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Alterations in the PI3K Pathway Drive Resistance to MET Inhibitors in NSCLC Harboring MET Exon 14 Skipping Mutations

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1 **Title**

2 Alterations in the PI3K pathway drive resistance to MET inhibitors in NSCLC
3 harboring MET exon 14 skipping mutations

4 **Short title**

5 PI3K pathway alterations drive resistance to MET TKIs in METex14 NSCLC

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49

50 Abstract

51 **Introduction**

52 MET TKIs (tyrosine kinase inhibitors) have demonstrated efficacy against advanced
53 NSCLC with mutations causing MET exon 14 skipping (*METex14* mutations), but
54 primary resistance seems frequent, as response rates are lower than for targeted
55 TKIs of other oncogene-addicted NSCLC. Given the known interplay between MET
56 and PI3K, we hypothesized that in *METex14* NSCLC, PI3K-pathway alterations might
57 contribute to primary resistance to MET TKIs.

58 **Methods**

59 We reviewed clinical data from 65 patients with *METex14* NSCLC, assessing PI3K-
60 pathway alterations by targeted NGS (mutations) and immunohistochemistry (loss of
61 PTEN). Using a cell line derived from a patient with primary resistance to a MET TKI
62 and cell lines harboring both a *METex14* mutation and a PI3K-pathway alteration, we
63 assessed sensitivity to MET TKIs used alone or with a PI3K inhibitor and investigated
64 relevant signaling pathways.

65 **Results**

66 We found a *PIK3CA* mutation in 2/65 samples (3%) and loss of PTEN in 6/26 (23%).
67 All three of the MET-TKI-treated patients with a PI3K pathway alteration had shown
68 progressive disease at first assessment. Likewise, MET TKIs had no effect on the
69 proliferation of *METex14*-mutated cell lines with a PI3K-pathway alteration, including

70 the PTEN-lacking patient-derived cell line. Treatment combining a MET TKI with a
71 PI3K inhibitor caused inhibition of both PI3K and MAPK signaling and restored
72 sensitivity to MET TKIs.

73 **Conclusion**

74 PI3K-pathway alterations are common in *MET*^{ex14} NSCLC and may confer primary
75 resistance to MET TKIs. In preclinical models, PI3K inhibition restores sensitivity to
76 MET TKIs.

77

78 **Introduction**

79 Splice site mutations located in the regions flanking exon 14 of the MET oncogene
80 (*METex14*) have been reported in 3% of patients with non-small cell lung cancer
81 (NSCLC) ^{1,2}. Elderly persons and smokers constitute a larger proportion of patients
82 harboring these mutations than of patients with alterations affecting other key
83 oncogene drivers such as EGFR or ALK³. *MET* exon 14 skipping, causing the
84 absence of the MET juxtamembrane domain (containing several negative regulation
85 sites), leads to increased MET receptor stability and activation⁴. This activation
86 results in recruitment of effector proteins such as GAB1, GRB2, SHC, STAT, and
87 PI3K, leading to activation of downstream signaling pathways including the PI3K,
88 MAPKinase, and JAK-STAT pathways ^{4,5}. As *METex14* mutations can confer
89 sensitivity to MET inhibition, several clinical trials have evaluated MET tyrosine
90 kinase inhibitors (TKIs) in *METex14* NSCLC. Reported objective response rates
91 (ORRs) range from 32% to 67.9% ⁶⁻⁹. These results are somewhat lower than
92 observed with targeted therapies for other oncogene-addicted NSCLC, such as
93 *EGFR*-mutated or *ALK*-rearranged NSCLC. This may be due to a higher frequency of
94 primary resistance mechanisms in *METex14* NSCLC, including concurrent molecular
95 alterations.

96 The PI3K signaling pathway is involved in several cell functions induced by activation
97 of the MET receptor, including cell migration and survival¹⁰. The MET receptor
98 interacts with class I PI3K isoforms directly through their SH2 domain and indirectly
99 through activation of GAB1, FAK, and RAS¹⁰. PI3K promotes phosphorylation of
100 PIP2 to PIP3, favoring plasma membrane recruitment of the PDK1 kinase, leading
101 notably to AKT phosphorylation. The PTEN phosphatase (Phosphatase and TENsin
102 homolog), on the other hand, causes PIP3 dephosphorylation and thus acts as the

103 main regulator of the PI3K pathway. The PI3K pathway is frequently dysregulated in
104 NSCLC. *PIK3CA* and *AKT1* mutations are found, respectively, in 4.1-5.2%¹¹⁻¹³ and
105 0.2-1.9%¹⁴⁻¹⁶ of lung cancers, and loss of PTEN has been reported in 24% of
106 NSCLC. Several papers report the role of PI3K pathway alterations in resistance to
107 cancer therapy, including TKI treatment^{17,18}. Given the role of the MET receptor in
108 direct activation of the PI3K pathway, we hypothesized that alterations in this
109 pathway might contribute to the resistance of *METex14* NSCLC to MET inhibitors.

