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More antitumor efficacy of the PI3K inhibitor GDC-0941 in breast cancer with PIK3CA mutation or HER2 amplification status *in vitro*

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PIK3CA is probably the most commonly mutated kinase in several malignant tumors. Activation of class I phosphatidylinositol 3′ kinase (PI3K) regulates tumor proliferation, survival, etc. This study sought to identify whether the pan-inhibitor has more antitumor efficacy in breast cancer cells with PIK3CA Mutation or HER2 amplification than basal-like cancer cells. The proliferation of breast cancer cells was measured by MTT assay in the presence of GDC-0941. Afterwards, we determined the visible changes in signaling in the PI3K/AKT/mTOR pathway. Finally, we examined GDC-0941 effects on cell cycle, apoptosis and motility. GDC-0941 exhibited excellent inhibition on three cell lines with PIK3CA mutation or HER2 amplification. In addition, GDC-0941 resulted in decreased Akt activity. GDC-0941 downregulated the key components of the cell cycle machinery, such as cyclin D1, upregulated the apoptotic markers and inhibited cell motility on three cell lines with PIK3CA Mutation or HER2 amplification. Antitumor activity of GDC-0941 treatment amongst tumor cell lines with PIK3CA mutation and HER2 amplification may have clinical utility in patients with these oncogenic alterations.

1. Introduction

Breast cancer is the most common malignancy among females. Simultaneouly, breast cancer is also the first human tumor for which targeted therapies have been developed. There has been a longstanding interest in inhibitors that target aberrant mitogen-activated protein kinase (MAPK) signaling. However in recent years, the PI3K/Akt pathway is a key cell survival pathway known to be deregulated in many cancers. The pathway has been shown to be activated by upstream signals such as IGF and EGF, and then regulate tumor proliferation, growth, survival, protein synthesis, and glucose metabolism. Its activation has also been associated with malignant transformation and apoptotic resistance (Bader et al. 2005).

PIK3CA, which encodes the p110α catalytic subunit of PI3K, is probably the most commonly mutated kinase in the human genome (15% of all cancers) and is also amplified in some tumors (Ligresti et al. 2009; Samuels et al. 2004). The mutant versions of the protein exhibit increased enzymatic activity independent of upstream signaling, constitutively stimulates signaling through the AKT pathway, and has oncogenic properties such as conferring anchorage-independent growth and increased cell invasion and metastasis (Kang et al. 2005). This frequent activation of the PI3K pathway in cancers has led to intensive efforts to identify therapeutics that abrogate PI3K signaling and hence may have utility in patients with cancers addicted to the PI3K pathway (Workman et al. 2006). Several inhibitors of PI3 kinase, including wortmannin (Schultz et al. 1995; Norman et al. 1996) and LY294002 (Vlahos et al. 1994), have been previously reported. Both of these inhibitors have been extensively used to elucidate the functional role of PI3K. However, their toxicity and lack of selectivity with respect to targets other than class I PI3K family members have limited their therapeutic potential. GDC-0941 is a pan inhibitor of PI3K with IC50 values in the low nanomolar range for all three of the class IA p110 isoforms (p110α, p110β, and p110δ). The compound shows potent growth inhibitory activity *in vitro* in a range of human tumor cell lines. GDC-0941 demonstrates an acceptable pharmaceutical profile and is currently being evaluated in human clinical trials for the treatment of cancer (Folkes et al. 2008).

However, a major question in the clinical development of GDC-0941 is the predictive ability, which patients can benefit from the inhitibors in breast cancer. As we know, preclinical studies can elaborate clinical diagnostic strategies and prioritization of biomarker candidates. Several pre-clinical studies of GDC-0941 have demonstrated that it has potent anti-tumor activities in cell lines that harbor activating PI3K mutations, PTEN, or HER2 amplification (Raynaud et al. 2009; O'Brien et al. 2010; Dan et al. 2010). To address this question in preclinical models, we applied GDC-0941 to inhibit the specific pathway–PI3K/Akt pathway in breast cancer cell lines with different PIK3CA and HER2 characteristic. We found GDC-0941 impeded potently the proliferation, growth, apoptosis, and motility of MCF7 (E545K mutation), T-47D (H1047R mutation), and SK-BR-3 (PIK3CA WT, HER2 amplication), but no change in MDA-MB-231 (PIK3CA WT, triple negative) *in vitro*.

