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# Inhibition of EGFR improves antitumor efficacy of vemurafenib in BRAF-mutant human melanoma in preclinical model

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| **Abstract:** | Background: Oncogenic activation of the epidermal growth factor receptor (EGFR) signaling pathway occurs in a variety tumour types, albeit in human melanoma the contribution of EGFR is still unclear.<br>Methods: The potential role of EGFR was analyzed in four BRAF-mutant, one NRAS-mutant and one wild-type NRAS-BRAF-carrying human melanoma cell lines. We have tested clinically available reversible tyrosine kinase inhibitors (TKI) gefitinib and erlotinib, irreversible EGFR-TKI pelitinib and a reversible experimental compound (PD153035) on in vitro proliferation, apoptosis, migration as well as in vivo metastatic colonization in a spleen-liver model.<br>Results: The presence of the intracellular domain of EGFR protein and its constitutive activity were demonstrated in all cell lines. We detected significant differences between the efficacies of EGFR-TKIs, irreversible inhibition had the strongest anti-tumour potential. Compared to BRAF-mutant cells, wild-type BRAF associated with relative resistance against gefitinib. In combination with gefitinib, selective mutant BRAF-inhibitor vemurafenib showed additive effect in BRAF-mutant cell lines. Treatment of BRAF-mutant cells with gefinib- or pelitinib attenuated in vitro cell migration and in vivo colonization.<br>Conclusions: Our preclinical data suggest that EGFR is a potential target in the therapy of BRAF-mutant malignant melanoma; however, more benefits could be expected from irreversible EGFR-TKIs and combined treatment settings. |
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# Inhibition of EGFR improves antitumor efficacy of vemurafenib in BRAF-mutant human melanoma in preclinical model

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**Abstract:**

Background: Oncogenic activation of the epidermal growth factor receptor (EGFR) signaling pathway occurs in a variety tumour types, albeit in human melanoma the contribution of EGFR is still unclear.

Methods: The potential role of EGFR was analyzed in four BRAF-mutant, one NRAS-mutant and one wild-type NRAS-BRAF-carrying human melanoma cell lines. We have tested clinically available reversible tyrosine kinase inhibitors (TKI) gefitinib and erlotinib, irreversible EGFR-TKI pelitinib and a reversible experimental compound (PD153035) on *in vitro* proliferation, apoptosis, migration as well as *in vivo* metastatic colonization in a spleen-liver model.

Results: The presence of the intracellular domain of EGFR protein and its constitutive activity were demonstrated in all cell lines. We detected significant differences between the efficacies of EGFR-TKIs, irreversible inhibition had the strongest anti-tumour potential. Compared to BRAF-mutant cells, wild-type BRAF associated with relative resistance against gefitinib. In combination with gefitinib, selective mutant BRAF-inhibitor vemurafenib showed additive effect in BRAF-mutant cell lines. Treatment of BRAF-mutant cells with gefinib- or pelitinib attenuated *in vitro* cell migration and *in vivo* colonization.

Conclusions: Our preclinical data suggest that EGFR is a potential target in the therapy of BRAF-mutant malignant melanoma; however, more benefits could be expected from irreversible EGFR-TKIs and combined treatment settings.

**Keywords:**

EGFR; small molecule tyrosine kinase inhibitors; human malignant melanoma; metastasis; xenograft

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## BACKGROUND

Malignant melanoma is an aggressive type of malignant skin lesions with worldwide increasing incidence [1], which is resistant to common cytotoxic therapies. Since malignant melanomas have a potential to form organ metastases in a very early phase of primary growth, a better understanding of their progression is urgently needed. In the past few years paradigm shift occurred in the treatment modalities: in contrast to the previous practice when major survival benefit could only be achieved with early detection and complete surgical removal, recently target based modalities have appeared on the horizon. Fifty to sixty percent of malignant melanomas carry mutation in the BRAF oncogene, vemurafenib and dabrafenib show activity against the V600E-mutant BRAF [2, 3]. Nevertheless, application of BRAF-inhibitors can induce cutaneous squamous cell carcinoma due to paradoxical activation of RAF signaling in cells carrying wild type BRAF [4]. Moreover, a number of BRAF mutant melanomas show limited response due to intrinsic resistance and initially responding patients often relapse because of acquired resistance [5]. To prevent these mechanism novel combinations are in development, recently clinical trials proved that the addition of MEK-inhibitors to V600E-selective BRAF-inhibitors was associated to significant improvement in progression-free survival among patients of BRAF-mutated metastatic melanoma [6, 7].

Hyperactivation of the epidermal growth factor receptor (EGFR) signaling components commonly presents in human melanoma (e.g. NRAS-BRAF-MAPK, PI3K-AKT), which suggests the potential role of EGFR itself as well. EGFR (Her-1 or c-erbB-1), a member of the c-erbB receptor tyrosine kinase family, is a glycoprotein (170 kDa) composed of an extracellular binding domain, transmembrane lipophilic segment, and an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment. EGFR becomes activated by homodimerization, a mechanism that could be promoted by ligand binding as well as by high receptor density due to overexpression [8]. Receptor activation normally leads to the recruitment and phosphorylation of several intracellular substrates, regulating various cellular activities such as differentiation, increased proliferation, survival and migration [9].

Aberrant activation of EGFR has been shown to correlate with poor prognosis in a wild range of malignant tumors, e.g. urinary bladder, cervix, esophagus, ovarian cancers, and tumors of the head and neck region [10]. Therapeutic inhibition of tyrosine kinase activity by small molecule substrates is a possible approach to interfere with such an aberrant activation of TK-

type receptors: small molecule tyrosine kinase inhibitors (TKIs) bind to the ATP cleft of the TK receptor and selectively block growth factor-stimulated signal activation via dimerization and autophosphorylation [11]. Inhibition of phosphorylation leads to depletion of the activated downstream effectors, resulting in attenuation of tumor progression.

Previously a number of experimental studies suggested the importance of EGFR function in malignant melanoma cells [12-16]. Furthermore, according to clinicopathological data, EGFR gene copy number alterations in primary cutaneous malignant melanomas were associated with poor prognosis [17]. Several genomic and proteomic analysis confirmed the potential role of EGFR in the progression of malignant melanoma [18-20], therefore EGFR-TKI strategy could serve as a potential anti-melanoma approach. Recent studies demonstrated in BRAF-mutant colorectal cells that selective inhibition led to feedback activation of EGFR [21]. Previous findings confirmed a similar mechanism in the development of adaptive resistance to vemurafenib in the case of BRAF-mutant malignant melanoma as well [22], therefore simultaneous application of BRAF and EGFR inhibitors could be a potential novel combination.

