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Original Article

Molecular determinants of response to PI3K/akt/mTOR and KRAS pathways inhibitors in NSCLC cell lines

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Abstract: Despite the impressive results obtained in the preclinical setting, all the inhibitors targeting two central cascades in cancer, the PI3K/akt/mTOR and the KRAS/MEK/ERK pathways, have shown, apart from very few exceptions, disappointing efficacy when translated to the clinic. One of the main reasons of their clinical failure seems to be the lack of a clear molecular determinant of response to these drugs. In this study, we tried to address this point by evaluating the cytotoxic activity of different inhibitors targeting the two pathways at different levels in a panel of ten NSCLC cell lines harboring alterations in PI3K, KRAS or both. We were not able to highlight a correlation between the presence of *KRAS* and *PI3K* mutations and a specific sensitivity to the different drugs used. Molecular analyses performed after equimolar treatments showed that, independently from the entity of the response, the drugs are able to modulate the activation of their targets. Interestingly, we found that *p53* mutational status separates the cell lines according to their sensitivity to PI3K pathway inhibitors treatments. The alterations considered in the PI3K/akt/mTOR and in the KRAS/MEK/ERK pathways in the different NSCLC cell lines are not sufficient to drive treatment choice but rather *p53* status is a potential biomarker for the activity of this class of drugs.

Keywords: Non-small cell lung cancer, p53, PI3K inhibitors, mTOR inhibitors

Introduction

The PI3K/akt/mTOR and RAS/RAF/MEK/ERK pathways are among the most downregulated pathways in cancer [1-7]. They both control several of the hallmarks of cancer including metabolism, survival, cell cycle, new vessels formation [8]. Several drugs inhibiting at different levels the two pathways are available and some of them in the current clinical practice [9, 10].

Among the proteins belonging to these pathways, KRAS still lacks drugs directly targeting its activity, although very recently different allosteric G12C mutated KRAS inhibitors have entered phase I-II trials in the clinic [11-13]. For other targets such as BRAF, MEK and ERK there are available inhibitors with different degrees of specificity, some of which have been extensively studied at clinical level. Two BRAF inhibitors, vemurafenib and dabrafenib have been approved for the treatment of late stage and BRAF mutated melanoma. In addition, vemurafenib received also the approval for the treatment of Erdheim-Chester Disease. Trametenib, a MEK inhibitor has been approved, as single agent or in combination with dabrafenib, for melanoma patients presenting mutations in the *BRAF* gene. No approvals exist yet for the ERK inhibitors. Ulixertinib, is just being tested in clinical trials as “first in class drug” [14].

The results from the trials performed, particularly in the case of PI3K inhibitors, were somehow below the expectancies [15], considering the central role of this pathway in cancer. While for BRAF there is clinical evidence of its activity in tumors with defects in *BRAF* gene [16, 17], for the other drugs currently approved, no clear evidence of their preferential activity in tumors harboring alterations in the gene they are targeting exists [18].

In the present study we evaluated the activity of different inhibitors of the pathways in 10 different non-small cell lung cancer (NSCLC) cell lines

Table 1. Major molecular alterations in the cell lines used

GENE CELL LINE	PIK3CA	PTEN	AKT1/2	KRAS	B-RAF	ERK	mTOR	TSC2	P53
H1975	G118D	WT	WT	WT	WT	WT	WT	H798Y	R273H
H596	E545K	WT	WT	WT	WT	WT	WT	WT	G245C
H460	E545K	WT	WT	Q61H	WT	WT	WT	WT	WT
LU-99	T1025A	WT	WT	G12C	WT	WT	WT	WT	WT
H358	WT	WT	WT	G12C	WT	WT	WT	WT	WT
H23	WT	WT	WT	G12C	WT	WT	WT	WT	M246I
A549	WT	WT	WT	G12S	WT	WT	WT	WT	WT
H727	WT	WT	WT	G12V	WT	WT	WT	WT	Q165S
H1299	WT	WT	WT	WT	WT	WT	WT	WT	del
H1437	WT	WT	WT	WT	WT	WT	WT	WT	R267P

and tried to correlate the *in vitro* activity with the molecular alterations present in the cells used.

Materials and methods

Cells, drugs and cytotoxicity assays

The human NSCLC cell lines used in these studies were: A549, NCI-H23, NCI-H358, NCI-H460, NCI-H596, NCI-H727, NCI-H1299, NCI-1437, NCI-H1975, obtained from ATCC and LU-99 obtained by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The medium used to culture all the NSCLC cell lines was RPMI1640 supplemented with 10% FBS. Cells were routinely tested for the presence of mycoplasmas and authenticated with the PowerPlex 16 HS System (Promega) every six months by comparing the STR profiles with those deposited in different databases.

