

Pharmacological suppression of the Ras/MAPK pathway in thyroid carcinoma cells can provoke opposite effects on cell migration and proliferation: The appearance of yin-yang effects and the need of combinatorial treatments

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Abstract. A major challenge in tumor therapy is the decrease or even the halting of cell proliferation and migration of cancerous cells. In the present study, we have analyzed the impact of a pharmacological blockade of the PI3K/Akt and MAPK/ERK1/2 signaling pathways on cell migration, proliferation and cell death in three human thyroid tumor cell lines that represent the main types of malignant thyroid carcinomas (B-CPAP, follicular; Cal-62, anaplastic; FTC-133, papillary thyroid carcinoma cells) and in which these pathways are constitutively activated. In general, pharmacological perturbation of PI3/Akt (application of MK-2206) and MEK/ERK1/2 (application of PD0325901 or U0126) signaling led to a cell line and drug-specific decrease in the proliferation and migration potential of thyroid carcinoma cells, although to a varying extent. However, one exception became apparent: in Cal-62 cells inhibition of the MEK/ERK1/2 module increased the migration rate up to 50%. This effect could be prevented by a simultaneous suppression of the PI3/Akt pathway, but also by application of the multiple kinase inhibitor sorafenib, a treatment that did not change the activation state of Akt. Thus, a pharmacological perturbation of canonical signaling pathways in thyroid carcinoma may induce drug-dependent yin-yang effects that are characterized by a simultaneous suppression of one (i.e., proliferation) and the activation of another (i.e., migration) cellular process. The appearance of

such phenomena should be taken into account when therapy plans are established.

Introduction

Well-differentiated papillary and follicular carcinomas typically have indolent behavior and can be effectively treated by surgery followed by radioiodine therapy (1). However, tumors that lose differentiation during progressive disease or already have developed as undifferentiated entities (i.e., anaplastic thyroid tumors) have lost the ability to trap iodine. As a consequence, these types of tumors do not respond to conventional radioiodine treatment and, thus, have a much less favorable prognosis (2). Hence, alternative concepts are urgently warranted to improve therapeutic undertakings. In recent years, small molecule kinase inhibitors have been introduced in therapeutic treatments of several types of cancers (3,4). Especially in regard to thyroid cancers focus has been directed to the Ras/ERK1/2 and PI3K/AKT pathways because both are activated in a high percentage of thyroid tumors. In more than 80% of papillary cancers, for example, genes of the MAPK pathway are affected (5). Activation of the classical MAPK pathway occurs mainly via genetic alterations of Ras, Raf or Ret genes (6). In addition to the activation of the MAPK pathway, RET/PTC or BRAF mutations can also result in the activation of PI3K. In thyroid cancer, moreover, the activation of Akt can also be a result of PI3K mutation and amplification, RET/PTC rearrangements, reduction or loss of PTEN expression (7).

Clinical studies with specific ERK1/2 or Akt inhibitors as well as with multikinase inhibitors such as sorafenib have already been undertaken with promising results. Sorafenib (BAY 43-9006, Nexavar) is a second-generation, orally active multikinase inhibitor that was recently approved for the treatment of patients with advanced renal cell carcinoma and patients with unresectable hepatocellular carcinoma (8). Some clinical studies have also demonstrated the effectiveness of sorafenib in advanced thyroid cancer (9,10). The action of

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sorafenib affects a broader range of signal transducers, such as vascular endothelial growth factor receptors, platelet-derived growth factor receptor- β , c-kit or RET (8). Sorafenib down-regulates activated Akt and ERK1/2 in neuroblastoma (11) and lymphoma cells (12), but, unexpectedly, can also initiate ERK1/2 (13,14) or Akt activation (15) that leads to increased tumor cell proliferation and migration. The use of the MEK inhibitors PD0325901 or U0126 is restricted to academic research, as these drugs produced undesirable side effects in clinical trials (16). Nevertheless, these compounds serve as valuable experimental paradigms to analyze the consequences of MEK/ERK1/2 inactivation on the cellular level.

In the present study we show that in thyroid carcinoma cells downregulation of Ras/MAPK and PI3K/Akt pathway activation predominantly suppresses cell migration and proliferation. In the anaplastic cell line Cal-62, however, inactivation of Ras/MAPK signaling positively affects the migration rate. This increase can be suppressed by PI3K/Akt pathway-dependent (MK-2006 treatment) and, most likely, -independent (sorafenib treatment) mechanisms.

Materials and methods

Cell lines and culture conditions. Human papillary thyroid cancer cell line B-CPAP and human follicular thyroid cancer cell line FTC-133 were from Professor G. Brabant (Department of Endocrinology, Christie Hospital, Manchester, UK). Human anaplastic thyroid cancer cell line Cal-62 was obtained from Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). B-CPAP cells were cultivated in RPMI-1640 medium, Cal-62 cells in DMEM, and FTC-133 cells in DMEM/Ham's F12. Media were supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin and L-glutamine.