In our preclinical study, we first examined the EGFR-TK-status at protein level in six human melanoma cell lines representing the major oncogenic driver mutations (mutant BRAF, mutant NRAS, double wild-type cells). Moreover, we studied the potential effect of specific EGFR TKIs in combination with vemurafenib on proliferation, apoptosis and migration of human melanoma cells *in vitro* as well as on *in vivo* growth and colonization of human melanoma xenografts.

## METHODS

### Cell lines and culture conditions

BRAF-mutant A2058 cell line was provided by L. A. Liotta (NCI, Bethesda, MD), HT168-M1 human melanoma was the derivative of A2048 with high metastatic potential [23], HT199 melanoma line was established by our group [24], WM983B melanoma cell lines were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). NRAS-mutant M24met melanoma line was kindly provided by B. M. Mueller (Scripps Research Institute, La Jolla, CA). Double wild-type MEWO and A431 squamous carcinoma cells (which served as a positive control for EGFR) are available from ATCC. Human melanoma cell lines were grown in medium RPMI-

1640, while A431 were cultured in DMEM containing 4500 mg/l glucose (Sigma Chemical Co., St. Louis, MO), both were supplemented with 5% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Sigma), at 37°C in a humidified atmosphere of 5% $CO_2$.

**Flow cytometric measurement of EGFR protein expression**

Cells from monolayer cultures were detached with 0.02% EDTA (Sigma), then washed with phosphate-buffer-saline (PBS) for 3x5 min, fixed and permeabilized by methanol for 15 min. After blocking nonspecific binding sites with 3% BSA for 15 min, cells were labelled for 45 min at 37 ºC with a mouse monoclonal antibody against the intracellular amino acid region of EGFR between 1020 and 1046, purchased from Becton-Dickinson (Sunnyvale, CA, 1:20 in PBS). After washing period RPE-conjugated goat polyclonal anti-mouse antibody (DakoCytomation, Glostrup, Denmark) was applied for 45 min at 37 ºC. Fluorescence was analyzed by flow cytometer (CyFlow SL-Green, Partec, Munster, Germany) using FlowMax software (Partec). Positive events from a total of $10^4$ cells were counted. Negative controls were prepared by primary antibody with isotype-matched nonimmune IgG (Sigma).

**Small molecule tyrosine kinase inhibitors**

EGFR-specific TKI gefitinib (ZD1839, Iressa®; [N-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine]) was kind gift of AstraZeneca. Erlotinib (OSI-774, Tarceva®; [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine]) and irreversible inhibitor pelitinib (EKB-569; [N-(4-(3-chloro-4-fluorophenylamino)-3-cyano-7-ethoxyquinolin-6-yl)-4-(dimethylamino)but-2-enamide]) were synthesized by Vichem Chemie Ltd., Budapest, Hungary. PD153035 [4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline] were purchased from Calbiochem (La Jolla, CA, USA). V600E-selective BRAF-inhibor vemurafenib (PLX4032, [N-(3-[5-(4-chlorophenyl)-1H-pyrrolo[2,3-b]pyridin-3-yl]carbonyl-2,4-difluorophenyl)propane-1-sulfonamide] was provided by Selleck Chemicals (Munich, Germany). All TKIs were suspended in DMSO (Sigma) and used at 0.01-100 μM concentrations in 0.5% DMSO-medium for *in vitro* studies. For *in vivo* metastasis assays vaporized inhibitors were suspended in Tween-20 and diluted in physiologic saline to reach a final concentration of 1%. The final applied doses of gefitinib, pelitinib and vemurafenib were 2 mg/kg and 20 mg/kg, 0.04 mg/kg and 0.4 mg/kg, 12.5 mg/kg and 25 mg/kg, respectively. (Applied *in vivo* doses were based on *in vitro* proliferation assays.)

**Immunofluorescence**

Melanoma cells of monolayer culture were fixed in paraformaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 1 min. After washing in PBS for 3x5 min and blocking with 1% bovine serum albumine (BSA; Sigma) and goat serum (9:1) for 30 min at room temperature, slides were incubated with primary phosphospecific rabbit anti-EGFR[pY$^{1068}$] antibody (Biosource, Nivelles, Belgium, 1:20 in PBS) for 45 min at 37°C. After washing, biotin-conjugated anti-rabbit IgG (Amersham, Buckinghamshire, UK) was applied for 40 min at 37°C (dilution 1:100). EGFR protein was visualized by streptavidin-FITC (dilution 1:100, Vector Laboratories, Burlingame, CA). Negative controls were prepared by primary antibody with isotype-matched non-immune IgG (Sigma). Cell nuclei were stained with propidium iodide (PI, Sigma). Slides were covered with Vectashield (Vector Laboratories) and examined with confocal microscopy (Eclipse C1 Plus, Nikon Optoteam, Vienna, Austria).

**Kinexus Kinex Kinetworks™ protein kinase screen**

WM983B control and treated cells with 25 μM gefitinib for 5 min or 30 min were prepared according to the recommendations of Kinexus Bioinformatics Corporation (Vancouver, BC, Canada; www.kinexus.ca). 1x10$^7$ adherent cells were washed twice with PBS, and 200 μl ice-cold lysis buffer was added to each sample (20 mM MOPS, pH 7.0; 2 mM EGTA; 5 mM EDTA; 30 mM sodium fluoride; 60 mM β-glycerophosphate, pH 7.2; 20 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 1% Nonidet P-40; 1 mM phenylmethylsulfonylfluoride; 3 mM benzamidine; 5 μM pepstatin; 10 μM leupeptin; 1 mM dithiothreitol; final pH of the homogenizing buffer was adjusted to 7.2). Scrapped cells were collected, sonicated four times for 10 seconds each time with 10-15 second intervals on ice, each homogenates were centrifuged at 90,000 x g 30 min at 4°C in ultracentrifuge. Supernatants were transferred to 1.5 ml microcentrifuge tubes, resuspended in SDS-PAGE Sample Buffer (31.25 mM Tris-HCl, pH=6.8; 1% SDS; 12.5% glycerol; 0.02% bromophenol blue; 1.25 % β-mercaptoethanol) to final protein concentration of 1.4 mg/ml, and shipped to Kinexus. Commercially available service of fluorescent labelling, hybridization onto KPCS-1.0 microarray with selected phospospecific antibodies (ERK1/2, MEK1/2, p38 MAPK), as well as scanning, imaging and quantitative analysis of the enhanced chemiluminescence signal of the detected proteins in the EGFR-pathway were performed by Kinexus. Images of

immunoblots were provided by Kinexus, and our further conclusions were based on their evaluations.