110

111 **Results**

112 **PI3K pathway alterations are common in *METex14* NSCLC**

113 Considering the involvement of the PI3K pathway in resistance to TKIs in several
114 oncogene-addicted cancer models, we focused retrospectively on PI3K-pathway
115 alterations in a multicentric cohort of NSCLC patients whose tumors displayed
116 *METex14* mutations. All tumor samples were obtained at diagnosis, before any
117 systemic treatment. Screening for mutations had been done using the CLAPv1
118 optimized next-generation sequencing (NGS) panel combined with fragment length
119 analysis, as previously described². All these mutations were previously described or
120 predicted to induce MET exon14 skipping (**Table S1**). The median age was 75.0
121 years (range 49-88); there were as many men as women (49%) and as many
122 smokers as never-smokers (55%). Most patients had been diagnosed at an
123 advanced stage (67%). Seventeen of them had received a MET TKI, either off-label
124 or as part of a clinical trial (**Figure 1A**).

125 The *METex14* mutations detected in this cohort were of the following types: deletions
126 in 30 patients (46%), substitutions in 28 patients (42%), and indels in 7 patients

127 (12%). Thirty-nine samples had been tested for *MET* gene amplification by FISH, of
128 which 5 (13%) were positive: one with low amplification (*MET/CEP7* ratio 1.8), one
129 with intermediate amplification (*MET/CEP7* ratio 3.2), and three with high
130 amplification (clusters in 53-78% of tumor cells) (**Table S1**).

131 Samples from all 65 patients had been tested for PI3K pathway alterations (*PIK3CA*,
132 *AKT*, and *PTEN* mutations) by targeted NGS, revealing two *PIK3CA* mutations (3%)
133 (**Figure 1B**) but no *AKT* or *PTEN* mutation. We further evaluated *PTEN* expression
134 by immunohistochemistry (IHC) performed on 26 available tumor samples (**Table**
135 **S3**). This revealed 6 samples (23%) with total loss of *PTEN* expression (**Figure 1C**
136 **and S1**).

137 In addition to the above, the observed mutations included 15 *TP53* mutations (27%),
138 two *KRAS* mutations (G13C), one *NRAS* mutation (Q61L), one *EGFR* mutation
139 (V774M), and one *SMAD4* mutation (D351N) (**Table S1**). Overall, six of the 26
140 samples tested by both NGS and *PTEN* IHC (23%) displayed a PI3K-pathway
141 alteration. This makes the PI3K pathway one of the most altered pathways in
142 *METex14* NSCLC.

143 **METex14 patients with concurrent alterations in the PI3K pathway display**
144 **primary resistance to MET TKIs**

145 To determine whether PI3K-pathway alterations might impact sensitivity to *MET*
146 inhibition, we analyzed responses to treatment in the 17 *METex14* NSCLC patients
147 having received a *MET* TKI.

148 Tumor samples from three of these patients appeared, on the basis of either NGS or
149 *PTEN* IHC, to have an impaired PI3K pathway (**Figure 1D**).

150 In 14 patients, no alteration of the PI3K pathway was detected. This means that NGS
151 revealed no *PIK3CA*, *AKT*, or *PTEN* mutation and that the corresponding tumor
152 samples were either PTEN positive (4 samples) or had not been analyzed by PTEN
153 IHC (10 samples). Of these 14 patients, nine displayed an objective response (64%),
154 one achieved disease stability for six months, and four showed progressive disease
155 at first assessment. Although none of these last four harbored a *PIK3CA* mutation,
156 one of them, identified as PTEN-positive, harbored an *EGFR* V774M mutation likely
157 to explain primary resistance to MET TKI. The PTEN status of the other three was
158 not available. The three PTEN-positive patients with no *EGFR*, *KRAS*, or *TP53*
159 mutation all responded to MET TKI treatment.