The MTS assay (Promega) was used to determine the activity of drugs *in vitro* as described [19]. The drugs used in the study were obtained from Selleckchem: PIK-75, BKM-120, BEZ-235, TORIN-1, MEK-162 and SCH772984. All drugs were dissolved in DMSO as stock solutions and further diluted in culture medium. For each experiment different concentrations of the drugs were used and the percentage of absorbance relative to untreated cells was calculated for each drug concentration (six replicates for each concentration). From these percentages, we derived the concentrations dependent curves. Each graph reports the mean and SD of at least three independent experiments. Concentrations inhibiting the growth by 50% (IC50) were calculated from the curves using Graphpad Prism Version 7.

Drug sensitivity data were retrieved from the Genomics of Drug Sensitivity in Cancer database (www.cancerrxgene.org/). The scatter plots showing the IC50 of NSCLC cell lines harboring wild type (wt) or mutated p53 were generated via the Genomics of Drug Sensitivity in Cancer database online platform.

Western blotting analyses

Proteins were extracted from exponentially growing cells and visualized as described [19]. Immunoblotting was carried out with the following antibodies: anti-S6 (Ser235/236) ribosomal protein #2211, anti-S6 ribosomal protein #2217, anti-4EBP1 #9644, anti-4EBP1 (Thr 37/46) #2855, anti-p70S6K #9202, anti-p70S6K (Thr 389) #9206 provided by Cell Signalling Technology. Anti-ERK #sc94, anti-ERK (Tyr204) #sc7383, were obtained from Santa Cruz Biotechnology.

Results

The major characteristics of the 10 cell lines used in this study are reported in **Table 1**. Considering the mutational status of KRAS and PI3K (*PIK3CA* gene) there were four cell lines with *KRAS* mutation, two with *PIK3CA* mutation, two with both *KRAS* and *PIK3CA* mutations and two wt. All the cell lines were wt for PTEN, AKT1 and 2, BAF, ERK and mTOR. One cell line only (H1975) had mutation in *TSC2* gene, while 6 out of 10 present a mutation in the *p53* gene. We tested, in this genetic background, the in vitro cell growth inhibitory activity of drugs inhibiting the PI3K/mTOR pathway and of drugs interfering with the RAS/RAF/MEK/ERK pathway.

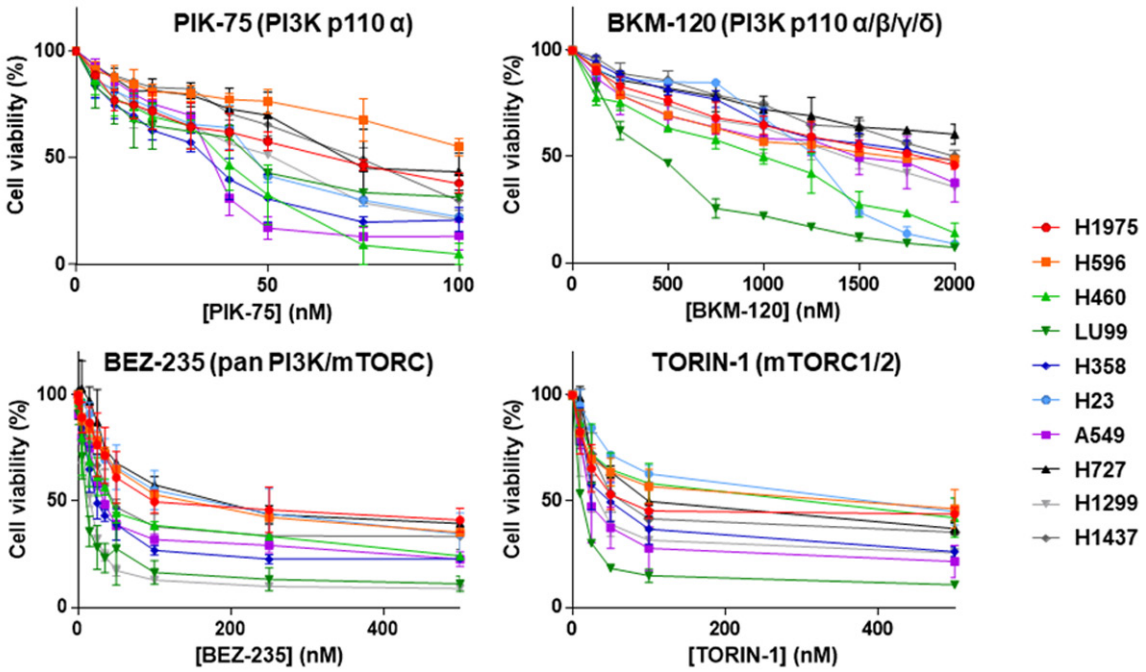

Figure 1. Activity of PIK-75, BKM-120, BEZ-235 and TORIN-1 in 10 NSCLC cell lines. Each graph reports the concentration dependent inhibition curves in the different cells. The values represent the mean +/- SD of three independent experiments, each consisting of six replicates.