**Flow cytometric measurement of apoptosis**

Melanoma cells of monolayer were previously treated for 48 hours with different concentrations EGFR-TKIs and/or vemurafenib, detached with EDTA, washed with PBS, then fixed with 70% ethanol (30 min, 4°C). Samples were washed twice with PBS and incubated with propidium-iodide and RNAse (CyStain PI Absolute T, Partec) for 4 hours at room temperature. The amount of DNA in cells was measured by flow cytometer (CyFlow, Partec), and the percentage of the apoptotic cells (sub-G1 fraction) was analyzed by FlowMax software.

**Cell proliferation assay**

Cell suspensions containing $5x10^4$ viable cells/ml were plated in 96-well dishes (Greiner, Frickenhausen, Germany), incubated for 24 hours and treated with gefitinib, PD153035, erlotinib, pelitinib at concentrations of 0.1-100 μM and/or 5 μM of PLX4032 in 200 μl serum-free or serum-containing medium for 48 hours. At the end of incubation, cell monolayers were fixed with 10% trichloroacetic acid and stained for 15 min with Sulforhodamine B (SRB). Wells were repeatedly washed with 1% acetic acid to remove excess dye. Protein-bound dye was dissolved in 10 mM Tris, and absorbance was measured at 570 nm using microplate reader (BioRad, Hercules, CA). 50% inhibitory concentrations (IC50) were calculated by Dose-Effect Analysis with Microcomputers software (Elsevier-Biosoft, Cambridge, UK).

**Modified Boyden-chamber migration assay**

Cell migration was assayed by a method reported previously [25]. Human melanoma cells were previously treated with different concentrations of gefitinib, pelitinib or vemurafenib for 24 hours at 37°C, harvested with 0.02% EDTA, washed twice with serum-free medium, and resuspended at a density of $10^6$ viable cells/ml in medium contained 0.1% BSA. Viability was assayed by trypan blue staining (Sigma). 20 μl of the cell suspension was placed on top of the 96-well CXF8 plates (polycarbonate filter with 8 μm pore size, Neuroprobe Inc., Cabin John, MD) and the lower compartment was filled with 30 μl of fibronectin in RPMI (100 μg/ml, Sigma). Cells were allowed to migrate for 6 hours (except M24met, where 24 hour incubation

period was applied) at 37 ºC in a humidified atmosphere of 5% $CO_2$. The cells on the upper surface of the filter were then removed mechanically and the membranes were stained with toluidine blue (Sigma). Six independent parallel samples were applied. Migrated cells were counted under a light microscope in 3 high-power fields per sample.

**Animal experiments for liver colonization**

SCID mice (C.B-17/lcr-Prkdc$^{scid}$/lcrlcoCrl) were bred and maintained in our specific pathogen-free colony and housed 10 to a cage. WM983B and HT168-M1 human melanoma cells from monolayer culture were detached, washed with serum-free medium and one-cell suspension was inoculated into the spleen of SCID mice with a number of $10^6$ or $5x10^4$ cells/animal, respectively. Fourteen days after intrasplenic injection animals were randomized (10 animals per group) and treated intraperitoneally daily for 21 days. Reversible EGFR-specific tyrosine kinase inhibitor gefitinib, irreversible pelitinib and V600E-selective BRAF-inhibitor vemurafenib were suspended in physiologic saline containing 1% Tween-20. After termination the weight of the primary tumors was measured. Livers were fixed in 10% neutral buffered formalin for 48 hours and the number of liver colonies was counted under a stereomicroscope.

**Ethics approval**

All animal experiments were conducted following standards and procedures approved by the Animal Care and Use Committee of the National Institute of Oncology, Budapest (license number: 22.1/722/3/2010).

**Immunohistochemistry**

Routinely fixed xenograft tumors were dehydrated in a graded series of ethanol, infiltrated with xylene and embedded into paraffin at a temperature not exceeding 60°C. Three to four micron thick sections were mounted on Superfrost slides (Thermo Shandon, Runcorn, UK) and were manually deparaffinized. Endogenous peroxidase activity was blocked by 3% $H_2O_2$ in methanol for 5 min at room temperature. Slides were immersed in 0.05 mM citrate buffer (pH=9) and exposed to 93°C for 10 min (MFX-800-3 automatic microwave, Meditest, Budapest, Hungary).

Slides were primarily treated with rabbit antibody (in dilution 1:100) against phospho-S6 ribosomal protein (pS6; Cell Signaling, Danvers, MA) and incubated overnight at 4°C. After

washing, secondary antibody Biotinylated Link (Dako) was used and incubated for 10 minutes at room temperature. For visualization a standard avidin-biotin peroxidase technique (ABC system, Dako) was used with aminoethyl carbazole as chromogen.

**Statistical analysis**

To determine statistical differences between different strata ANOVA (analyses of variance) were used with the *post hoc* Scheffé-test where parametric methods were available. For the animal experiments we used non-parametric Kruskal-Wallis test with *post hoc* analysis. Statistical significance was determined when *P* values were under 0.05. Statistical analysis was performed by Statistica 11.0 software (StatSoft, Tulsa, OK).

## RESULTS

**EGFR-signalization in human melanoma cells**

Fixed and permeabilized cells were labeled with antibodies specific for the intracellular domain of the EGF receptor, and the ratio of positive cells was evaluated by flow cytometry. Expression of EGFR detected by antibody against the intracellular domain showed 52-88% positivity in the studied human melanoma cell lines (Fig. 1).

In our previously published work we demonstrated tyrosine kinase activation and inactivation by immunofluorescence microscopy using a phosphospecific antibody [26]. By the application of EGFR-pY$^{1068}$-specific antibody we detected constitutively phosphorylation of EGFR without exogenous EGF stimulation in HT168-M1 and WM983B human melanoma cell lines (Fig. 2A, C). Furthermore, the EGFR signal could be inhibited by the EGFR-specific TKI, gefitinib **(**Fig. 2B, D).

Kinexus Kinex Kinetworks$^{TM}$ phosphoprotein assay confirmed that EGFR-specific inhibition by gefitinib affected elements of the EGFR-pathway in WM983B cells: activation of MEK1/2 and Erk1 were blocked at both endpoints, while Erk2 and p38a MAPK were blocked at 5 min, albeit the inhibitory effect was weakened at 30 min (Fig. 2E-F). Of note, that although p38a MAPK is involved in the EGFR signaling via RAC1, the major inducing stimuli are hypoxia and stress [27].

