen and treated for 24h with DMSO, selumetinib or PD184352. Cells were analysed for phospho-MLC (pMLC), pERK and ERK2. The intensity of pMLC was quantified using ImageJ. (d) A375 cells in fibrillar collagen were treated for 24h with DMSO or selumetinib. Fixed cells were stained for pMLC, F-actin was stained using Alexa Fluor® 594-phalloidin. (e) Collagen adhesion assay

of WM266-4 and A375 cells treated either with the vehicle DMSO or with 1 μ M PD184352 or 1 μ M selumetinib, before they were allowed to adhere to collagen.

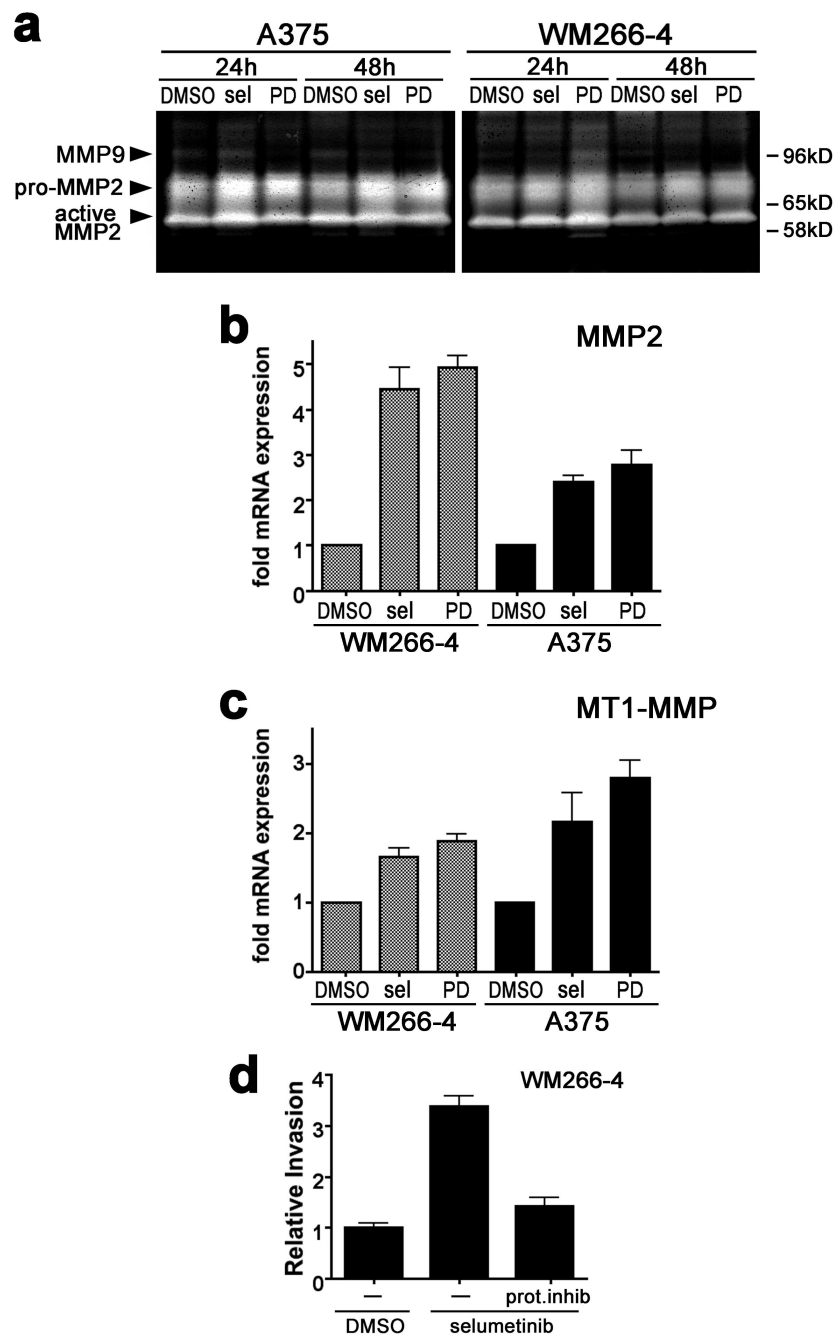
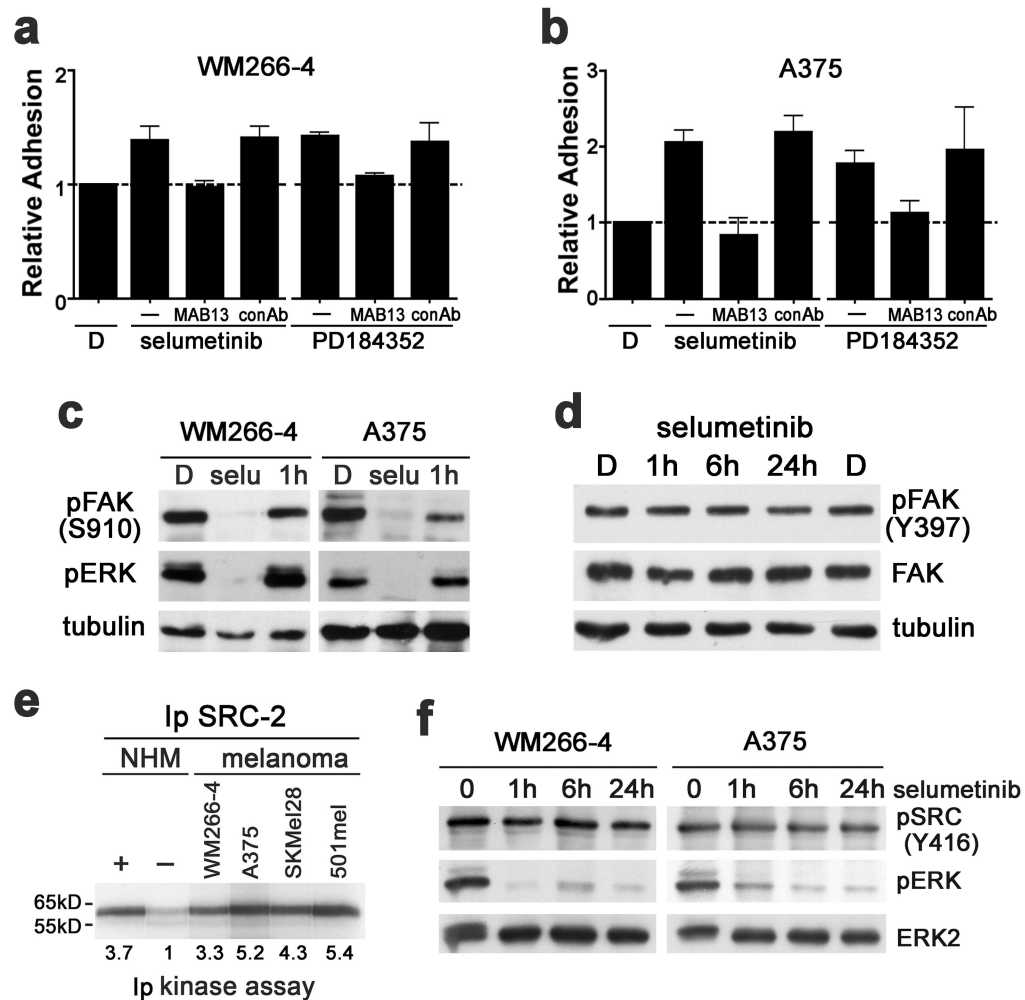
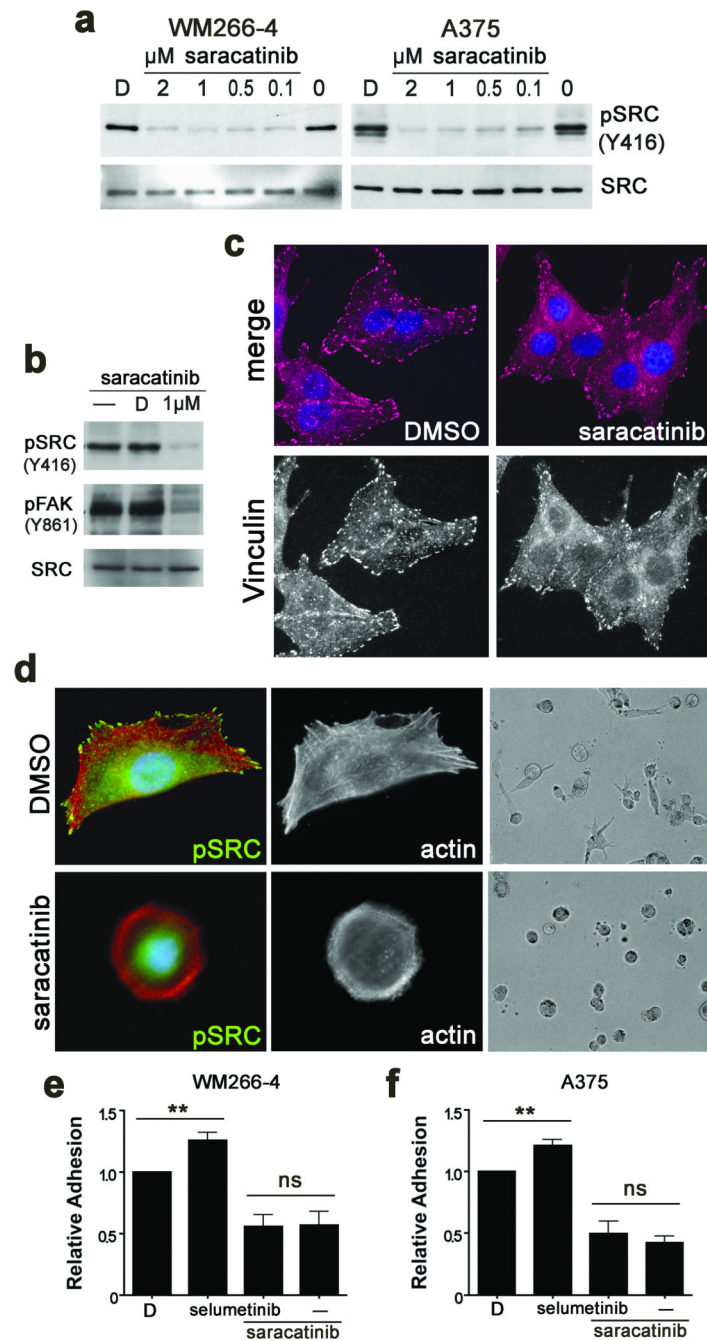