Cell extraction and western blot analysis. Cell solubilisation in the presence of protease and phosphatase inhibitors and western blot analyses were carried out as described (17).

Drug treatment. Stock solutions and working concentrations were as follows: for MK-2206 (Selleck Chemicals), 10 mM and 1 μ M, for PD0325901 (Axon Medchem) 10 mM and 2 μ M, for sorafenib (Enzo Life Sciences) 10 mM and 5 μ M, for U0126 (Promega) 10 mM and 20 μ M, for wortmannin (Santa Cruz) 1 mM and 100 nM. All stock solutions were prepared in DMSO and stored at -20°C. In a typical experiment, cells were cultivated for three days in the absence or presence of the compound that was added once after 36 h or twice after 24 and 48 h.

Cell proliferation assay. To analyze cell proliferation, 500 cells in 100 μ l culture medium were seeded in 96-well plates (12 wells per time-point and treatment). Drugs were added immediately after 24 as well as after 48 h in 100 μ l of culture medium. Addition of pure DMSO served as a medium control. Cells were fixed in 4% formaldehyde in PBS just before treatment (T0) or after 72 h of treatment (T1). Cells from both time-points were then stained with DAPI (1 μ g/ml in PBS) in order to determine microscopically the cell number in a defined area of each well.

Collective cell migration assay. Cell suspensions (5,000 cells/ μ l) in a total volume of 3 μ l were seeded on defined regions in Petri dishes and allowed to adhere for two to three hours. After floating the dishes with culture medium, the adherent and confluent cells occupied a circular area. Diameters of areas (12 per dish) were determined microscopically subsequently after floating (T0) and after a 72-h culture period (T1). Compounds were added at T0 as well as 24 and 48 h later.

Cell toxicity assay. Cytotoxicity was analyzed with the LDH Cytotoxicity Assay Kit from Roche. Briefly, 10,000 cells (in 100 μ l culture medium) were seeded per well in 96-well plates. Twenty-four hours later, cells were exposed to different drug concentrations for 24-72 h. After treatment, LDH activity was determined in the cell culture supernatants. In parallel, cells that had been treated identically were lysed in order to determine total LDH activity, a value that allows to quantify cell proliferation.

Results

ERK1/2 and Akt activation in human thyroid cancer cells. Human papillary cell line B-CPAP, anaplastic cell line CAL-62 and follicular cell line FTC-133 were maintained for 48 h in the presence or absence of serum, or for 48 h in the absence and finally for 20 min in the presence of serum. Under serum-free and serum-containing culture conditions, no considerable differences in the expression levels of pERK1/2 or pAkt were observed (Fig. 1). Stimulation of quiescent cells with a serum pulse did not lead to an increased pERK1/2 and pAkt activity, suggesting a constitutive activation of Akt and ERK1/2 in thyroid cancer cells to an almost maximal extent.

Pharmacological inhibition of kinases. We first optimized the conditions to achieve maximal inhibition rates after drug application. Drugs were applied for 72 h either without further medium change, with one medium change after 36 h or with two medium changes after 24 and 48 h (Fig. 2). The most efficient inactivation of ERK1/2 kinases was achieved when U0126 or PD0325901 were added daily or at least once after 36 h (shown for U0126 in Fig. 2A). An almost complete inactivation of Akt over the whole three-day incubation period was obtained with a single addition of 1 μ M MK-2206 in B-CPAP and with a twice repeated addition in FTC-133 cells. In contrast, in Cal-62 only an incomplete inhibition was detected, even when Mk-2206 was renewed daily (Fig. 2B) or its concentration was increased to 2 μ M (not shown). Treatment with multikinase inhibitor sorafenib did not affect the activation of Akt and ERK1/2 in Cal-62 and FTC-133 cells, but strongly reduced the pERK1/2 expression level in B-CPAP cells at a concentration of 5 μ M (Fig. 2C). PI3K inhibitor wortmannin efficiently inhibited pAkt expression, but due to the instability of the drug, this effect was stable only for short time periods after application (Fig. 2D, compare incubation times of 4 and 24 h). Such types of experiments also allow to analyze the presence of crosstalk between PI3K/Akt and the Ras/MAPK pathways. Indications for crosstalks of the two pathways were mostly observed after a more frequent drug application such as the upregulation of pERK1/2 upon MK-2206 application in B-CPAP cells or the downregulation of pERK1/2 upon