**Effect of EGFR-TKIs on the *in vitro* proliferation of human melanoma cells**

The inhibitory potential of gefitinib on the phosphorylation of EGFR suggested that EGFR-TKIs may have an effect on malignant melanoma at cellular level (Fig. 2). The inhibition of EGFR significantly decreased *in vitro* proliferative capacity of the human melanoma cells in serum-free and serum-containing media (Table 1A and B). The most potent inhibitor was irreversible EGFR-TKI pelitinib (IC50 values were in the range of 0.27-2.16 μM). In the case of gefitinib, IC50 values were between 0.25-17.2 μM; wild-type EGFR receptor expressing NRAS-mutant M24met cell line the [12] and double wild-type MEWO showed relative resistance to such inhibitor treatment. In BRAF-mutant lines the effect of PD153035 was relatively weaker compared to that of gefitinib, while M24met and MEWO showed total resistance. Regardless of oncogenic mutation status, all studied human melanoma cell lines were resistant to erlotinib, while the proliferation of the reference non-melanoma cell line, A431 was inhibited successfully. Generally IC50 values of human melanoma cells were higher than that of EGFR-amplified A431 human squamous cell line. Furthermore, in BRAF-mutant melanoma cells vemurafenib enhanced the inhibitory effect of gefitinib, while it proved to be ineffective in wild-type BRAF-carrying cells, which property was more significant in serum-containing media (Table 1A and B).

**Apoptosis induction by EGFR-TKIs in human melanoma cell lines**

To investigate the effect of EGFR inactivation on cell survival/apoptosis, we treated human melanoma cells with small molecule TKIs for 48 hours, and after propidium-iodide staining, samples were analyzed by flow cytometry. Since erlotinib proved to be ineffective to interfere with cell viability, we have not tested its potential for apoptosis induction. Measurement of sub-G1 fractions (Fig. 3) revealed that significant induction of apoptosis have not occurred in the range of the IC50 values for proliferation inhibition in most of the studied cell lines. After treatment with higher concentrations of gefitinib (25 and 50 μM) a strong, dose-dependent induction of apoptosis was shown in BRAF-mutant melanoma cells (Fig. 3A). Irreversible inhibitor pelitinib was already effective at lower concentrations, 5 μM led to 23-30% of sub-G1 fraction (Fig. 3B). PD153035 showed the weakest pro-apoptotic effect (Fig. 3C). Similarly to the proliferation assay, all the three reversible EGFR-TKIs were less capable to induce intense apoptosis in the wild-type BRAF-expressing M24met and MEWO cell lines; however the irreversible inhibitor pelitinib showed higher activity. Of note that vemurafenib have not induced significant apoptosis, its inhibitory effect was rather realized through the

blockade of cell cycle in G1-phase, and furthermore this effect was detectable only in BRAF-mutant cell lines (data not shown).

**Effects of EGFR-TKIs on the *in vitro* migration of human melanoma cells**

To investigate the effect of gefitinib and pelitinib on cell migration, we used modified Boyden-chamber assay using fibronectin as chemoattractant. Similarly to vemurafenib, pre-treatment with the EGFR-specific TKIs significantly reduced 6 hour migration of the BRAF-mutant human melanoma cells (Fig. 4). The inhibitory capacity of gefitinib and pelitinib proved to be dose-dependent. Pre-treatment with 10 μM concentration of pelitinib completely abolished viability of HT168-M1 cells, therefore migration assay could not be performed.

**EGFR-TKI strategy inhibited liver colonization of BRAF-mutant WM983B and HT168-M1 xenografts**

Based on our *in vitro* results, we examined the *in vivo* effect of gefitinib in combination with vemurafenib and pelitinib alone on the liver colonization of WM983B and HT168-M1 human melanoma cells in SCID-mice. Fourteen days after intrasplenic inoculation of WM983B or HT168-M1 cells, mice were treated intraperitoneally with gefitinib or pelitinib daily for three weeks, at doses of 2 mg/kg, 20 mg/kg or 0.04 mg/kg, 0.4 mg/kg, respectively. Based on the *in vitro* IC50 values, we applied equivalent *in vivo* dose, 2 mg/kg of gefitinib or 0.4 mg/kg of pelitinib, and we administered 10-fold higher or lower concentrations, respectively. Vemurafenib was applied at clinically relevant doses (12,5 mg/kg or 25 mg/kg). The weight of the primary tumors was measured during the autopsy and the number of liver colonies was determined under stereomicroscope after formaldehyde-fixation. Contrary to the *in vitro* results in the case of HT168-M1 cells, gefitinib did not inhibit primary tumor and liver colonization (data not shown) as compared to the irreversible inhibitor pelitinib, which reduced liver colonies at a dose of 0.4 mg/kg (Fig. 5A). In the case of the other BRAF-mutant WM983B melanoma cells, gefitinib significantly ($p<0.05$) inhibited liver colonization at the dose of 2 mg/kg as well as 20 mg/kg in a dose-dependent manner (Fig. 5B). Compared to control group, vemurafenib significantly affected liver colonization, however additive effect of the combination did not reach significance, only a statistical trend appeared. In the TKI-treated groups primary tumor sizes did not differ significantly from that of solvent-treated control, however a tendency of decrease was observed: 18% and 27% in the case of 2 mg/kg and 20 mg/kg of gefitinib and 9% and 14% in the case of 0.04 mg/kg, 0.4 mg/kg of pelitinib,

respectively. Interestingly, vemurafenib have not shown any effect on primary tumors either alone or in combination (data not shown).

Ribosomal S6 protein is the part of the translational machinery, one of the effectors of EGFR/KRAS/MAPK-pathway, therefore its phosphorylation status is highly dependent of signalization activity. By immunohistochemical examination of the primary WM983B xenograft tumors we confirmed *in vivo* inhibitory effect of gefitinib, since compared to solvent control, gefitinib-treated cells showed lower pS6-positivity (Fig 5C-D).

## DISCUSSION

Aberrant activation of the tyrosine kinase EGFR was demonstrated in several common solid tumors, resulting in increased proliferation, survival, invasiveness and metastasis. Constitutive activity of EGFR has been shown to correlate with poor prognosis in urinary bladder, cervical, esophageal, ovarian cancers and head and neck tumors [10]. EGFR-inhibitory strategy has already been approved in cancers of the head and neck region, colon cancers and non-small cell lung cancer [28]. Nevertheless, the effectiveness of EGFR inhibition may be influenced by oncogenic mutations in the downstream signaling pathway, for instance by the V600E mutant BRAF or by mutant NRAS, which are the two most common driver mutations in malignant melanoma cases [29]. On the other hand, activation of EGFR and vemurafenib resistance linked to the signal of microphthalmia-associated transcription factor [30].