Figure 3. MEK inhibition promotes protease dependent melanoma cell invasion. (a) Zymogram of conditioned media from WM266-4 and A375 cells cultured on a thick collagen gel and treated with DMSO, 1 μ M PD184352 or 1 μ M selumetinib for 24h and 48h. (b) Quantitative PCR for MMP-2 and (c) MT1-MMP from WM266-4 and A375 cells grown on a thick collagen gel treated with either DMSO, 1 μ M PD184352 or 1 μ M selumetinib for 24h. (d) Relative invasion assay of WM266-4 cells either untreated, or treated with 1 μ M selumetinib in the absence or presence of a protease inhibitor cocktail (prot.inhib) containing GM6001, calpeptin, leupeptin and aprotinin.

**Figure 4.**

Selumetinib increases integrin-mediated collagen adhesion in melanoma cells. (a) and (b). Competitive collagen adhesion assay. WM266-4 (a) and A375 (b) cells treated with either DMSO, 1 μ M PD184352 or 1 μ M selumetinib were either left untreated or pre-incubated with a beta1-integrin specific antibody (MAB13) or a control antibody, before they were allowed to adhere to collagen. (c) Western blot of phosphoS910-FAK, pERK and beta-tubulin (loading control) of A375 and WM266-4 cells, which were treated with selumetinib for 24h, before the drug was washed out for 1h, during which time pERK recovers. (d) Western blot of phosphoY397-FAK and FAK of A375 cells treated with selumetinib for the indicated times. Beta-tubulin served as loading control. (e) In vitro kinase assay for total Src-kinase activity using a pan Src-kinase family antibody in melanoma cells and melanocytes. Melanocytes were left untreated (-) or stimulated with growth factor supplement (+). (f) Western blot of WM266-4 and A375 cells for phosphoY416-SRC and pERK treated with selumetinib for the indicated times. ERK2 serves as loading control.

**Figure 5.**

Saracatinib inhibits integrin-mediated collagen adhesion in melanoma cells. (a) Western blot for phospho-SRC (p-Y416) and total SRC of WM266-4 and A375 cells treated with the indicated concentrations of saracatinib or left untreated. (b) Western blot of A375 cells treated with DMSO or 1 μM saracatinib for 24h. Cell lysates were analysed for phospho-SRC (p-Y416), phospho-FAK (p-Y861) and total SRC, which was used as loading control. (c) Immunofluorescence of A375 cells for vinculin using Cy3-labelled secondary antibodies. Cells were either treated with DMSO or with 1 μM saracatinib. (d) Immunofluorescence of A375 cells for phospho-SRC using Cy2-labelled secondary antibodies (merge, left panel). Cells were either treated with DMSO or with 1 μM saracatinib. F-actin was stained using

Alexa Fluor® 594-phalloidin (middle panel). A375 cells were cultured on a thick collagen gel and treated with DMSO or 1 μ M saracatinib for 24h before bright field images were taken (right panel). (e) and (f) Collagen adhesion assay of WM266-4 and A375 cells treated either with the vehicle DMSO or with 1 μ M selumetinib in the absence or presence of 1 μ M saracatinib. Cells were pre-treated before they were allowed to adhere to collagen for 1h.