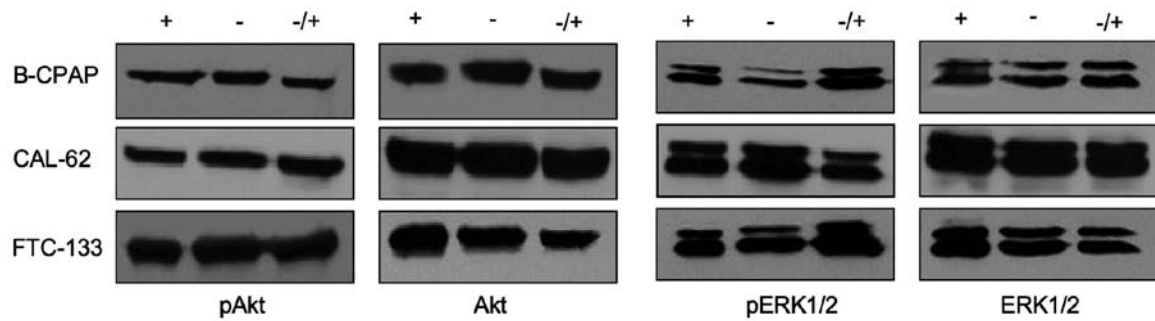


Figure 1. Western blot analysis of Akt and ERK1/2 activation in thyroid carcinoma cells. Thyroid carcinoma cells B-CPAP, Cal-62 and FTC-133 were cultured for 48 h in DMEM, 10% FCS (+), DMEM (-) or DMEM + 10% FCS (-/+, only during the final 20 min of culture). Cell lysates were analyzed for the presence of total or phosphorylated (p) Akt or ERK1/2 proteins. Equal amounts of protein (50 μ g) were loaded per lane.