Although EGFR-family pathway has more influent on epidermal tissue and cancers, it was previously described that heregulin (ligand of ErbB3 and ErbB4 receptors) stimulated the proliferation of both melanocytes and malignant melanoma cells [13]. Since heregulin contains similar domains to EGF, the oncogenic effect of EGFR (ErbB1) could not be excluded in malignant tumor type that shares neuroectodermal origin, malignant melanoma or glioblastoma multiforme. In the latter the role of EGFR seems to be more clear [31]. Among numerous other tyrosine kinases Tworkoski et al. showed the activity of EGFR in human malignant melanoma cell lines [20]. A previous work using standardized ATP-based chemosensitivity assay showed significant response of human melanoma cells to gefitinib, however, the extracellular domain of EGFR could be detected only in minority of tumor samples [14]. These findings are in concordance with our results that all the studied human melanoma cell lines expressed the intracellular domain of the receptor that harbours the

tyrosine kinase domain. In mutant BRAF-carrying melanoma cell lines EGFR-TKI treatment led to significant response in the signaling cascade, inhibited phosphorylation level of EGFR itself which resulted inactivation of the major elements in the downstream signal (e.g. MEK1/2, Erk1/2, p38a MAPK) in 30 minutes. These short-term alterations in the EGFR-signal may be the explanation of the detected long-term biological responses. Furthermore, we are the first to categorize the effectiveness of EGFR-TKIs in human melanoma according to the molecular pattern: treatment blocked proliferation activity of BRAF-mutant cells, however wild-type BRAF-carrying human melanoma cells showed relative insensitivity against gefitinib.

Inhibition of EGFR leads to the inactivation of PI3K/Akt survival signal, which results in increased apoptosis [32-34]. Our results confirmed previous studies showing that albeit a minority of malignant melanoma cells expresses extracellular domain of EGFR, gefitinib still proved to be an apoptosis-inducing agent [15, 35]. This observation suggests the involvement of intracellular domain in the survival signal, while the presence of the extracellular region is not essential. Another novel statement of our current work is that irreversible inhibition of EGFR by pelitinib had a more potent effect on apoptosis as well as on proliferation than the already clinical administered agents. Previously Djerf Severinsson et al. showed that pan-ErbB tyrosine kinase inhibitor canertinib also had better anti-tumor activity in malignant melanoma [36]. Additionally, our results served the first evidence that irreversible inhibition could work in NRAS-BRAF double wild-type as well as NRAS-mutant melanoma cells.

The relative resistance of NRAS-mutant M24met cell line to gefitinib could be explained by previous observations that confirmed the inactivity of EGFR in those cells [12], and the receptor was not capable to react to exogenous EGF stimulation despite the gene was amplified [17]. Probably the relative resistance of double wild-type MEWO cells is caused by EGFR-independent signalization, since this line is NF1 mutant, which resulted loss of NRAS-suppression [37]. In our present study we have not only confirmed the experimental work of Djerf et al. [35], but according to the driver oncogenic mutation status we have systematically explored the potential of EGFR-TKI strategy in malignant melanoma. Moreover, based on previous theories in colorectal cancer that selective BRAF(V600E) inhibition led to feedback activation of EGFR [21], we have first shown that the efficacy of EGFR-TKIs can be enhanced by vemurafenib.

EGFR not only plays a role in the regulation of proliferation and survival but in cell migration as well. Selective EGFR-TKI treatment resulted in inhibition of adhesion, migration and invasion in several tumor cell lines, such as cutaneous squamous cell carcinoma, carcinoma of the head and neck region, malignant mesothelioma, hepatocellular carcinoma and prostate cancer [38-43]. Moreover, numerous data of animal experiments is available that gefitinib had an *in vivo* inhibitory effect of metastasis formation in mice using hepatocellular carcinoma, head and neck cancer, squamous cell carcinoma of the vulva and prostate carcinoma cells [44-48]. We are the first to show that in BRAF-mutant melanoma cells selective inhibition of EGFR prevented both *in vitro* motility and *in vivo* metastasis formation, and that the irreversible inhibitor pelitinib could open a new option for those cells which showed relative resistance against the reversible inhibitor gefitinib (e.g. NRAS-mutant, NRAS-BRAF double wild-type cells). At the same time, in contrast to previous findings, our vemurafenib-treatment has not impacted migratory activity of human melanoma cells [49], which effect was irrespective of BRAF and NRAS status; however, we applied other cell lines and shorter incubation period than the cited work.

Genetic analysis of tumors of vemurafenib-relapsed melanoma patients revealed several acquired resistance mechanisms. These includes among others overexpression of previously overseen growth factor pathways of melanoma involving EGFR [22], EGFR3 [50], EGFR2/HER2, AXL and PDGFRβ receptors [51]. Studies revealed also acquired genetic alterations such as ERBB4, besides FLT1, PTPRD, RET, TERT and RUNX1T1 [51]. These data all conclude to the same direction that in human melanoma cells inhibition mutant BRAF frequently results in the (re)-activation of the EGFR receptor family signaling pathway. Moreover, recent clinical trials confirmed that in combination downstream elements of the TK-signal should be feasible targets: MEK-inhibitors improved the antitumor effect of mutant BRAF-specific inhibitors [6, 7]. Our data suggests that these pathways are already active in mutant BRAF-expressing human melanoma cells and themselves serve targets for therapeutic interventions which can further be exploited later upon vemurafenib resistance. A phase II study of gefitinib showed minimal clinical efficacy as a single-agent in unselected patients with metastatic melanoma [51], which can be explained by the different EGFR activities in various molecular subgroups of human melanoma. Beside of others our data also suggests revisiting the clinical application of EGFR-TKIs, since several new agents are now available.

CONCLUSIONS

In summary, our study suggests that EGFR is a potential target in the therapy of BRAF-mutant malignant melanoma; however, more benefits could be expected from irreversible EGFR-TKIs and combined treatment settings.

## LIST OF ABBREVIATIONS:

ATP: adenosine triphosphate
DNA: deoxyribonucleic acid
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
FCS: fetal calf serum
GTP: guanosine triphosphate
Ras: protein product of RAS gene
RAS: rat sarcoma gene
RNAse: ribonuclease
SD: standard deviation
TKI: tyrosine kinase inhibitor

## COMPETING INTERESTS.

The authors declare that they have no conflict of interest.


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TITLES AND LEGENDS TO FIGURES

Fig. 1. **Expression of the intracellular domain of EGFR protein in human melanoma cell lines** (flow cytometry, 3 parallel samples, data are mean ± SD).