2. Investigations and results

2.1. GDC-0941 Induced growth inhibition in breast cancer cell lines with different PIK3CA and HER2 characteristic

To determine the effect of GDC-0941 on breast cancer cell lines, a variety of breast cancer cell lines, including PIK3CA E545K

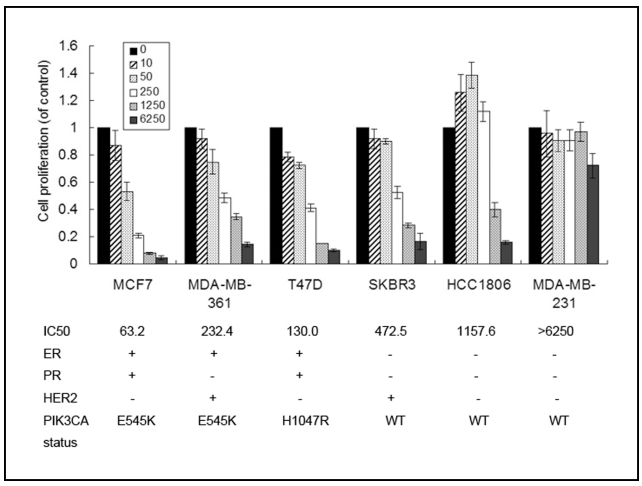

Fig. 1: Inhibition ratio of cell growth by GDC-0941. Breast cancer cell lines MCF7, MDA-MB-361, T-47D, SK-BR-3, HCC1806 and MDA-MB-231 were grown overnight without serum (serum starvation), and the next day cells were treated with different GDC0941 (0, 10 nM, 50 nM, 250 nM, 1250 nM, and 6250 nM) for 6 days, and cell viability was determined by MTT.

mutant, PIK3CA H1047R mutant, HER2-overexpressed, triple-negative and PIK3CA wild breast cancer cell lines (Fig. 1), were cultured with different concentrations of GDC-0941 (0, 10 nM, 50 nM, 250 nM, 1250 nM and 6250 nM). Cell proliferation was measured by MTT assay after 6 days of culture. As shown in Fig. 1, the proliferation of most of cell lines in the presence of GDC-0941 was inhibited in a concentration-dependent manner. These cell lines, including MCF7, MDA-MB-361, T-47D and SK-BR-3, had an IC50 of < 500 nM. HCC1806 had a higher value of IC50 (1157.6 nM). However, only a high dosage (6,250 nM) had a slight effect and did not result in 50% inhibition of viability on MDA-MB-231 (Fig. 1). Together, these results indicate that GDC-0941 has an excellent inhibition effect on the proliferation of PIK3CA E545K mutant and PIK3CA H1047R mutant cell lines such as MCF7, MDA-MB-361 and T-47D, and also inhibits effectively the growth of HER2-overexpressed cell lines such as SK-BR-3. These cell lines harboring oncogenic mutations in PIK3CA, or HER2 amplification, might be more sensitive than cell lines without these alterations.

2.2. GDC-0941 Treatment downregulated PI3K/Akt signaling

Because Akt is considered as an key node in the PI3K/AKT pathway, we studied the level of Akt phosphorylation after the MCF7, SK-BR-3 T-47D and MDA-MB-231 cells were treated with 500 nM GDC-0941. In the absence of IGF1, we observed slight changes in Akt phosphorylation (Fig. 2A). IGF1 is considered to be the amplification factor of the PI3K/AKT/mTOR pathway (LeRoith et al. 1995; Butt et al. 1999). Thus, we treated four cells types with 60 ng/ml IGF1 for 20 min, and afterwards these cell types, MCF7, SK-BR-3, T-47D and MDA-MB-231, were treated with GDC-0941 for 24 h. In the presence of IGF1, GDC-0941 caused a detectable decrease in the phosphorylation of Akt in part compared to control cells in all four cell lines (Fig. 2B).