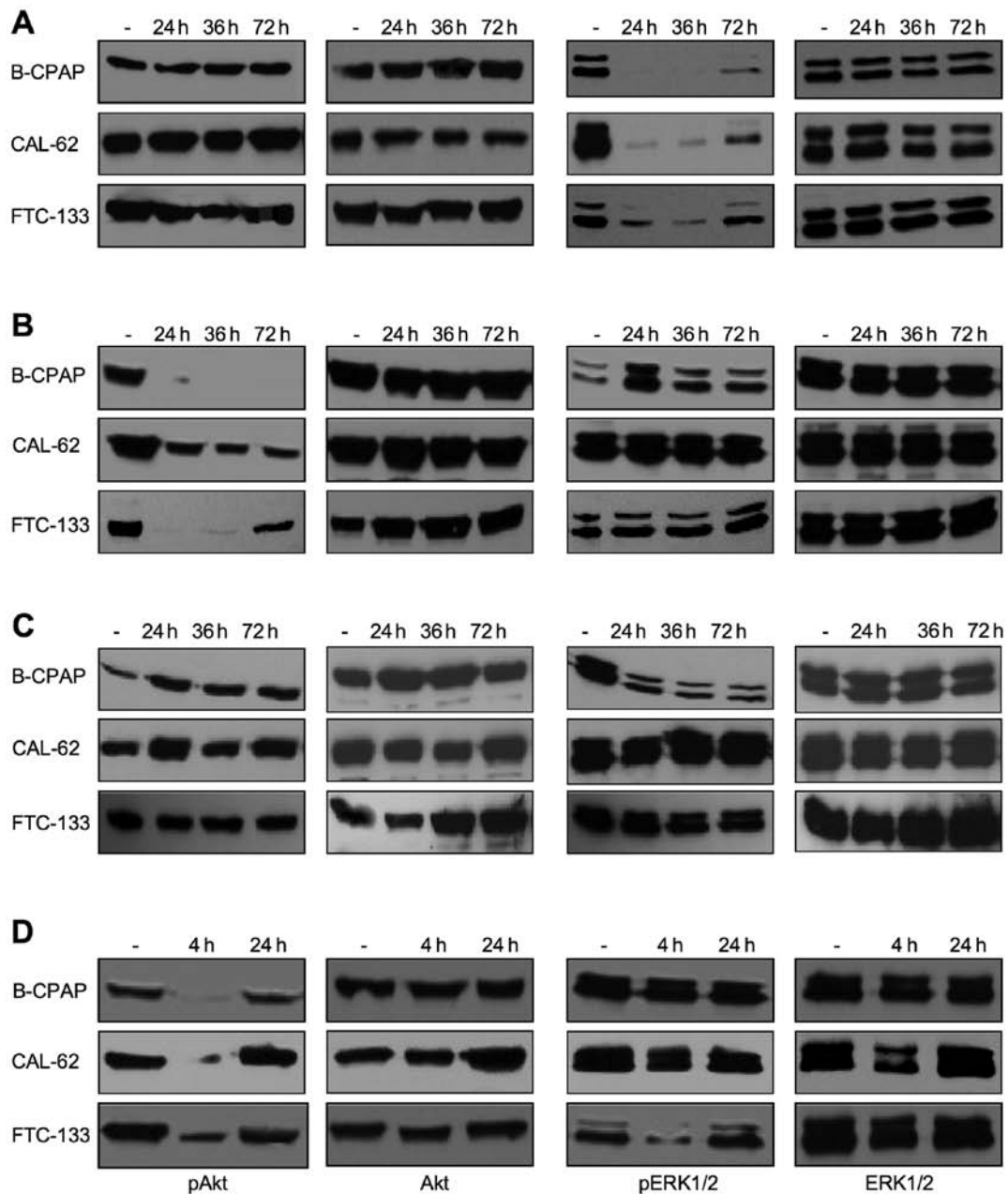


Figure 2. Western blot analysis of pharmacological inhibition of Akt and ERK1/2 activation in thyroid carcinoma cells. Cells were treated with 20 μ M U0126 (A), 1 μ M MK-2206 (B) 5 μ M sorafenib (C) or 100 nM wortmannin (D) under the following conditions. (A-C) Cells were cultured for 72 h without (-) or with additives, whereby the culture medium was exchanged every day (24 h), after half of the culture period (36 h) or remained unchanged (72 h). (D) Cells were cultured 24 h in the absence (-) or for 4 or 24 h in the presence of 100 nM wortmannin. Cell lysates were then analyzed for the activation, i.e., phosphorylation (p) level of Akt or ERK1/2 proteins. Equal amounts of protein (50 μ g) were loaded per lane.

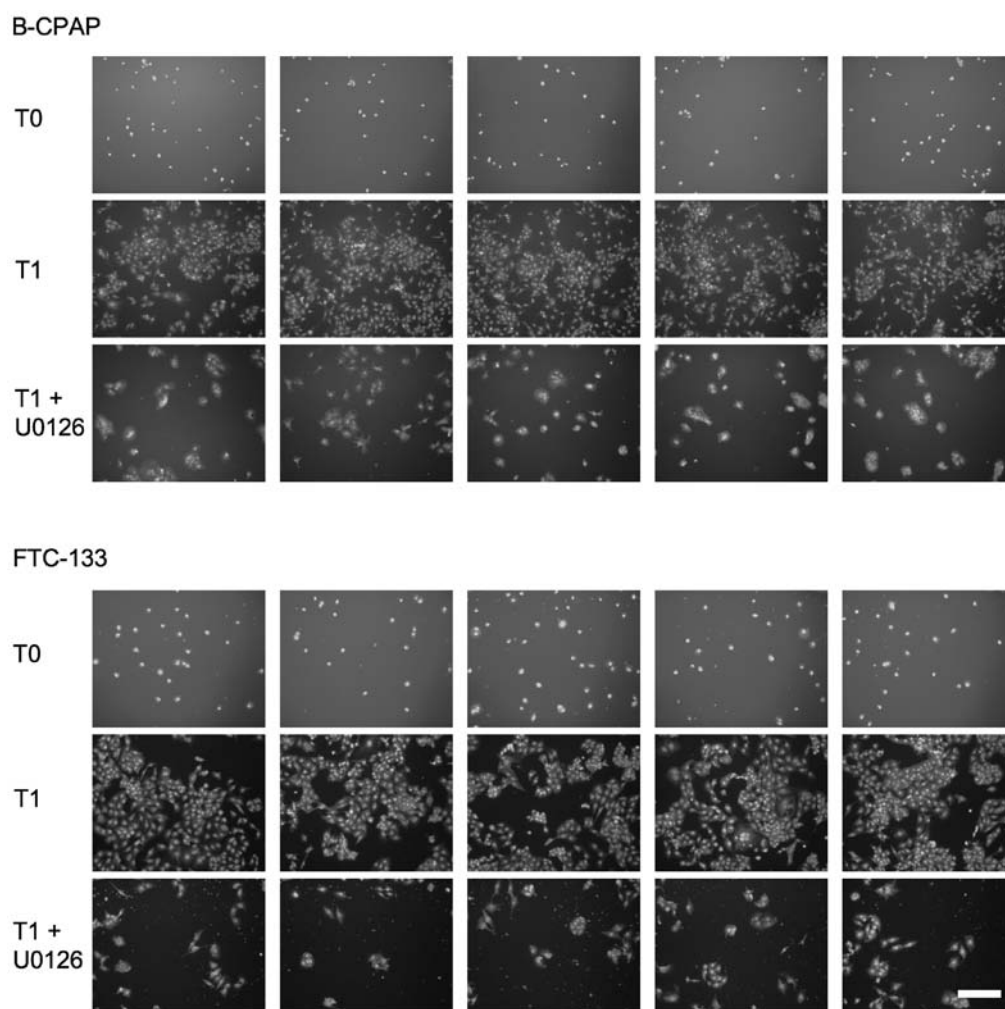


Figure 3. Impact of MEK/ERK1/2 inhibition on proliferation of thyroid carcinoma cells. Low-density B-CPAP or FTC-133 cells were cultured in 96-well plates for 0 (T0) or 72 h (T1) in the absence or presence of 20 μ M U0126, fixed with 4% formaldehyde and stained with DAPI. Representative micrographs of comparable areas in 5 different wells are shown for each time-point and treatment. Bar, 200 μ m.

wortmannin treatment in FTC-133 cells (Fig. 2). Based on these results, in three-day lasting functional assays, drugs were replaced every 24 h and applied at a concentration of 1 μ M for PD0325901, MK-2206 and sorafenib and at 20 μ M for U0126. Because of its low stability, wortmannin was not included in long-lasting experiments.