160 In contrast, all three patients with an impaired PI3K pathway, including one with a
161 *PIK3CA* mutation and two with total loss of PTEN expression, showed progressive
162 disease at first radiological assessment (**Figure 1 D, E**). The clinical courses of these
163 three patients are detailed below.

164 Patient CHUL#11, harboring a c.3082+1G>T MET mutation, was a 49-year-old
165 former-smoker woman diagnosed with stage IV NSCLC. She received first-line
166 platinum-based chemotherapy, but progression was observed after four cycles of
167 treatment. The patient then received a MET TKI as part of a clinical trial. Treatment
168 was stopped at first radiological assessment because of rapid disease progression
169 (**Figure 1E**). The patient further received an anti-PD1 agent followed by a new
170 course of chemotherapy with no efficacy. She died nine months after diagnosis.
171 PTEN IHC performed on a right cervical lymph node obtained before treatment with
172 the MET TKI revealed loss of PTEN expression (**Figure 1C**).

173 Patient CHUL#18, harboring a c.3067_3082+1delinsC MET mutation, was a 75-year-
174 old woman diagnosed with advanced adenocarcinoma. She received six cycles of
175 platinum-based chemotherapy with maintenance pemetrexed for one year. She then
176 received a MET TKI as part of a clinical trial. The treatment was stopped at first
177 radiological assessment because of new bone and adrenal gland metastases
178 (**Figure 1E**). The patient died three months later. PTEN IHC performed on a
179 pretreatment bronchial biopsy revealed loss of PTEN expression (**Figure S1**).

180 Patient CHUL#20 harboring a c.3082+1G>A MET mutation was a 75-year-old
181 woman diagnosed with metastatic pulmonary sarcomatoid carcinoma. The patient
182 was not eligible for chemotherapy and therefore received a MET TKI as first-line
183 therapy. The treatment was stopped at first radiological assessment because of
184 lymph node progression (**Figure 1E**). The patient died within two weeks after
185 discontinuing treatment. Targeted NGS performed on the lung tumor revealed an
186 activating *PIK3CA* mutation (N1044K).

187 These three cases suggest that in *METex14* NSCLC patients, alterations in the PI3K
188 pathway are associated with primary resistance to MET TKIs.

189 **PTEN loss is associated with sustained activation of the PI3K pathway and**
190 **resistance to MET TKIs in *METex14* cancer cells**

191 To decipher the role of PI3K-pathway alterations in resistance to MET TKIs, we
192 derived a cell line from a pleural effusion sample from patient CHUL#11, collected at
193 progression on third-line therapy. The tumor sample from this patient harbored a
194 *METex14* mutation and displayed no PTEN expression detectable by IHC (**Figure**
195 **1C**). Targeted NGS confirmed in this cell line (named ZORG) the presence of the
196 *METex14* mutation c.3082+1G>T, identical to that found in the patient tumor sample.

197 As expected, the mutation leads to exon 14 skipping, as validated by fluorescence
198 RT-PCR with primers located in MET exons 13 and 15. As shown in **Figure 2A**, the
199 amplicon amplified from ZORG cells migrated as expected to 128 bp, as observed
200 with cell lines known to harbor *METex14* mutations (Hs746T and H596). The
201 amplicons from MET wild-type cell lines (A549, PC9, and GTL16) migrated to 286 bp.
202 In addition, the dissociation temperature of the amplicon from ZORG cells (74.8°C)
203 was in agreement with that of MET lacking exon 14. Finally, western blotting revealed
204 a lack of PTEN in ZORG cells (**Figure 2B**).

205 MET expression and downstream signaling were investigated by western blotting in
206 ZORG cells stimulated or not by the MET ligand HGF (Hepatocyte Growth Factor).
207 As shown in **Figure 2C**, unstimulated *METex14* and ERK displayed no
208 phosphorylation, while HGF stimulation induced MET phosphorylation and
209 subsequent activation of ERK. In contrast, as an expected consequence of PTEN
210 loss, AKT was activated by its ligand even in the absence of MET activation.
211 Stimulation by HGF led to overactivation of the PI3K pathway. All further experiments
212 were performed under HGF supplementation.