The 40S ribosomal protein S6 is one of the major downstream components of the mTOR signaling pathway. This protein is phosphorylated or activated via the phosphorylation of S6K1 and might be regulated directly by activated Akt (Carracedo et al. 2008). We investigated whether the PI3K inhibitor GDC-0941 was able to inhibit the phosphorylation of S6. Similarly after the MCF7, SK-BR-3, T-47D and MDA-MB-231 cells were treated with 500 n M GDC-0941 for 24 h, we observed that 500 n M

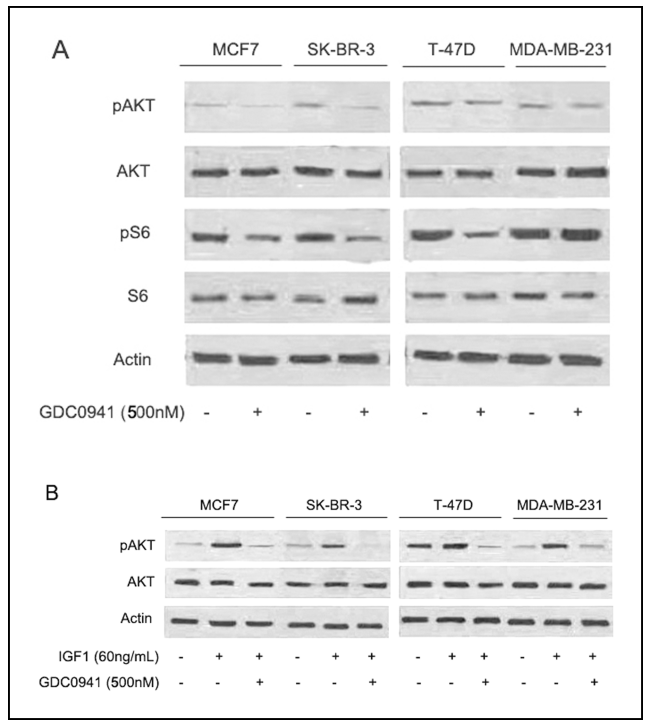

Fig. 2A: GDC-0941 induced the changes of Akt phosphorylation and S6 phosphorylation. A, breast cancer cell lines were grown overnight without serum (serum starvation), and the next day cells were treated with 500 nM GDC0941 for 24 h. B, breast cancer cell lines were grown overnight without serum (serum starvation), and the next day cells were treated with 500 nM GDC0941 for 24 h, and then were treated by 60ng/mL IGF1 for 20 min. Cell lysates were immunoblotted with antibodies against phospho-S6 ribosomal protein (Ser240/244), total S6, phospho-Akt (Ser473), total Akt, Actin, Fig. 2B: GDC-0941 induced the changes of Akt phosphorylation and S6 phosphorylation. A, breast cancer cell lines were grown overnight without serum (serum starvation), and the next day cells were treated with 500 nM GDC0941 for 24 h. B, breast cancer cell lines were grown overnight without serum (serum starvation), and the next day cells were treated with 500 nM GDC0941 for 24 h, and then were treated by 60 ng/mL IGF1 for 20 min. Cell lysates were immunoblotted with antibodies against phospho-S6 ribosomal protein (Ser240/244), total S6, phospho-Akt (Ser473), total Akt, Actin.

of GDC-0941 inhibited markedly the phosphorylation of S6 in MCF7, SK-BR-3 and T-47D cells (Fig. 2A). However, GDC-0941 did not further decrease S6 phosphorylation in MDA-MB-231 cells compared to no treatment.

2.3. The effects of GDC-0941 on the levels of cell-cycle-associated protein and apoptotic markers

To investigate the effect of GDC-0941 on the cell cycle, we determined the G1/S-specific cyclin D1 protein levels. The results of western blotting and cyclin D1 activity are shown in Fig. 3. Our data indicate that the level of cyclin D1 was decreased in the GDC-0941-treated MCF7, SK-BR-3 and T-47D cells compared to control cells, but no visible change was detected in MDA-MB-231 cells. The PI3K/AKT pathway also regulates apoptosis through multiple mechanisms, so we also detected induction of cleaved PARP, a key substrate of activated caspase and an early indicator of apoptosis. Notably, we observed a substantial increase in cleaved PARP accumulation upon GDC-0941 treatment in MCF7, SK-BR-3 and T-47D cells, but no change in the MDA-MB-231 cells.

2.4. GDC-0941 Treatment resulted in impaired cell motility

In wound-healing assays, the distance moved by a wounded cell monolayer on plastic after treatment with GDC-0941 (500 nM).