Fig. 2. G**efitinib inhibited the activity of EGFR-signal in HT168-M1 and WM983B human melanoma cells.** Immunofluorescent detection of p-EGFR (green) using antibody against pY1068, nuclei were labeled by PI (red) (A-D). Commercially available Western-blot analysis of Kinexus Kinex Kinetworks™ demonstrated the effect of gefitinib on WM983B cells, bands represent ERK1/2, MEK1/2 and p38a MAPK – marked by white lines (E). The analysis of Kinexus Kinex Kinetworks™ protein kinase assay confirmed that EGFR-specific inhibition blocked activation of MEK1/2 and Erk1 at both endpoints, while Erk2 and p38a MAPK were blocked at 5 min, albeit the inhibitory effect was weakened at 30 min (F).

Fig. 3. **Induction of *in vitro* apoptosis by EGFR-TKIs in human melanoma cell lines (flow cytometry).** Comparing gefitinib (A), PD153035 (B) and pelitinib (C), the most effective drug was irreversible inhibitor pelitinib while PD153035 had the lowest capacity to induce apoptosis (3 parallel samples, data are mean ± SD).

Fig. 4. **Effects of gefitinib (A) and pelitinib (B) pre-treatment on the *in vitro* migration of human melanoma cell lines.** The inhibition of EGFR reduced migratory capacity of melanoma cells expressing mutant BRAF, while wild-type BRAF-expressing cells were unaffected. Vemurafenib-treated cells (C) served as reference. (*$p<0.05$ compared to solvent control; **no viable cells after pre-treatment, migration assay was not performed; 6 parallel samples, data are mean ± SD).

Fig. 5. **Effects of EGFR-TKI treatment on *in vivo* liver colonization of human melanoma cells.** Pelitinib (irreversible TKI) inhibited colonization of HT168-M1 (A). Gefitinib (reversible TKI) inhibited WM983B, while the inhibitory effect of vemurafenib was as explicit as significant additional effect has not presented, only tendency has appeared (B). The inhibitory effect of ZD1839/gefitinib was confirmed by immunohistological examination of the primary WM983B xenograft tumors: compared to solvent control (C) gefitinib-treated tumors (D) showed lower positivity to antibody against ribosomal phospho-S6 protein. (*$p<0.05$ compared to solvent control; 10 animals per group, data are mean ± SD).

Table 1. **Effect of EGFR-TKIs on the *in vitro* proliferation of human melanoma cell lines with different molecular background and A431 as positive control** (50% inhibitory concentrations in μM, 5 parallel samples). The most effective drug was pelitinib, while all melanoma cell lines showed resistance against erlotinib. Asterisks indicate synergistic inhibitory effect of 5 μM vemurafenib with gefitinib in cells harboring V600E-mutant BRAF (NA: not available).

A) Serum-free media

| | **gefitinib (ZD1839)** | **gefitinib + vemurafenib (PLX4032)** | **erlotinib (OSI-774)** | **pelitinib (EKB-569)** | **PD153035** |
|---|---|---|---|---|---|
| A431 | 0.33 | NA | 0.05 | 0.22 | 0.4 |
| M24met (NRAS – Q61R) | 17.2 | >25 | >100 | 2 | ~100 |
| MEWO (wt NRAS-NRAF) | >25 | >25 | >100 | 0.64 | >100 |
| A2058 (BRAF – V600E) | 4.72 | 2.15* | >100 | 1 | 3.3 |
| HT168-M1 (BRAF – V600E) | 1.1 | 1.085 | >100 | 2.16 | 3.6 |
| HT199 (BRAF – V600E) | 0.979 | 0.507* | >100 | 0.27 | 8.05 |
| WM983B (BRAF – V600E) | 0.25 | 0.078* | >100 | 0.38 | 1.93 |

B) Media contained 2.5% of serum

| | **gefitinib (ZD1839)** | **gefitinib + vemurafenib (PLX4032)** | **erlotinib (OSI-774)** | **pelitinib (EKB-569)** | **PD153035** |
|---|---|---|---|---|---|
| A431 | 0.01 | NA | 0.01 | 0.43 | 0.05 |
| M24met (NRAS – Q61R) | >25 | >25 | >100 | 0.94 | >100 |
| MEWO (wt NRAS-NRAF) | >25 | >25 | >100 | 0.127 | >100 |
| A2058 (BRAF – V600E) | 5.361 | 4.543* | >100 | 1.1 | 8.51 |
| HT168-M1 | 3.87 | 1.213* | >100 | 1.08 | 15 |

| | | | | | |
|---|---|---|---|---|---|
| (BRAF – V600E) | | | | | |
| HT199 (BRAF – V600E) | 9.989 | 0.199* | >100 | 1.02 | 30.23 |
| WM983B (BRAF – V600E) | 3.553 | 1.888* | >100 | 0.22 | 4.51 |

Figure 1

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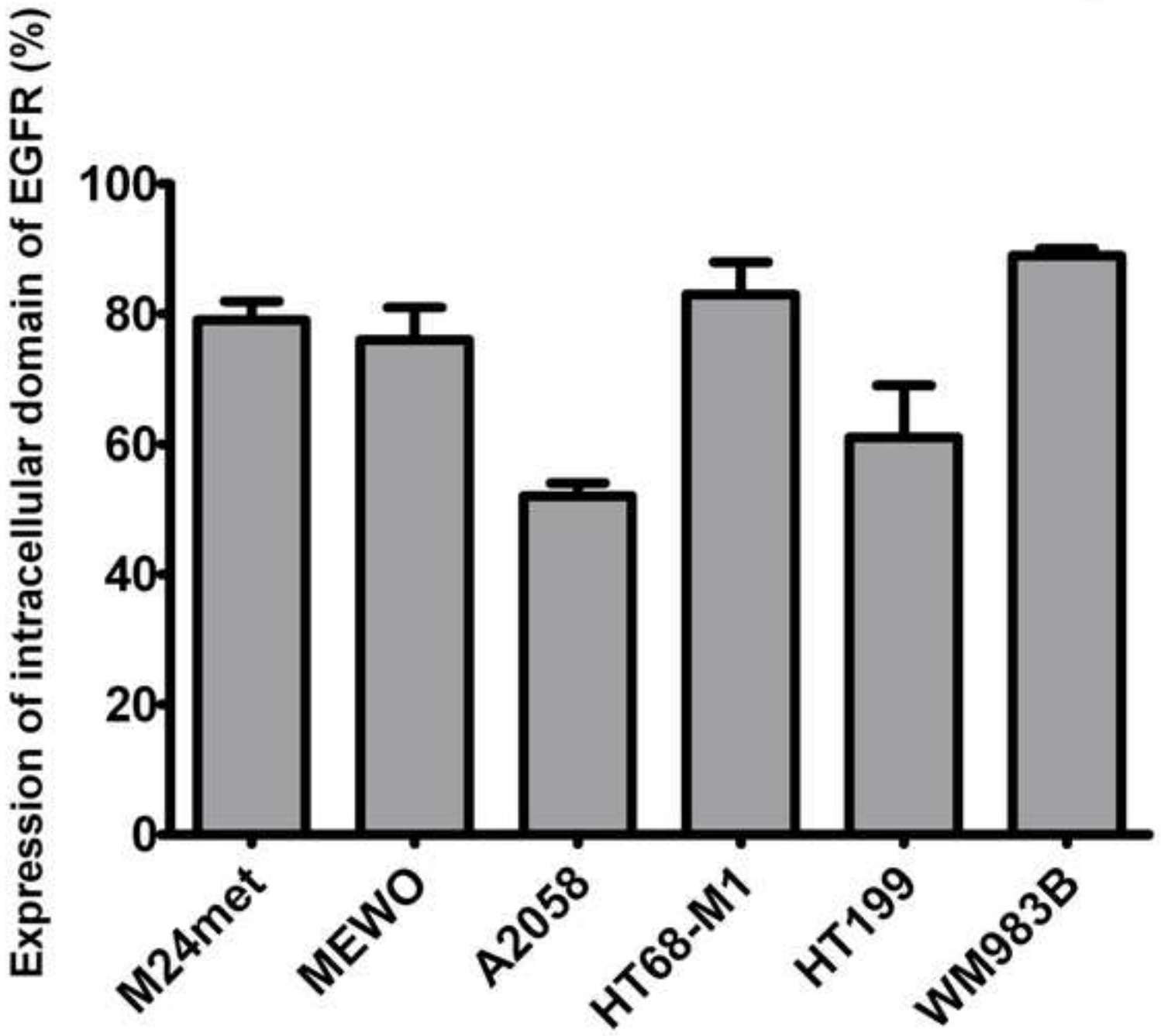
Fig. 1



