Title: Subcellular localization of novel fluorescent Epidermal Growth Factor Receptor (EGFR) and hepatocyte growth factor receptor (MET) targeted inhibitors in cancer cells

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Introduction: Epidermal growth factor receptor (EGFR) inhibitors of the quinazoline class have emerged as potent agents in personalized lung cancer therapy. It is known that EGFR is not only localized on the cell surface, but also inside the cell. However, little attention is paid to the cellular localization of EGFR inhibitors, which could perhaps influence their biological activity. Additionally, EGFR inhibitors can bind to multiple kinases interfering with cellular pathway of some oncogenes, such as MET in EGFR inhibitors-resistant tumors. We hypothesize that the target multiplicity of EGFR inhibitors can be visualized by cell live imaging, reflecting the macromolecular binding profile of these inhibitors.

Methods: One quinazoline-based molecule (anti-EGFR) carrying a red fluorescence, called "AB64", and one molecule of crizotinib (anti-MET inhibitor) green labeled with nitrobenzoxadiazole (NBD), called "AB20", were synthetized. Four cell lines were used to analyze the subcellular distribution of MET and EGFR: A549-wild type, A549-GFP-EGFR, NIH3T3-wild type and NIH3T3-EGFR. MitoTracker green was used for the colocalization experiment in A549 cells. Visualization was performed using Zeiss LSM780 laser scanning confocal microscope. Observations were made in a monolayer of cells which were treated with AB64 or/and AB20 (5 or 10 μM) with/without MitoTracker for 15 minutes prior the live imaging.

Results: The red fluorescence of AB64 (anti-EGFR inhibitor) was observed in the perinuclear region of all cell lines and it was not present on their cellular surface. More interestingly, AB64 co-localized with the mitochondria in A549 cells. In order to look if AB64 co-localized with MET, the green fluorescent AB20 (anti-MET inhibitor) was co-stained with the red AB64. The co-staining of AB64 and AB20 showed high co-localization. Prior to this double staining, AB20 was observed to be confined to the perinuclear region where it was more largely intracellular distributed than AB64 in all cell lines.

Conclusions: We were able to synthetize two new fluorescent molecules showing that the inhibitors of the quinazoline class: **(1)** has a unique property of colocalization in the mitochondria,

which may interfere with the mitochondrial functions like perhaps apoptosis or redox signalling: **(2)** can simultaneously bind to MET kinase sites, showing kinase off-targets effects in living cells.