Impact of kinase inhibition on cell proliferation. Low density thyroid carcinoma cells were allowed to proliferate in 96-well plates for three days in the absence or presence of compounds. Different sets of cells were stained with DAPI before and after the 72-h incubation period and cell numbers were determined microscopically (Fig. 3 and Table I). Perturbation of the Ras/MAPK pathway led to a strong reduction in cell number, whereby inhibition rates of 70-90% were observed for the different cell lines after U0126 treatment (Fig. 3 and Table I). For PD0325901, inhibition rates varied between 53% for FTC-113 cells and 84% for B-CPAP cells (Table I). Also with the multikinase inhibitor sorafenib proliferation of thyroid carcinoma cells could be efficiently inhibited in the range of 70 to ~90% (Table I). Disruption of the PI3K/Akt pathway with MK-2206 inhibited the proliferation rate by ~65% in B-CPAP and by ~40% in Cal-62 and FTC-133 cells.

Table I. Impact of kinase inhibition on the proliferation rate of thyroid carcinoma cells.

Cell line	U0126	PD0325901	Sorafenib	MK-2206
B-CPAP	90 \pm 8 ^c	84 \pm 2 ^c	91 \pm 17 ^c	65 \pm 24 ^b
Cal-62	81 \pm 11 ^c	74 \pm 27 ^b	68 \pm 6 ^c	42 \pm 14 ^b
FTC-133	72 \pm 8 ^c	53 \pm 13 ^b	69 \pm 6 ^c	43 \pm 16 ^b

Cells were seeded at low density in 96-well plates and cultured for 72 h in the absence of drugs or in the presence of 20 μ M U0126, 1 μ M PD0325901, 5 μ M sorafenib, 1 μ M MK-2206. For counting, fixed cells were stained with DAPI at the beginning and end of the culture period and the number of cells present in a defined area determined microscopically for 10 wells per time-point and treatment. Relative inhibition rate (in comparison to untreated cells in %) was calculated from three independent experiments and mean values \pm SD are shown. Statistical significance was evaluated with an unpaired two-sample Student's t-test. ^ap<0.05; ^bp<0.01; ^cp<0.001.

Impact of kinase inhibition on collective cell migration. Thyroid carcinoma cells were seeded at high density on defined areas into Petri dishes and allowed to adhere for two

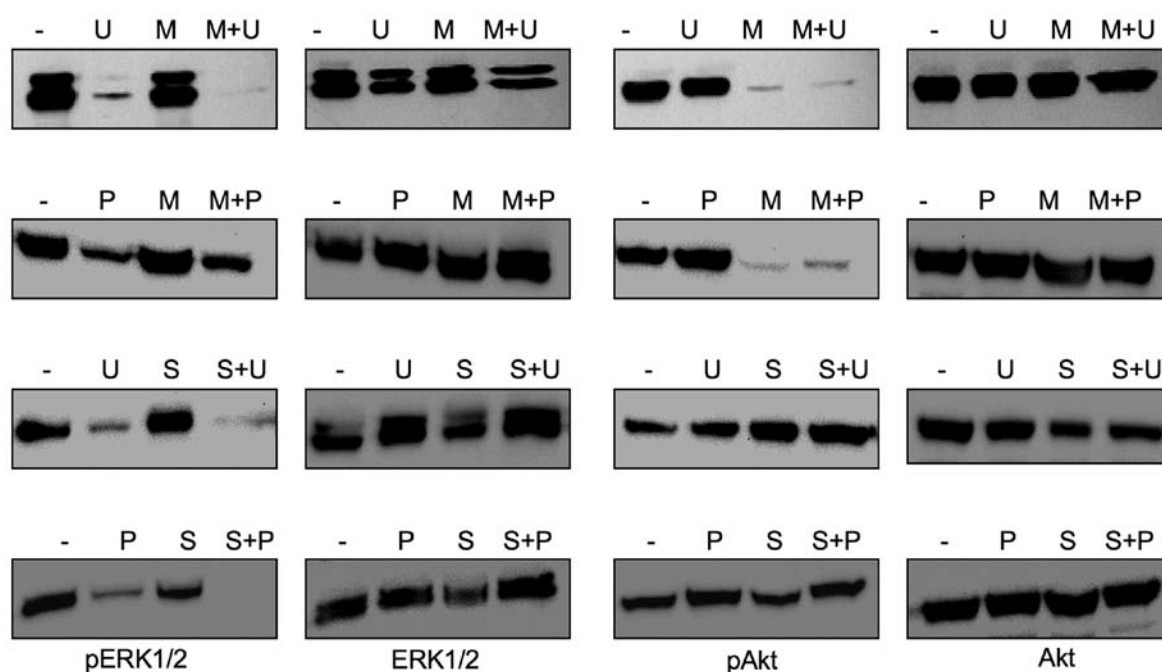


Figure 5. Western blot analysis of Akt and ERK1/2 activation in double-treated Cal-62 carcinoma cells. Cells were treated for 24 h with 1 μ M MK-2206 (M), 1 μ M PD0325901 (P), 5 μ M sorafenib (S) or 20 μ M U0126 (U) as single additives or at the indicated combinations. Cell lysates were then analyzed for the activation, i.e., phosphorylation (p) level of Akt or ERK1/2 proteins. Equal amounts of protein (50 μ g) were loaded per lane.