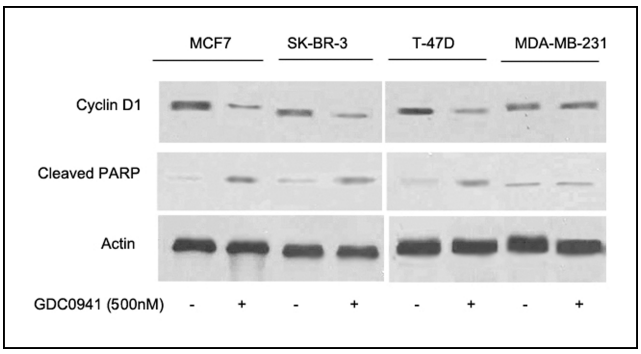

Fig. 3: Western blots showing effects of 24-h GDC-0941 treatment on key regulators of the G1-S cell cycle transition and apoptotic markers in MCF7, SK-BR-3, T47D and MDA-MB-231 cells. The cells were grown overnight without serum (serum starvation), and the next day cells were treated with 500 nM GDC0941 for 24 h. Cell lysates were immunoblotted with antibodies against cyclin D1, cleaved PARP and actin.

The results (Fig. 4) are reported as migration indices, which represent the distance moved by the GDC-0941-treated cancer cells to close the wound, expressed as a proportion of the distance moved by control non-treated cells. In MCF7, SK-BR-3 and T-47D cells, the GDC-0941-treated cells significantly decreased migration, respectively, when compared with non-treated controls ($p < 0.001$, Fig. 4B). However, in MDA-MB-231 cells, the GDC-0941 treatment did not induce significantly reduction in the migration. These finding suggest that GDC-0941 treatment result in impaired cell motility in PIK3CA E545K mutant and PIK3CA H1047R mutant cell lines such as MCF7 and T-47D, and also inhibit effectively the motility of HER2-overexpressed cell lines such as SK-BR-3, but not in triple-negative and PIK3CA wild cell lines such as MDA-MB-231.

3. Discussion

The PI3K/AKT pathway is one of the most frequently deregulated pathways in human cancer, nearly every major node can be activated and lead to constitutive signaling (Yuan et al. 2008). For example, it is activated through mutations that target the PI3KA, PTEN, Akt, TSC1 or TSC2 genes and amplification of HER2 (Vivanco et al. 2002; Di Cristofano et al. 2000). PI3K activation can produce diverse changes in cell physiology and inhibits apoptosis, indicating that PI3K/Akt pathway mutations may have broad consequences for tumorigenesis and treatment responses. Because of its gain-of-function mode of action, this pathway is also a compelling target for new therapeutics. Intensive efforts are underway to develop pan- and isoform-selective inhibitors of PI3K, dual PI3K and mTOR (Workman et al. 2006). Most notable of these inhibitors are mutations or amplifications of PIK3CA, the gene encoding the p110a catalytic subunit of the class I PI3K. So we have focused in this report on a pan-inhibitor of class □PI3K in breast cancer and identifying whether the pan-inhibitor have more antitumor efficacy in breast cancer cells with PIK3CA Mutation or not.

Recently, GDC-0941 has been shown to be a potent, selective and orally bioavailable inhibitor of PI3K, and this inhibitor is currently undergoing phase I clinical trials in cancer patients. GDC-0941 has equal potency against both of the common activated oncogenic hotspot p110 mutants that were tested, namely the helical domain E545K mutant and the C-terminal kinase domain H1047R mutant (21). Our results suggest that GDC-0941 exhibits excellent inhibition efficacy on MCF7 and T-47D cell lines, whether or not they include PIK3CA E545K or H1047R mutants. These results confirm the potency of this inhibitor against both of the common activated oncogenic hotspot p110 mutants, namely, the helical domain E545K mutant and the C-terminal kinase domain H1047R mutants (Folkes et al. 2008). In addition, GDC-0941 also exhibited excellent inhibition efficacy on SK-BR-3 cells with HER2-positive characteristic. However, GDC-0941 had little effect on the growth of basal-like cell lines such as MDA-MB-231.

In PI3K/AKT signaling axis, we found that levels of phosphor-Akt were generally decreased by GDC-0941 treatment in all cell lines, independent of oncogenic alterations in PIK3CA and HER2. This finding suggests that pAKT may not be an universal predictive biomarker of single-agent GDC-0941 response. In contrast, we found that the cell lines with PIK3CA mutant and HER2-positive characteristic showed substantial GDC-0941-mediated downregulation of mTORC1-regulated proteins such as phosphor-S6, this reponse was absent in basal-like cell lines such as MDA-MB-231. This suggests that although PI3K may signal through multiple upstream mechanisms in cell lines with oncogenic alterations such as PIK3CA and HER2, the ability to couple these mechanisms to mTORC1 signaling may be a hallmark for these cell lines.