213 The sensitivity of ZORG cells to MET TKIs was then analyzed in viability assays
214 using MTT. Four MET TKIs (the type-I inhibitors crizotinib, capmatinib, and tepotinib
215 and the type-II inhibitor foretinib) were tested at increasing doses. The viability of
216 ZORG cells was practically unaffected by capmatinib or tepotinib at doses as high as
217 10 μ M Crizotinib (a multitarget inhibitor of MET, ROS, and ALK) and foretinib
218 displayed low viability-reducing activity on ZORG cells, with a half-maximal inhibitory
219 concentration (IC₅₀) above 1 μ M following a 72-h exposure to the drug. By
220 comparison, all four TKIs reduced the viability of Hs746T cells, known to harbor both
221 *METex14* mutation and amplification, with an IC₅₀ below 0.1 μ M (**Figure 2D**).

222 Investigation of signaling pathways by western blotting showed all four MET TKIs to
223 inhibit MET phosphorylation in both ZORG and Hs746T cells. MET TKIs nearly
224 abolished both AKT and ERK phosphorylation in Hs746T cells, but had little effect in
225 ZORG cells (**Figure 2E**). Overall, these results suggest that PTEN loss is associated
226 with resistance to type-I and type-II MET TKIs in *METex14* cells through sustained
227 activation of the PI3K pathway.

228

229 **Inhibition of PI3K restores sensitivity to MET TKIs in *METex14* cells showing**
230 **PTEN loss**

231 To further demonstrate the role of PI3K-pathway alterations in resistance to MET
232 TKIs, we tested whether PI3K inhibition might restore sensitivity of ZORG cells to
233 MET TKIs. Proliferation of ZORG cells was unaffected by treatment with capmatinib,
234 one of the most selective and potent MET TKIs¹⁹, and was only slightly slowed down
235 by GDC0941, a specific inhibitor of PI3K α/δ (**Figure 3A**). When these two inhibitors
236 were combined, however, proliferation of ZORG cells was significantly reduced. The
237 same inhibitor combination had no effect on cell lines with no MET alteration, such as
238 A549 (*KRAS* G12S mutation) and PC9 (EGFR E746-A750 deletion). For ZORG cells,
239 the IC₅₀ for capmatinib shifted from over 10 μ M, when the inhibitor was used alone,
240 to 0.034 μ M, when it was used in combination with GDC0941 (1 μ M) (**Figure 3B**).

241 Analysis of the downstream signaling pathway revealed that capmatinib or GDC0941
242 added alone only slightly reduced the phosphorylation levels of ERK and AKT,
243 whereas the combination of both inhibitors fully abolished AKT activation and
244 drastically inhibited ERK activation (**Figure 3C**). Overall, these results demonstrate
245 that ZORG cells, which display both a *METex14* mutation and PTEN loss, rely on

246 both the PI3K and MET pathways for cell growth, and that combining a MET inhibitor
247 with a PI3K inhibitor is required to achieve cell growth arrest.

248

249 **Inhibition of PI3K restores sensitivity to MET TKIs in *METex14* cells displaying**
250 **a *PIK3CA* mutation**

251 Given the action of the PI3K-MET inhibitor combination on *METex14* cells with
252 concurrent PTEN loss, we investigated whether the same is true when PI3K
253 activation is due to a *PIK3CA* mutation. For this we used the lung adenocarcinoma
254 cell line H596, known to harbor the activating E545K *PIK3CA* mutation along with a
255 *METex14* mutation.

256 We first confirmed in a viability assay that H596 cells are resistant to crizotinib, as
257 previously shown⁵, and to the MET TKIs capmatinib, tepotinib, and foretinib (**Figure**
258 **4A**). We then studied the effect of MET and PI3K inhibitors, added alone or in
259 combination, on cell growth. Like that of ZORG cells, H596 cell proliferation was
260 unaffected by capmatinib alone. It was slowed down in the presence of GDC0941
261 alone. In the presence of both inhibitors combined, full cell growth arrest was
262 achieved (**Figure 4B**).

263 Signaling pathway analysis revealed only a slight effect of capmatinib on AKT and
264 ERK activation. When the MET and PI3K inhibitors were combined, the levels of both
265 phospho-AKT and phospho-ERK decreased markedly (**Figure 4C**). For H596 cells,
266 the IC₅₀ for capmatinib shifted from over 10 μ M, when the inhibitor was used alone,
267 to 0.06 μ M, when it was used in combination with GDC0941 (**Figure 4D**). Taken
268 together, these results demonstrate that inhibition of both MET and PI3K is required

269 to achieve full cell growth arrest and to suppress activation of the PI3K and
270 MAPKinase pathways in *METex14* cells harboring a concurrent *PIK3CA* mutation.