Figure 1 reports the concentration versus growth inhibition curves of the 4 PI3K/mTOR pathway inhibitors tested in the panel of NSCLC cell lines.

As schematically represented in **Figure 2A**, we could not detect any obvious correlation between their activity and the mutational status. In particular, the two cell lines harboring *PIK3CA* mutation (H1975 and H596) were unexpectedly among the most resistant to the treatment with the alpha isoform specific inhibitor PIK-75. The same was true for the pan PI3K inhibitor BKM-120, for the double PI3K/mTOR inhibitor BEZ-235 and for the mTOR inhibitor TORIN-1. No correlations were observed even considering the two additional cell lines harboring both *PIK3CA* and *KRAS* mutations.

Interestingly, the cytotoxic profile of the dual PI3K/mTOR inhibitor was more similar to the profile of the mTOR inhibitor than to the one of the PI3K inhibitor. We noticed, however, that all the four drugs showed a preferential activity towards cell lines expressing a wt p53 (**Figure 2B**).

The role of the p53 status was further investigated in the Genomics of Drug Sensitivity in Cancer database (https://www.cancerrxgene.org/) where about 1000 human cancer cell lines were screened with almost 400 compounds. Both BEZ-235 and BKM-120 were among the tested drugs while PIK-75 and TORIN-1 were not tested. We therefore selected different drugs but with the same targets of PIK-75 (Apelisib, PI3K p110α) and TORIN-1 (AZD2014, mTORC1/2). The analysis has been restricted to lung cancer cell lines. As shown in Supplementary Figure 1, BEZ-235 and Apelisib showed a preferential activity (although not statistically significant) in cells with a mutated *p53* (as experimentally observed in the NSCLC panel used). On the other hand, BKM-120 and AZD2014 were active independently from *p53* status.

Analyzing the effect of the four drugs on the level and phosphorylation status of the proteins involved in the pathway, we observed that, although at different level, all the compounds were able to act on their target in all the cell lines (**Figure 3**). In both sensitive and less sensitive cells, at least at the concentrations used, a change in the phosphorylation status was found for p70S6K, S6 and 4EBP1 proteins. A decrease in the phosphorylation status of ERK was also detected for all the compounds in all the cell lines irrespectively on the growth inhibitory activity of the drugs.