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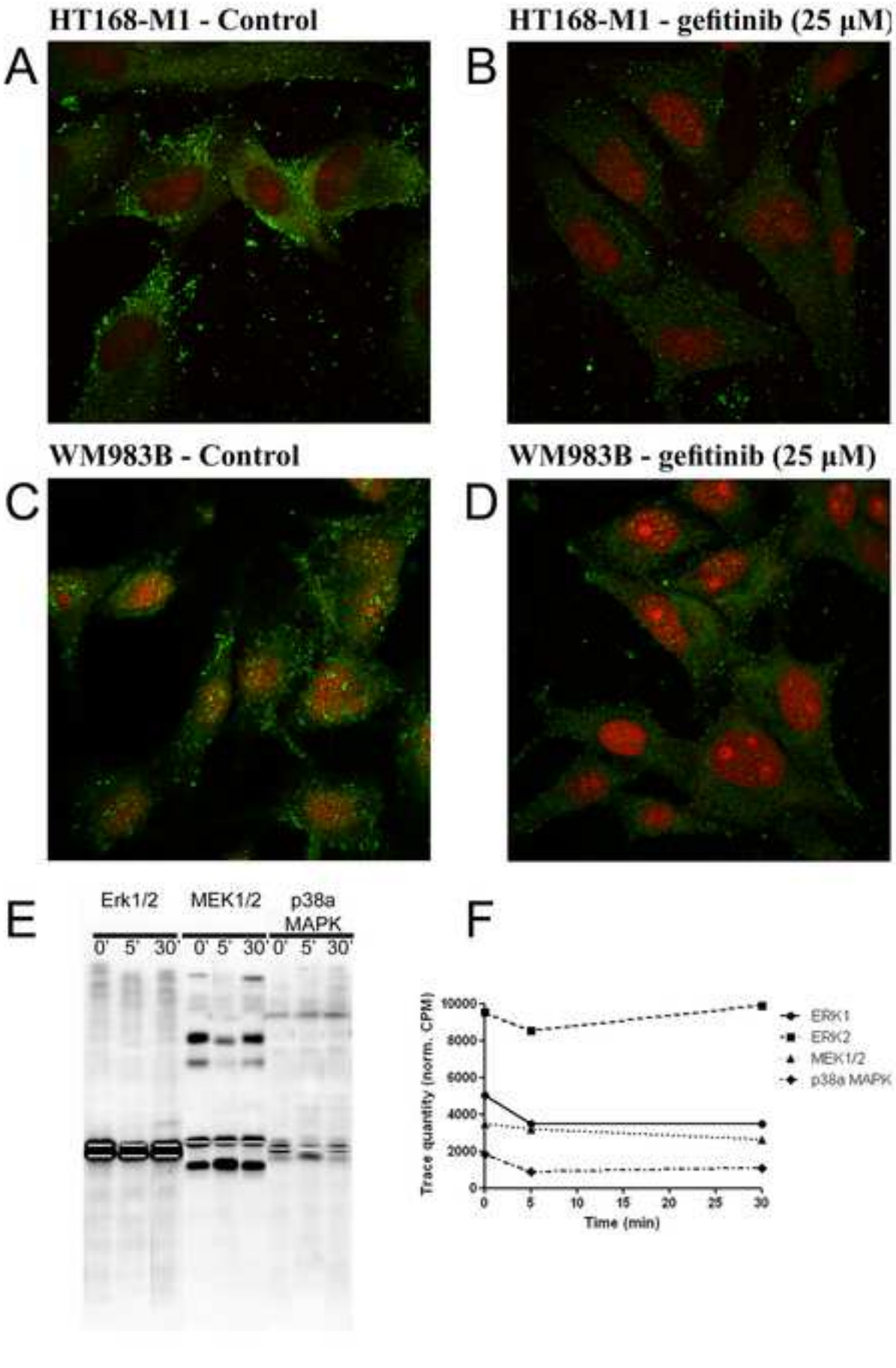