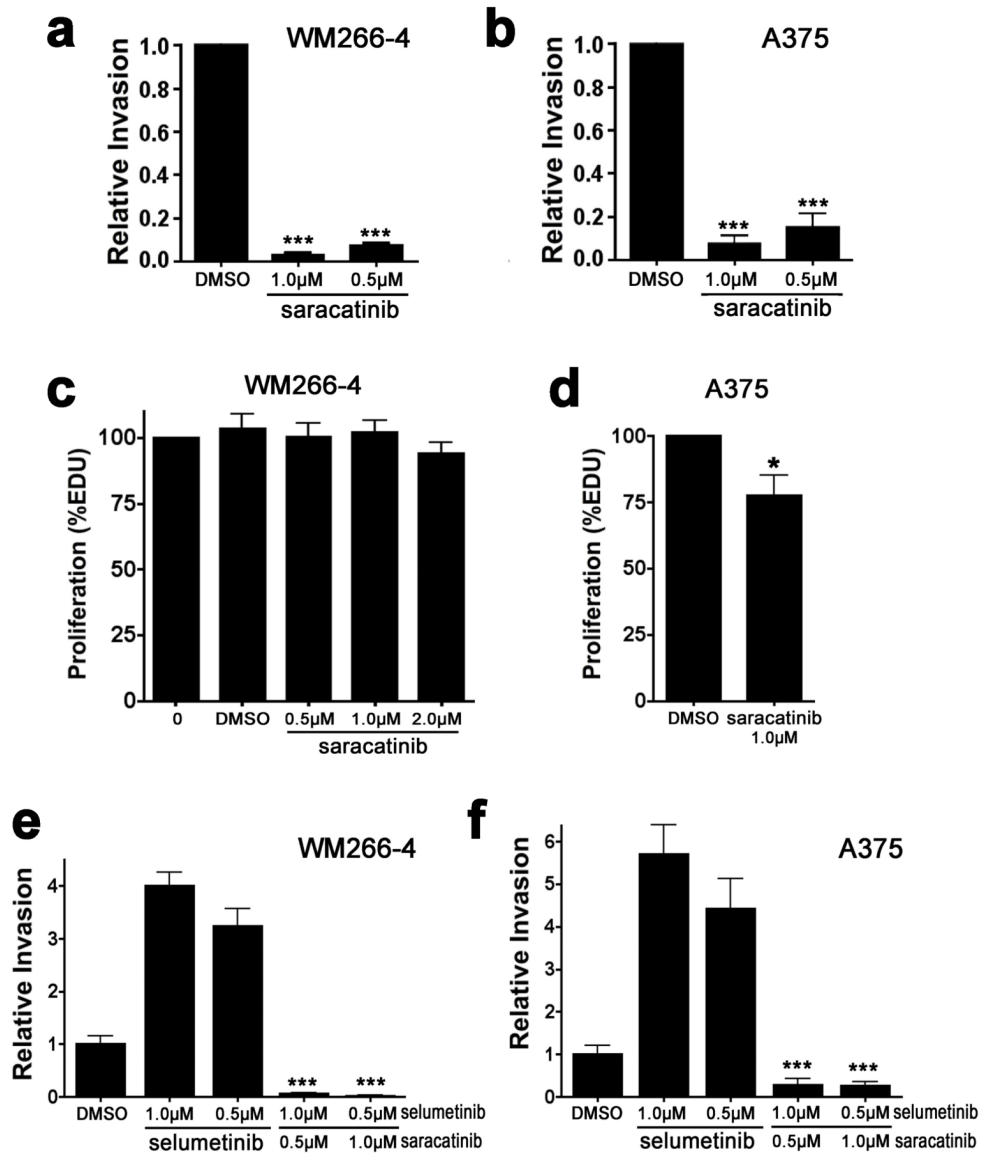


Figure 6. Saracatinib suppresses melanoma cell invasion. (a) Relative invasion assay of WM266-4 and (b) of A375 cells. Cells were analysed in an inverted collagen invasion assay either in the absence or in the presence of DMSO or 1 μ M or 0.5 μ M saracatinib. Invasion was quantified after 24h. (c) Proliferation assay. WM266-4 cells were treated with the indicated drugs and EdU incorporation was measured 24h after drug addition. EdU incorporation into untreated cells was set 100%. (d) EdU proliferation assay of A375 cells using 1 μ M saracatinib. EdU incorporation into untreated cells was set 100%. (e) Relative invasion assay of WM266-4 and (f) of A375 cells. Cells were analysed in an inverted collagen invasion assay either in the absence or in the presence of DMSO or 1 μ M or 0.5 μ M selumetinib in combination with saracatinib. Invasion was quantified after 24h.