Since the PI3K/AKT pathway has also been shown to play a key role in regulating the G1-S cell cycle transition (Gottschalk et al. 2001), we examined a key component of the cell cycle machinery. We found that the cell lines with PIK3CA mutant and HER2-positive characteristic showed substantial decreases in G1/S-specific cyclin D1 protein levels, but no change was observed in the basal-like MDA-MB-231 cells. Cleaved PARP is considered as a key substrate of activated caspase and an early indicator of apoptosis. Similarly, we observed a substantial increase in cleaved PARP accumulation upon GDC-0941 treatment in all three cell lines with PIK3CA mutation and HER2 amplification but no change in basal-like cell line. Finally, we examined whether GDC-0941 could inhibit the motility of all three sensitive cell lines. Our experiments showed that all three sensitive cancer cell lines examined were sensitive to GDC-0941, showing substantial reductions in cell motility. The basal-like cell lines, by contrast, were unable to migrate to any considerable extent in wound-healing assays.

At present, PI3K is considered as an attractive target in the treatment of breast cancer. Our data show that GDC-0941 (a pan inhibitor of PI3K) has broad antitumor activity amongst tumor cell lines with PIK3CA mutation and HER2 amplification, suggesting its clinical utility in patients with these oncogenic alterations. These results may be useful clues on the promise of personalized cancer therapy with PI3K inhibitors.

4. Experimental

4.1. Cell lines and cultures

All following human breast cancer cell lines were obtained from the American Type Culture Collection: MCF7, MDA-MB-361, SK-BR-3, T-47D, HCC1806, MD-MB-231. Cell lines were propagated in RPM1 1640 containing 10% fetal bovine serum with antibiotics and supplements (50 μg/mL gentamicin, pyruvate, 10 mmol/L HEPES, and glucose to 4.5 g/L) in a humidified 37 °C incubator containing 5% CO_2.

4.2. Reagents and antibodies

GDC-0941 was a gift provided by Kingmed Diagnostics Center (Guangzhou, China). Antibodies against phospho-S6 ribosomal protein (Ser240/244), S6 ribosomal protein, phospho-Akt (Ser473), Akt, cyclin D1(DSC6) and cleaved PARP(Asp214) were purchased from Cell Signaling Technology (Beverly, Mass., USA). Antibodies against IRS1 and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

4.3. Growth assay

For MTT experiments, 2000 cells/well were plated in 96-well Optilux dishes for 24 h for triplicate wells, and then grown overnight in RPM1 1640 without serum overnight (serum starvation). The next day cells were treated with GDC-0941 in RPM1 1640 containing 5% fetal bovine serum. After 6 d