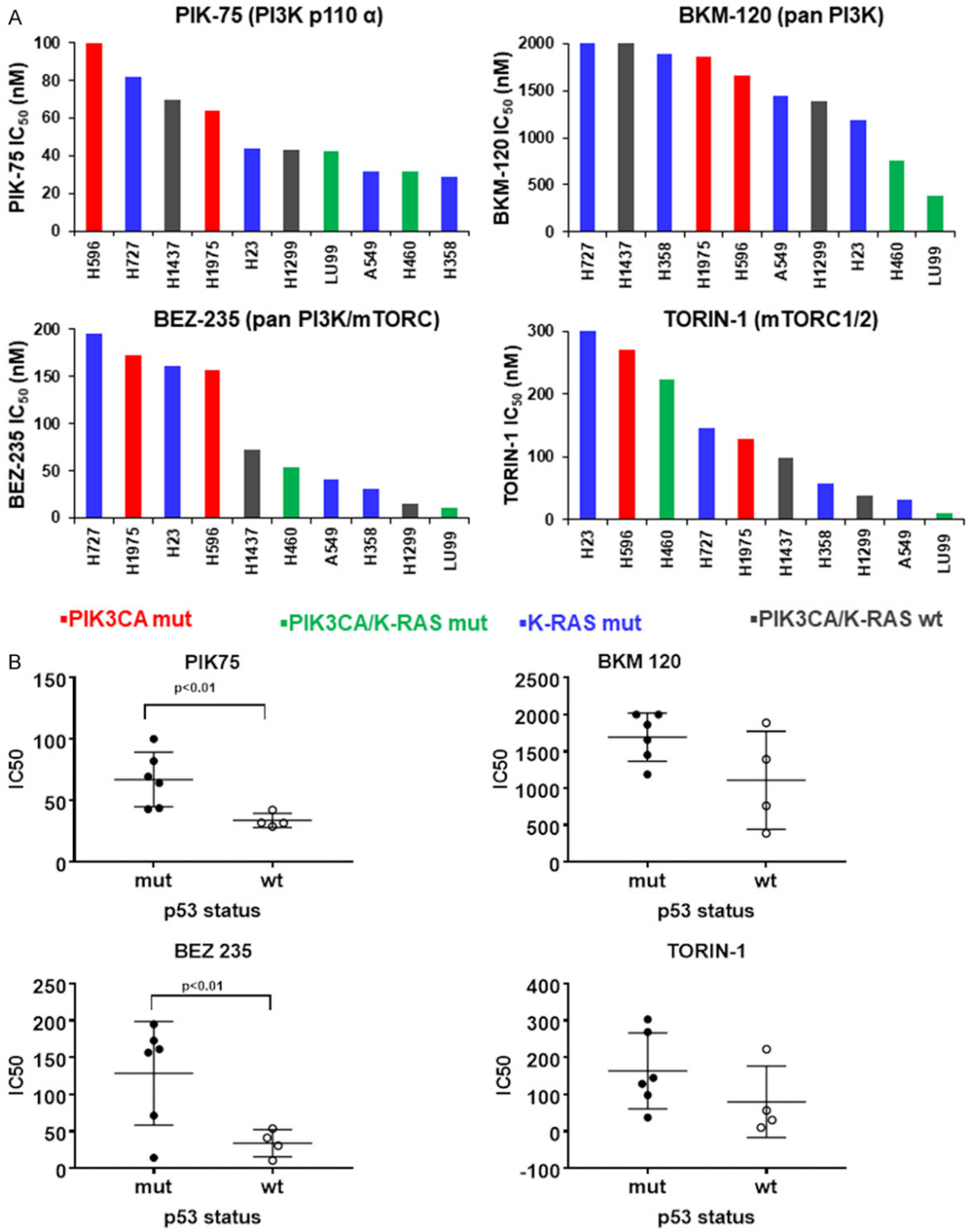

Figure 2. A. Correlation between the IC50 values calculated for each drug in each cell line and the mutational status. Each colour identifies a specific mutational status in the PI3K/KRAS pathways. B. *p53* status (mutant or wt) and response to treatment reported as IC50 in the different cell lines.

We then tested two inhibitors of the RAS/RAF/MEK/ERK pathway, namely MEK-162, inhibitor of MEK, and SCH772984, inhibitor of ERK, on the same cell lines. Quite surprisingly and unexpectedly, we found that all the cell lines but one (H727) were extremely resistant to these two