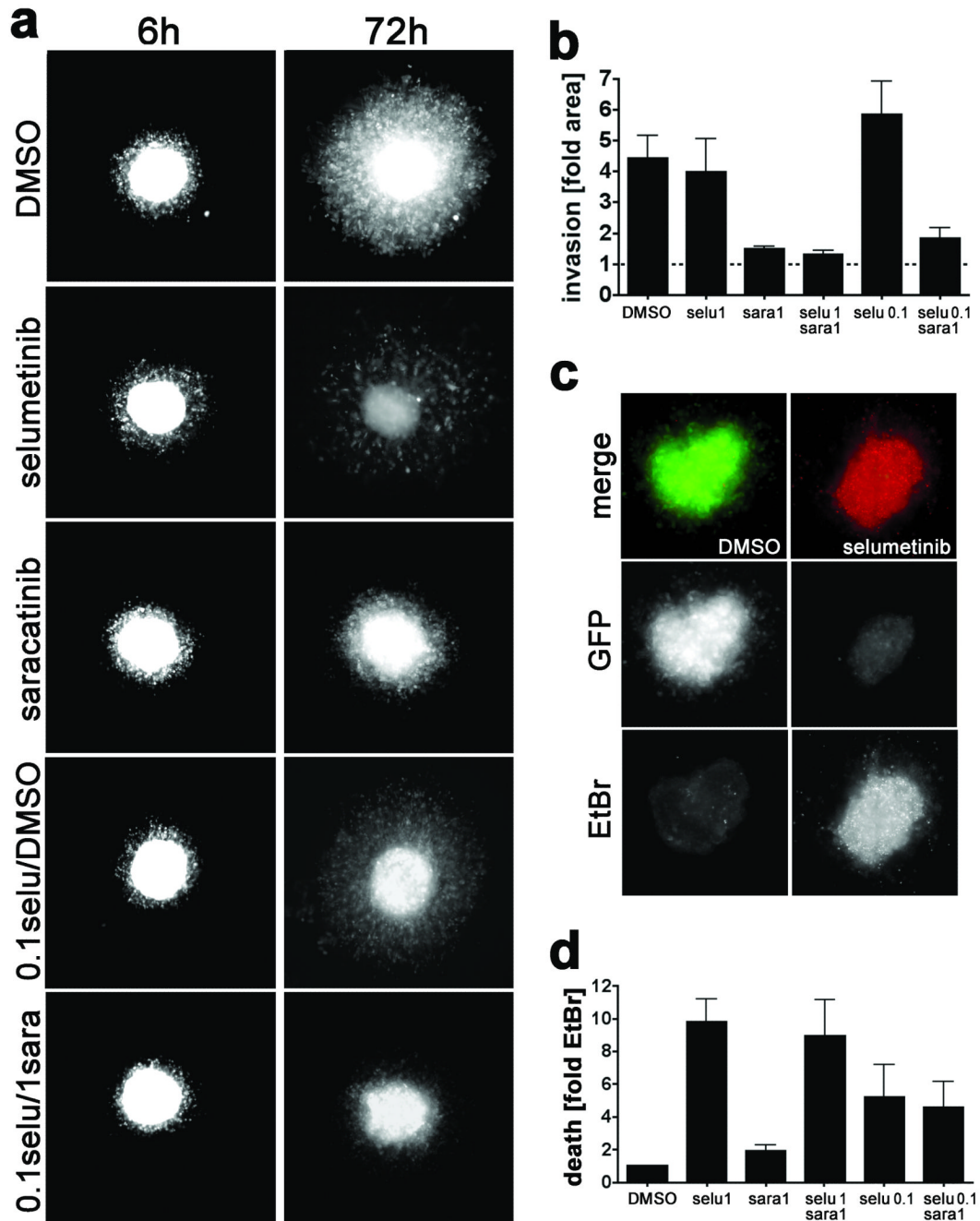


Figure 7.

Combination of selumetinib and saracatinib suppresses melanoma cell growth and invasion. (a) GFP-expressing A375 cells were cultured as spheres for 3 days before they were embedded in 3D collagen. After 16h, a front-wave of cells had started to invade the collagen, at which time the spheres were either treated with DMSO or with 0.1 or 1 μ M selumetinib in the presence or absence of 1 μ M saracatinib. Images shown are from 6h after drug addition and 72h after the addition of the drugs. (b) Quantification of the invasive activity of cells within the sphere. The total area invaded by the cells relative to the initial sphere size was quantified using ImageJ. (c) EtBr staining of spheres. Non-fixed spheres of GFP-A375 cells were stained with EtBr and images were taken. Shown are DMSO and 1 μ M

selumetinib treated cells. (d) The intensity of EtBr staining in treated spheres relative to DMSO treated spheres was quantified using ImageJ.