Figure 3

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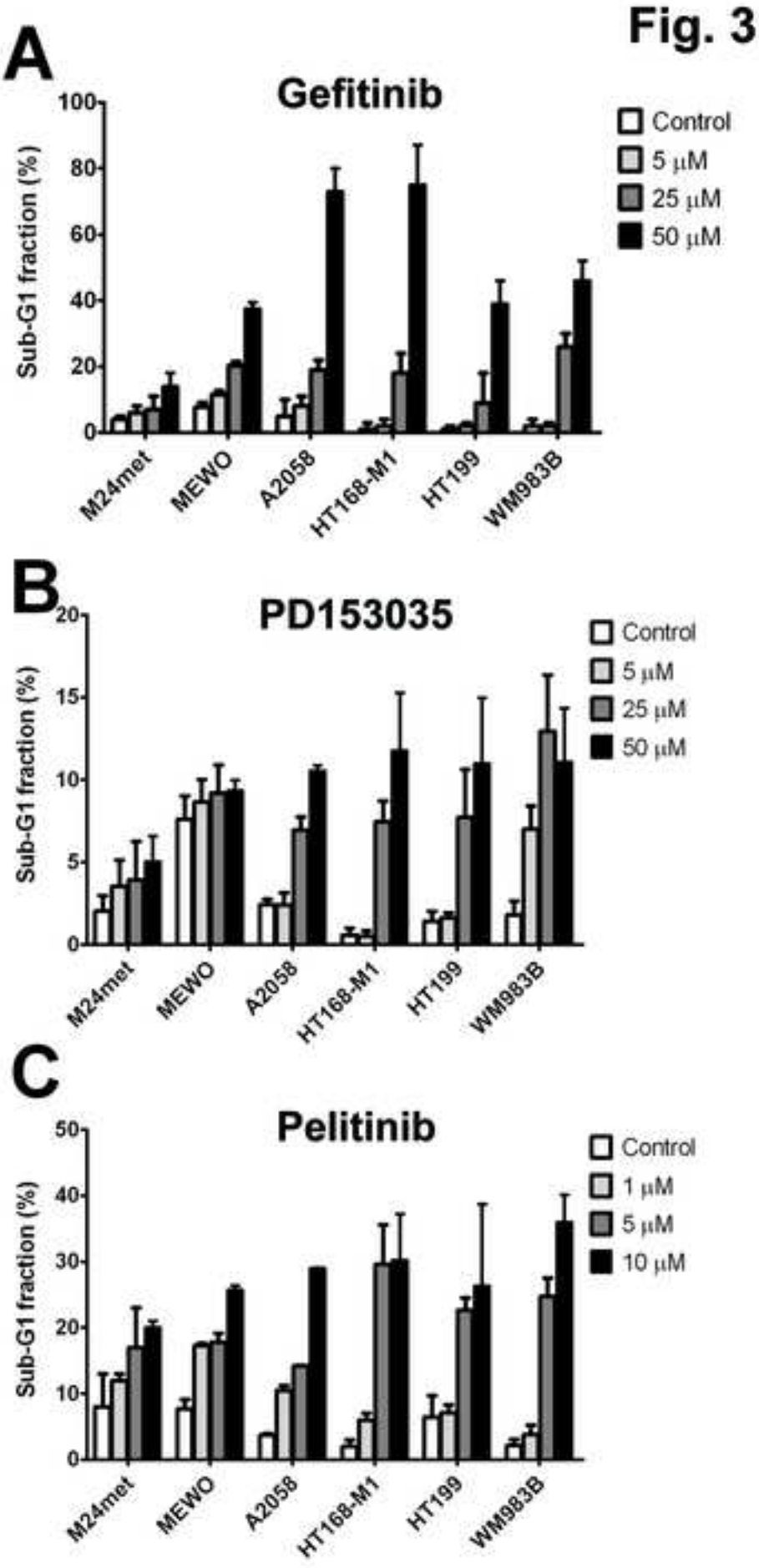

Figure 4

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Fig. 4

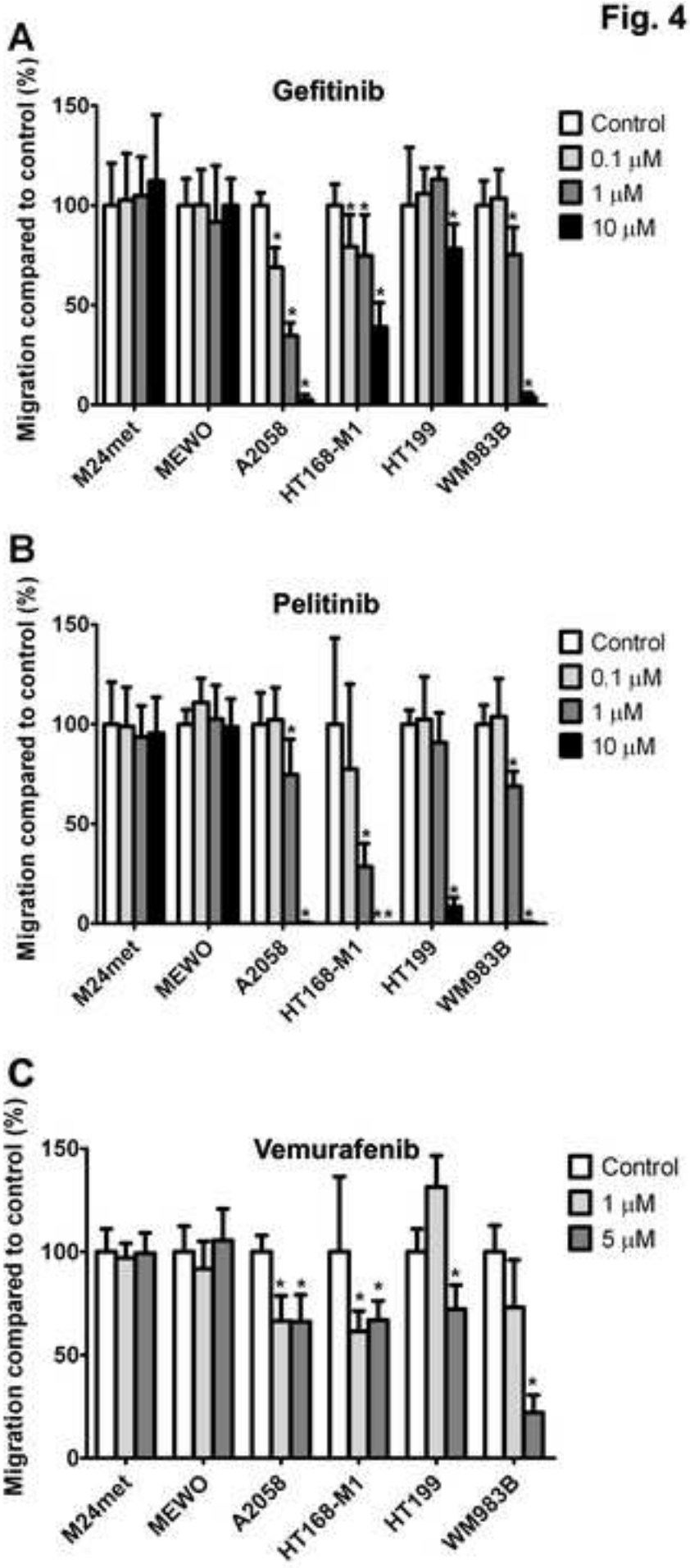

Figure 5

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