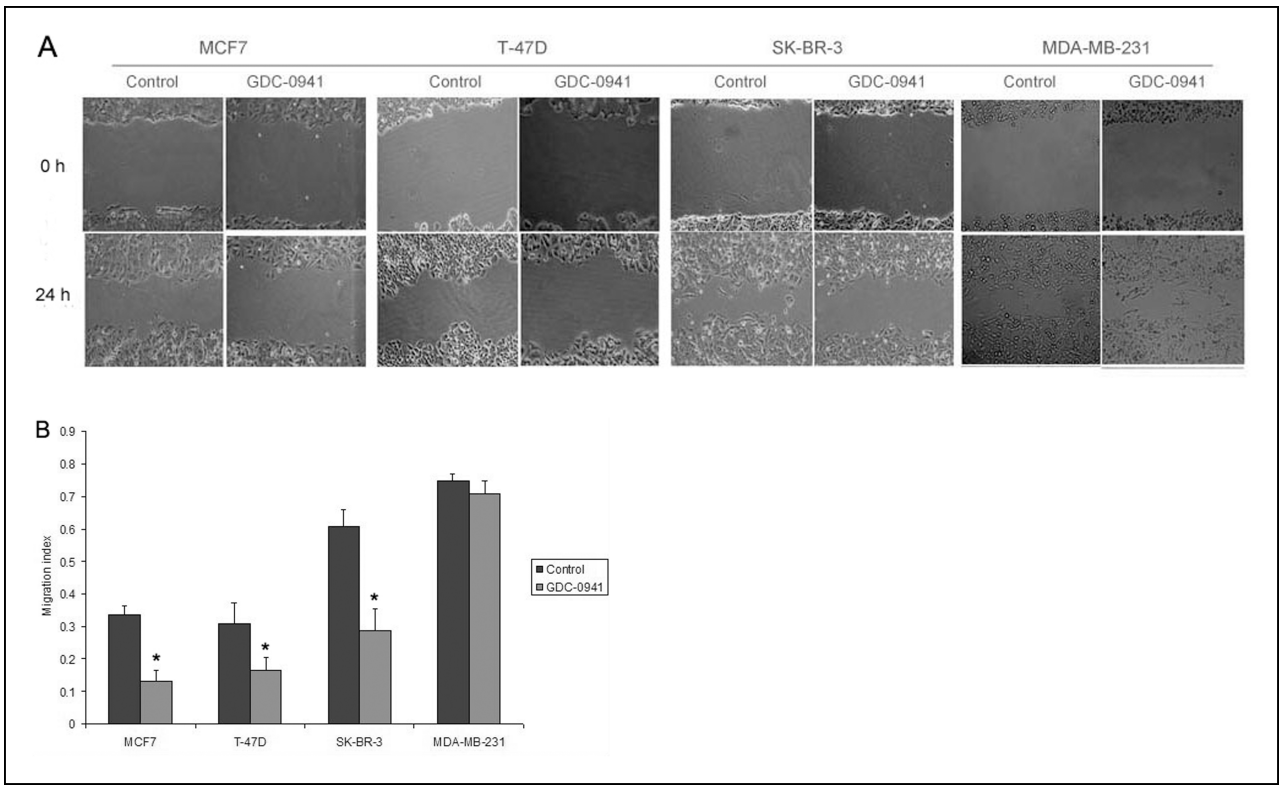

Fig. 4A: GDC-0941 treatment for 24 h inhibits wound healing. A, data and representative images are shown for four cell lines. B, bars represent the migration index of each treatment, expressed as a value relative to the distance moved by the cell monolayer in no treatment control. $*P < 0.01$, Fig. 4B: GDC-0941 treatment for 24 h inhibits wound healing. A, data and representative images are shown for four cell lines. B, bars represent the migration index of each treatment, expressed as a value relative to the distance moved by the cell monolayer in no treatment control. $*P < 0.01$.

MTT solution (*Invitrogen*, Carlsbad, Calif., USA) was added to each well. The reaction was stopped by removal of MTT after 3 h of incubation at 37 °C. 2-Propanol was added to dissolve crystals. Absorbance at 570 nm and 650 nm was recorded at a fluorescent microplate reader. The concentration of drug resulting in 50% inhibition of cell viability (IC50), IC50 values were calculated by the CalcuSyn software (Biosoft, Cambridge, UK). All experiments were repeated at least three times.

4.4. Western blotting

1×10^6 cells were plated in 6-well dishes, after 24 h the cells were washed with PBS and grown overnight in RPM1 1640 without serum. The next day cells were treated with drugs for 24 h. To prepare lysates for the breast cancer cell line panel, cells were washed with cold PBS and extracted with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, before use add protease inhibitor cocktail (Sigma, St. Louis, MO., USA); phosphatase inhibitor cocktail (Sigma, 1 mM PMSF; 1 mM NaPPi; 10 mM NaF; 1 mM Na3VO4 and 10 mMβ-glycerophosphate). Lysates were cleared by centrifugation and protein concentration was determined by Bradford assay (Bio-rad, Hercules, Calif., USA). Lysates were mixed with 2x reducing sample buffer, boiled and stored at −80 °C. Bio-Rad gel and gel running was used to separate proteins, and tranferred to a 0.2 μm polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat drymilk in Tris-buffered saline with 0.02% Tween 20 and immunoblotted with antibodies. Actin served as internal control.

4.5. Wound scratch assay

Cells were seeded at a density 5×10^5 per well in 6-well plates in complete medium. After 24 h the cells were washed with PBS and grown overnight in RPM1 1640 without serum. The next day the monolayers were scratched with a 200-μl plastic pipette tip to create a uniform wound and the cells were treated with drugs. At the same time photographs taken immediately (time zero) and 24 h after wounding for all cells, respectively. The distance migrated by the cell monolayer to close the wounded area during this time period was measured. Results were expressed as a migration index—that is, the distance migrated after 24 h (control or targeted) relative to the distance that cells fused together completely (Thompson et al. 2007). Experiments were carried out in triplicate and repeated at least three times.

4.6. Statistical analysis

Statistical significance of all data was analyzed by one-way ANOVA using the SPSS Statistics 17.0 software. *P* value smaller than 0.05 ($P < 0.05$) was considered significant.

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