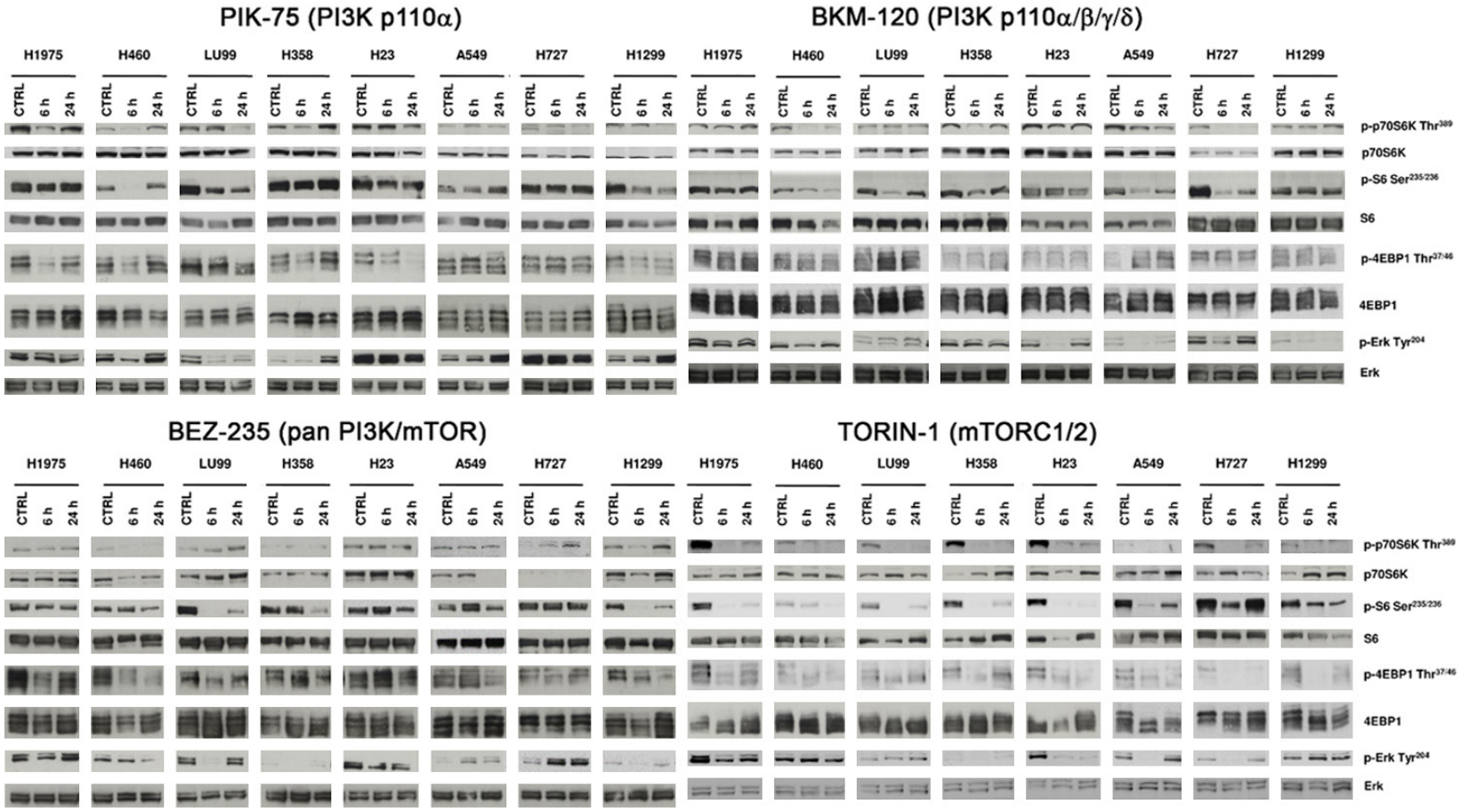

Figure 3. Representative western blot analysis showing the ability of the four PI3K/mTOR pathway inhibitors to modify the phosphorylation of proteins involved in the pathways transduction in eight NSCLC cell lines. Proteins were extracted 0, 6 and 24 hours after treatment start. Cells were treated with a concentration corresponding to the IC50 of the drug determined in the most sensitive cell line. The different cell lines were run on different gels.

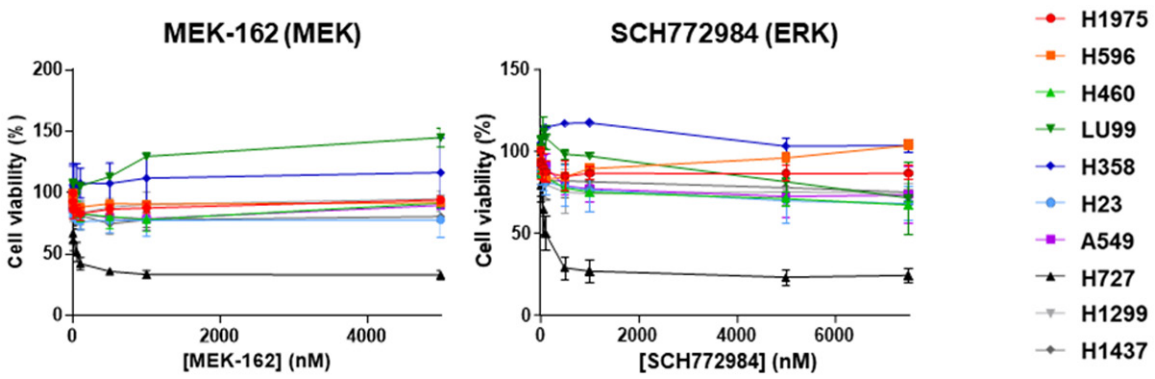

Figure 4. Activity of MEK-162 and SCH772984 in 10 NSCLC cell lines. Each graph reports the concentration dependent inhibition curves in the different cells. The values represent the mean +/- SD a three independent experiments, each consisting of six replicates.

drugs (**Figure 4**). For these compounds, we could not prove/find any correlation with the mutational status in both PI3K and KRAS pathways. The status of *p53* was also not able to influence (differently from what observed with the PI3K/mTOR inhibitors) the response of these cells to these inhibitors. When we checked the effects of the two inhibitors on the proteins involved in the different pathways, we found that for both MEK-162 and SCH772984 there was a clear inhibition of ERK phosphorylation in all the cell lines (**Figure 5**), indicating that the two drugs reached and inhibited their target in all the cell lines. The only difference we found between the sensitive cell line H727 and all the other nine cell lines, was possibly the ability of both inhibitors to induce a decrease in the phosphorylation level of S6 in H727 cells, a decrease that was not appreciable in the other cells investigated.

Discussion

The knowledge that the PI3K/mTOR pathway is one of the most altered in human cancer [5-7], prompted the design and generation of several inhibitors of the major proteins involved in the pathway. Since the class I PI3K (the most altered in cancer) is present in different isoforms, both isoform selective and pan inhibitors have been designed [5, 15, 20, 21]. In addition, several dual inhibitors, able to inhibit both the PI3K and mTOR activity, have been identified and widely studied [22, 23]. Inhibitors of the pathway have been studied in the clinic for long time in several tumors and overall their efficacy has been below the expectancies [24, 25]. There are several factors contributing to the underwhelming behavior of the PI3K inhibitors among which there are intrinsic or acquired resistance, tumor heterogeneity at molecular level and the undesired effects mostly associated with the off-target effects of the drugs [15, 24, 26-29].