Table II. Impact of drug treatment on collective cell migration of thyroid carcinoma cells.

Cell line	U0126	PD0325901	Sorafenib	MK-2206
B-CPAP	29 \pm 9 ^c	53 \pm 7 ^c	79 \pm 12 ^c	39 \pm 8 ^b
Cal-62	+15 \pm 12 ^c	+10 \pm 17	84 \pm 12 ^c	46 \pm 7 ^c
FTC-133	7 \pm 5 ^b	23 \pm 9 ^c	53 \pm 3 ^b	24 \pm 6 ^c

Cells at high density (15,000 cells in 3 μ l) were seeded onto twelve defined regions per Petri dish and allowed to adhere for two to three hours. Dishes were then floated with culture medium (T0) and cells were treated for three days (T1) with or without additives. Diameters of circular areas occupied by cells (Fig. 4) were determined at T0 and T1. Two to seventeen experiments were performed for each cell line. Values (given as mean \pm SD) represent inhibition rates in percent (in respect to circle diameter of controls). Statistical significance was evaluated with an unpaired two-sample Student's t-test. ^ap<0.05; ^bp<0.01; ^cp<0.001.

visible upon MK-2206 or sorafenib treatment. Eventhough statistical significance is only present for U0126-treatment, the data shown in Fig. 4B demonstrate that in more than half of the experiments performed with PD0325901 an increased migration also took place.

We thus wondered, if the stimulating effect of U0126 and PD0325901 on the migration of Cal-62 cells could be suppressed by a simultaneous inhibition of the PI3K/Akt pathway, because western blot analyses revealed that the combined treatment of thyroid carcinoma cells with U0126 and MK-2206 led to an almost complete downregulation of pAkt and pERK1/2 expression (Fig. 5). Indeed, the migration rate of U0126/MK-2206 dual-treated Cal-62 cells was substantially inhibited not only

Table III. Impact of single or dual drug treatment on collective cell migration of Cal-62 cells.

A.			
Additive	-	U0126	PD0325901
-	0	+23±16 ^b	+4±18
MK-2206	46±7 ^c	34±6 ^c	52±8 ^c
Sorafenib	84±12 ^c	54±7 ^c	33±21 ^b
B.			
Additive	-	U0126	PD0325901
-	0	+36±6 ^b	+18±5 ^b
Sorafenib	14±4 ^b	2±7	12±9
2 μM			
Sorafenib	7±9	1±5	8±2
1 μM			

Cal-62 cells were seeded and treated as described in legend of Fig. 4. Values are given as mean \pm SD of up to five (A) or three (B) independent experiments and represent inhibition rates in percent (in respect to diameters of circles in control experiments). Values for single or double treatments are shown. Statistical significance in respect to control treatment without additives was evaluated with an unpaired two-sample Student's t-test: ^ap<0.05; ^bp<0.01; ^cp<0.001.

in respect to U0126- or PD0325901-single-treated, but also in comparison to untreated control cells (Table IIIA). Moreover, although sorafenib did not affect Akt activation in U0126- or