A clear biomarker able to identify patients more likely to respond to PI3K inhibitors has not been found. The presence of mutations in the *PIK3CA* gene encoding for the p110 subunit of PI3K does not seem, for example, to play any role in the response to the pan inhibitor GSK 458 [18]. Similar data have been obtained using other compounds in NSCLC or colorectal cancer cells [30, 31]. Our data obtained *in vitro* confirm the lack of correlation between the presence of mutations in the *PIK3CA* gene and the response to isoform specific, pan PI3K or dual (PI3K/mTOR) inhibitors of the pathway. The lack of correlation does not depend on the ability of the compounds to reach and inhibit the target. We have in fact demonstrated that all the drugs in all the cell lines, independently from their ability to inhibit the growth, effectively decrease the phosphorylation of proteins downstream to the PI3K. Recently it has been reported that the presence of double mutations in the *PIK3CA* gene could increase the sensitivity to PI3K alpha inhibitors [32]. This is a potentially important information that could help in better stratifying patients. Unfortunately, in the panel of NSCLC cell lines we used, no one presents a double mutation in the *PIK3CA* gene.

The only clear marker of response in our panel of NSCLC cell lines remains p53. The fact that cells expressing a wt *p53* show increased

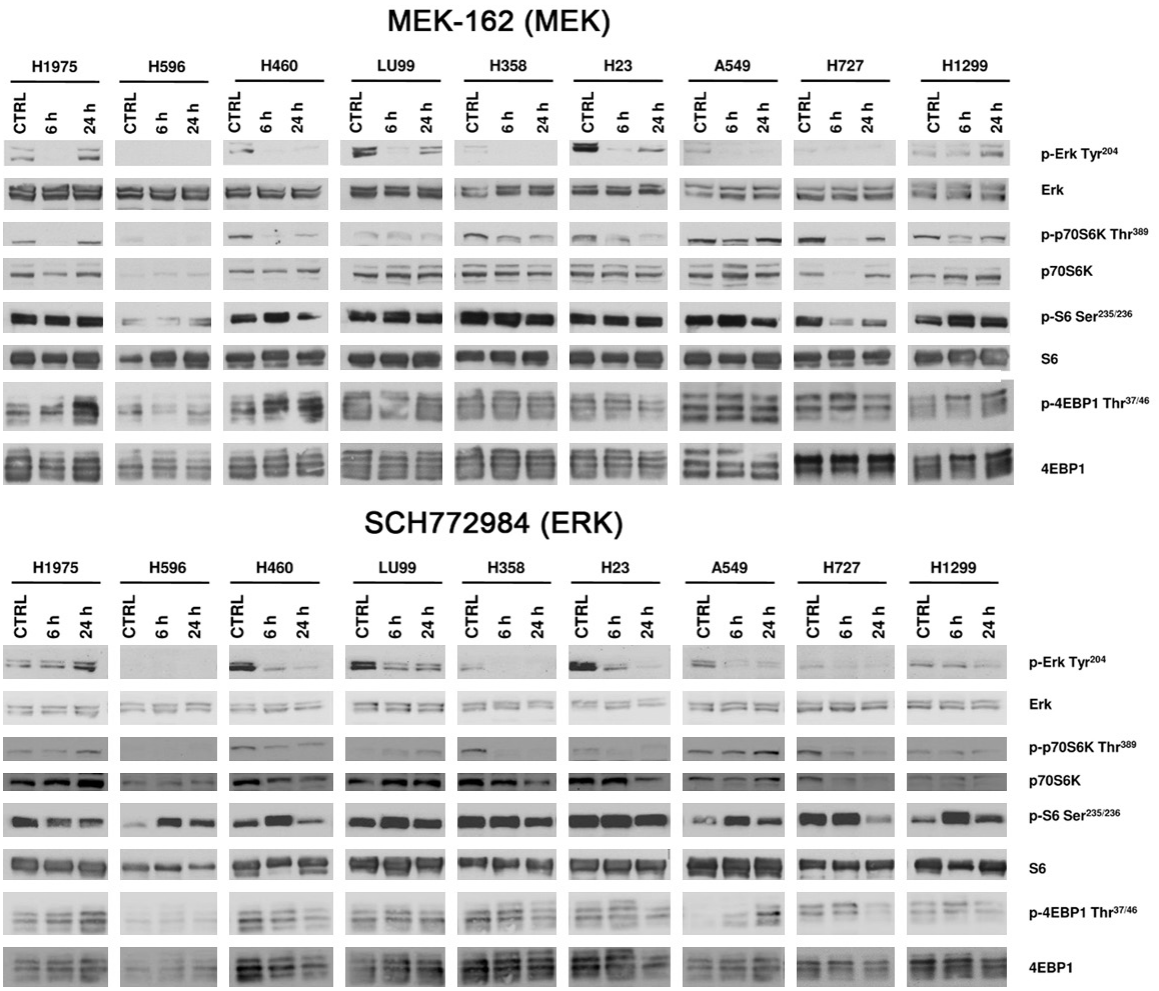

Figure 5. Representative western blot analysis showing the ability of MEK-162 and SCH772984 to modify the phosphorylation of ERK and other proteins as indicated in the figure in eight cell lines 6 and 24 hours after treatment start. Cells were treated with a concentration corresponding to the IC50 of the drug determined in the most sensitive cell line. The different cell lines were run on different gels.

response to the different PI3K inhibitors could potentially be an additional reason for the low therapeutic index of the clinically tested inhibitors, particularly in NSCLC were the fraction of patients with mut *p53* is particularly high [33]. A role for p53 (through the activation of p21) in the activity of BEZ-235, one of the compounds used here, was recently postulated in thyroid cancer cells [34] and indirectly by the evidence that restoration of p53 can enhance the activity of everolimus in hepatocellular and NSCLC cell lines [35]. p53 was also shown to be determinant for the activity of another PI3K/mTOR inhibitor (PF-04691502) in Head and Neck cancer cell lines [36]. We checked for additional alterations in genes belonging to the p53 pathway (according to the KEGG p53 signaling pathway) in our cell lines. We found that among the 68 genes analysed no one correlated with the response in vitro (Supplementary Figure 2) thus enforcing the central role of p53 in our panel.

We also tried to verify whether the panel of NSCLC cell lines could have a different response to the inhibition of the parallel pathway RAS/RAF/MEK/ERK. Strikingly, we found that 9 out of 10 cell lines were resistant to MEK and ERK inhibitors. This finding was not limited to the use of the inhibitors MEK-162 or SCH772984, as we have obtained the same results using different inhibitors such as ulixertinib (data not shown) suggesting that this finding is not drug specific but rather class specific. We do not have a clear explanation for these results, particularly considering that in all the cell lines and for all the drugs a clear decrease in ERK phos-

phorylation was achieved, thus implying that the lack of activity is not due to a reduced ability to reach or inhibit the target. We recently showed that LKB1 is a potential marker of response to ERK inhibitors in NSCLC cells, a finding obtained using isogenic pair of cells lacking or not LKB1 expression [37, 38]. In this relative broad panel of cell lines, LKB1 alone does not seem to predict response to ERK inhibitors, although we know that *LKB1* mutation is required but not sufficient for the activity of these inhibitors [37]. Recent evidence suggests that combination of inhibitors acting on the same pathways can have high activity in NSCLC cell lines [39, 40] and this could in part help in explaining the lack of a direct correlation between the activity of targeted inhibitors as single agents and the presence of target alterations in the tumor.

In conclusion, we showed here that *p53* but not *PIK3CA* mutational status could influence the response to PI3K/mTOR inhibitors. Unfortunately, we could not retrieve clinical data for NSCLC patients treated with PI3K/mTOR inhibitors (due to the limited data available), an information that would have strengthened our preclinical data. The search for potential biomarkers able to identify those patients likely to respond is crucial for this class of drugs whose potential as anticancer agents is enormous and not yet fully exploited.

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Disclosure of conflict of interest

None.