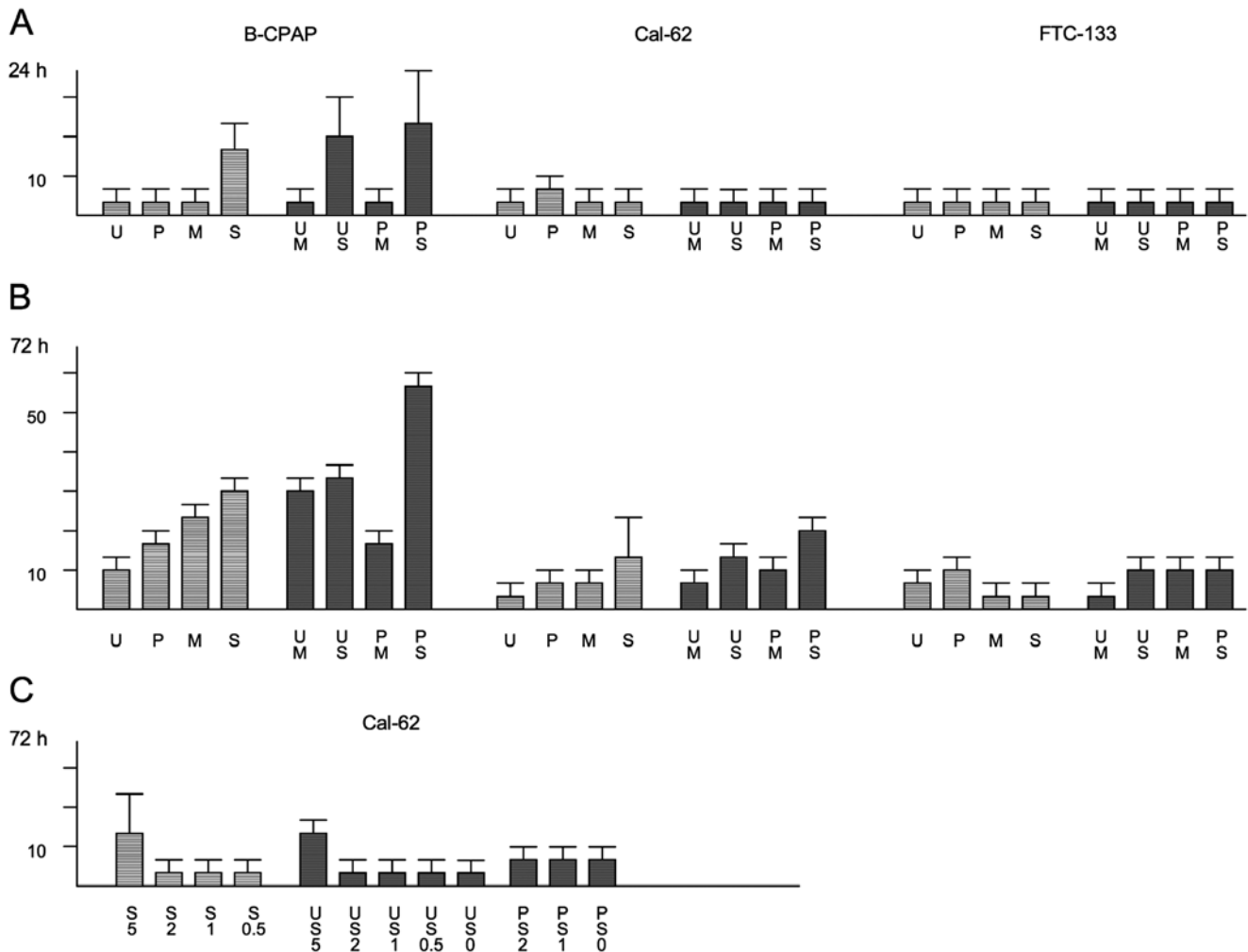


Figure 6. Cytotoxic effects of kinase inhibitors on thyroid carcinoma cells as determined by LDH assay. Cells were cultivated for 24 h (A) or 72 h (B and C) either in the absence of additives or in the presence of 1 μ M MK-2206 (M), 1 μ M PD0325901 (P), 5 μ M sorafenib (S) or 20 μ M U0126 (U) as single additives or at the indicated combinations and concentrations [given in μ M for sorafenib (C)]. Relative LDH activity present in the cell culture supernatant (rate of cytotoxicity) are shown. Data are means \pm SD from two to three independent experiments.

PD0325901-treated Cal-62 cells (Fig. 5), its presence led to a substantial inhibition of cell migration (Table IIIA). To rule out that the inhibition observed results mainly from cytotoxic drug effects, LDH assays were carried out. After a 24-h incubation period none of the single or dual drug treatments induced a noteworthy cytotoxic effect in Cal-62 cells, although dual-treated B-CPAP cells were considerably affected (Fig. 6A). After a 72-h incubation period, however, a moderate cytotoxic effect was observed in sorafenib-treated Cal-62 cells. Therefore, it is possible that under these conditions the proliferation and migration suppressing potential of this compound is partially due to a cytotoxic effect. Thus, in a final set of experiments, we applied sorafenib at concentrations of 1 and 2 μ M, i.e., at concentrations that did not induce considerable cytotoxic effects in Cal-62 cells after a 72-h incubation period neither as a single agent nor in combination with 20 μ M U0126 or 2 μ M PD0325901 (Fig. 6C). Under these conditions, the migration-promoting effect of U0126 was completely compensated, but an additional inhibition, as could be monitored for higher sorafenib concentrations, was not observed (Table IIIB). Altogether, these data suggest that the migration-stimulating effect of PD0325901 and U0126 can be

suppressed by Akt-dependent and -independent mechanisms, even though an inactivation of Akt downstream signaling molecules cannot be completely excluded.

Discussion

Our study demonstrated that the migration and proliferation of human thyroid carcinoma cells is partially mediated via constitutive active PI3K/Akt and RAS/MAPK signaling. Pharmacological inhibition of this signaling considerably inhibits proliferation in all three cell lines, although to varying extent (Table I). In general, also the migration rate of thyroid carcinoma cells is suppressed in the presence of the different kinase inhibitors, with the remarkable exception of an increased migration of Cal-62 cells in the presence of U0126- or PD0325901. This increase can be prohibited by MK-2206 treatment, i.e., via Akt inactivation, or by application of sorafenib, a compound that does not perturb Akt activation level in Cal-62 cells.