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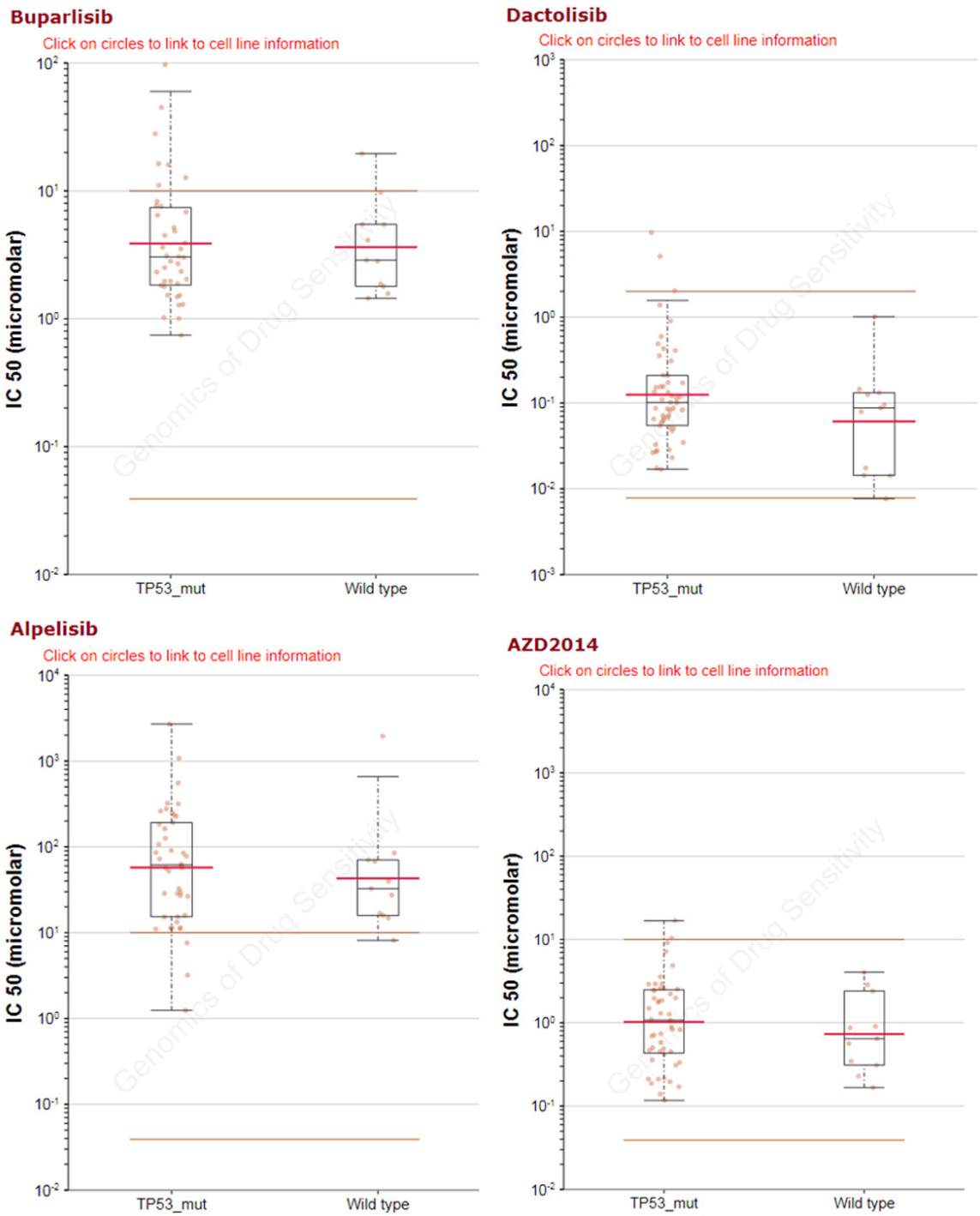
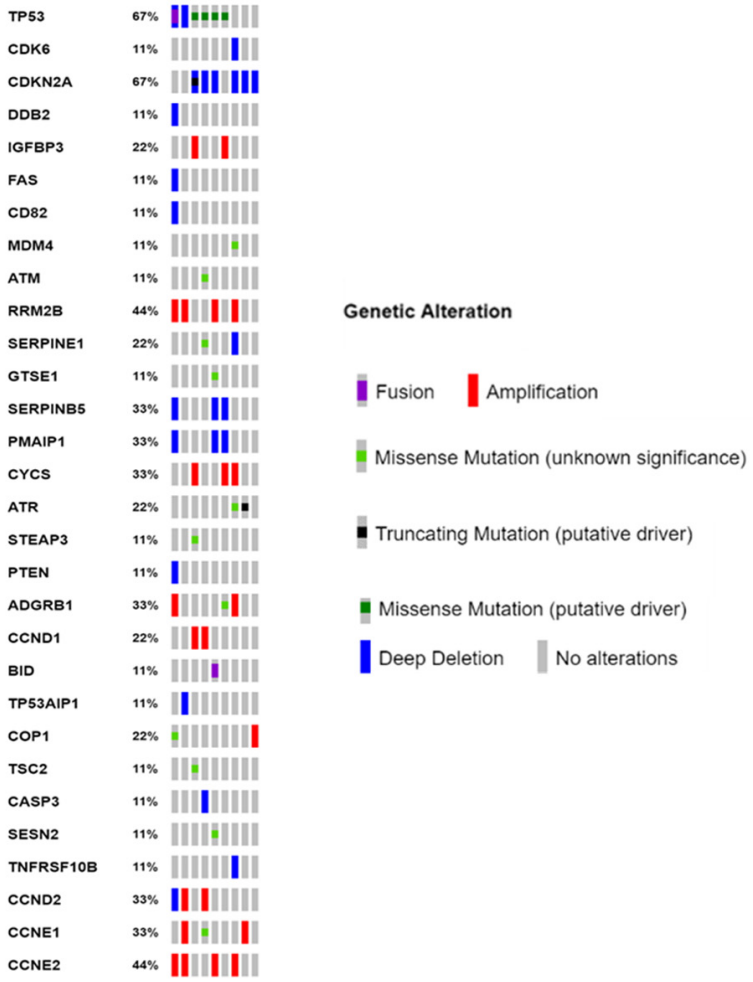


Supplementary Figure S1. Scatter plots showing the IC50 of NSCLC cell lines harboring wt or mutated *p53*.



Supplementary Figure S2. Graphical representation of the cell lines mutational status of genes belonging to the p53 pathway. In addition to those reported in the figure, the following were found not mutated in all cell lines: *CDK2, CDK4, CDKN1A, GADD45G, CHEK1, CHEK2, SESN3, GADD45A, RCHY1, BBC3, SESN1, SFN, APAF1, IGF1, MDM2, GADD45B, SHISA5, PIDD1, RPRM, BAX, RRM2, PERP, ZMAT3, SIAH1, THBS1, TP73, CASP8, CASP9, PPM1D, CCNB3, CCNB1, CCND3, CCNG1, CCNG2, CCNB2, EI24, TP53I3, CDK1*.