Crosstalk of signaling pathways in thyroid carcinoma cells. PI3K/Akt and Ras/MAPK signaling pathways, although

first described as linear conduits were soon shown to be connected with each other in a complicated fashion that comprises cross-activation and -inhibition processes as well as negative feedback loops and pathway convergence (18). In our experiments we found evidence for the presence of cross-inhibitory circuits of PI3K/Akt on Ras/MAPK in B-CPAP and of cross-activating circuits in FTC-133 cells (Fig. 2). As such crosstalk depends inter alia on substrate availability that can vary between different cell types or cell lines it is not surprising that the interactions we observed exhibit a cell line-specific pattern that can not be generalized (Fig. 2). Kandil and colleagues (19) also recently reported that cross-activation of Akt varies between different thyroid cancer cell lines upon treatment with the MEK/ERK inhibitor AZD6244. At the present level of knowledge, it is mainly assumed that the PI3K/Akt and Ras/MAPK pathways can negatively regulate each other and, in addition, the latter one can activate the former (18). Nevertheless, and thereby underlining our data, in a few reports it has been shown, that the PI3K/Akt pathway can activate ERK1/2 signaling (20-22).

Yin-yang effects in thyroid carcinoma cells. Although we have evidence for the presence of crosstalk between PI3K/Akt- and Ras/MAPK pathways in thyroid carcinoma cells, it does not illuminate the yin-yang effect we detected in Cal-62 cells treated with MEK inhibitors:

i) Treatment of Cal-62 cells with MEK inhibitors leads to an almost complete inhibition of pERK1/2 expression, but leaves pAkt expression substantially unaffected (Fig. 2). Vice versa, treatment with an Akt inhibitor strongly decreases pAkt expression without affecting the expression level of pERK1/2 (Fig. 2). Thus, no obvious crosstalk of the two pathways seems to be present in Cal-62 cells.

ii) The proliferation rate of Cal-62 cells is strongly suppressed by MEK inhibitors, but only mildly by MK-2206 (Table I), whereas the migration rate is significantly enhanced by MEK inhibitors and strongly suppressed by MK-2206 (Table II). Thus, the increased surface area observed in the collective cell migration experiments cannot be simply the result of an increased cell mass, or, more generally, of a growth process.

iii) Increased migration of Cal-62 cells due to the presence of MEK inhibitors can be suppressed by MK-2206 or sorafenib, i.e., in the presence of low as well as high expression levels of pAkt. To our knowledge, sorafenib treatment does not affect Akt downstream signaling molecules. Thus, it is likely that the stimulatory effect of MEK inhibitors can be suppressed via Akt-dependent and -independent signaling mechanisms.

Collectively, it seems feasible that interference with a single pathway can inhibit one cellular process, here the cell's proliferative capacity, and at the same time promote another, here the cell's migratory potential. In our example such an effect can be interpreted by assuming the existence of an (hyper)activated Ras/ERK1/2 pathway that suppresses cell migration but can itself be suppressed by MEK inhibitors. Based on the above, one could expect that Cal-62 cells migrate with a submaximal velocity, and indeed, although we have not performed a systematic analysis, Cal-62 shows the lowest velocity amongst the three cell lines we have used, i.e., a 25 or 40% reduced migration rate in comparison to FTC-133 or B-CPAP cells, respectively. This

resembles a scenario that hyperactivation of the Ras/ERK1/2 pathway can trigger a decreased proliferation rate, i.e., cell cycle arrest (23). An inhibitory effect of the MAPK cascade on cell proliferation is also active in K562 erythroleukemia cells, which differentiate into megakaryocytes upon phorbol ester stimulation, a process that is: i) accompanied by growth retardation and ERK activation, and ii) completely suppressed in the presence of MEK inhibitors (24). The aspect that Ras/ERK1/2 pathway activation not only activates (25,26), but may also suppress cell migration adds another facet to the complex pattern of MAPK signaling. To overcome the stimulatory effect of MEK inhibitors on Cal-62 cells, we have performed dual treatments to interfere with more than one signaling pathway and, thereby, successfully suppressed cell proliferation as well as migration. Such a strategy, in clinical practice known as a subtype of combination therapy (27), has been used in a number of trials to attack the fatal potential of tumor cells more efficiently. For example, in thyroid carcinoma cells, dual Ras/MAPK and PI3K/Akt/mTOR pathway inhibition leads to significant higher growth retardation even in a synergistic manner when compared to single pathway inhibition (28,29). Beside the advantage of a combinatorial treatment to increase the inhibitory action of a single drug such a strategy also decreases the risk of unfavorable yin-yang effects.

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