271

272 ***METex14* mutations contribute to resistance to PI3K inhibition in *PIK3CA*-**
273 **mutated cells**

274 Since PI3K-pathway alterations confer resistance to MET TKIs in *METex14* cells, we
275 investigated whether *METex14* mutations might similarly confer resistance to a PI3K
276 inhibitor in *PIK3CA*-mutated cells.

277 For this purpose, we chose the epithelial cell line T47D, harboring a H1047F-
278 activating *PIK3CA* mutation and expressing low levels of endogenous MET. To
279 observe the specific effect of *METex14* on the biological responses of such cells, we
280 created the T47DMETex14 cell line by stable transfection with a vector expressing a
281 mutant MET (*METex14*, c. 2887_3028del).

282 First we confirmed the presence of the exogenous *METex14* in the T47DMETex14
283 cells and its absence in the parental T47D cells (**Figure S2A**). We then evaluated the
284 sensitivity of these cells to MET TKIs. We found both T47D and T47DMETex14 cells
285 to be resistant to all tested MET TKIs (**Figure S2B**).

286 We further studied the effect of MET and PI3K inhibitors, alone and in combination,
287 on cell proliferation. In T47D cells, we found GDC0941 alone to suppress cell growth.
288 Adding capmatinib had no additional impact. Proliferation of these cells thus appears
289 to depend strictly on the PI3K pathway (**Figure 5A**). In contrast, treating
290 T47DMETex14 cells with GDC0941 only slowed down cell growth. Capmatinib alone
291 slightly increased cell growth. To achieve full cell growth arrest, combining GDC0941

292 with capmatinib was required. This suggests that proliferation of these cells relies on
293 both the PI3K and MET pathways.

294 Analysis of the signaling pathways revealed that PI3K activation depends partly on
295 MET activation in T47DMETex14 cells but not in T47D cells. ERK activation was
296 found to depend strictly on MET activation. It proved necessary to combine MET and
297 PI3K inhibitors to fully inhibit both AKT and ERK activation (**Figure 5B**). For
298 T47DMETex14 cells, the IC50 for capmatinib shifted from over 10 μ M, when the
299 inhibitor was used alone, to 0.007 μ M, when it was used in combination with
300 GDC0941 (**Figure 5C**).

301 Taken together, these results demonstrate that *METex14* can induce MET addiction
302 even in the presence of a concurrent oncogene mutation such as a *PIK3CA*
303 mutation, and that this leads to resistance to a MET or PI3K inhibitor used alone.
304 Combined inhibition thus appears required in the case of concurrent alterations
305 activating MET and a second signaling pathway.

306

307 **Discussion**

308 *METex14* mutations define a new subgroup of NSCLC patients who may benefit from
309 targeted therapies against MET. Several MET TKIs have demonstrated efficacy in
310 ongoing clinical trials for patients with *METex14* NSCLC, with a 32-67.9% ORR rate
311 and a 7.3-10.8 months median PFS⁶⁻⁹. Yet the observed ORR rate is lower than for
312 TKI treatment of EGFR-mutated or ALK-rearranged NSCLC, usually found to be 70-
313 80%. Accordingly, the proportion of *METex14* NSCLC patients showing progressive
314 disease as best response to MET TKIs ranged up to 32%⁶⁻⁹, suggesting that these
315 cancers are not driven by MET-dependent mechanisms only.

316 Primary resistance of oncogene-addicted cancers to targeted therapies may have
317 several causes, including concurrent genomic alterations²⁰. In the present study,
318 based on a cohort of 65 *METex14* NSCLC patients, we find that alterations in the
319 PI3K pathway, including PTEN loss and *PIK3CA* mutations, are common and can be
320 associated with resistance to MET TKIs. We confirm here, using a patient-derived
321 cell line combining a *METex14* mutation with PTEN loss, that PI3K-pathway
322 alterations confer resistance to type-I and type-II MET TKIs. Importantly, we show
323 that adding a PI3K inhibitor to the treatment can restore sensitivity of these cells to
324 the MET TKI. Similar results are obtained with *METex14* cells harboring a concurrent
325 *PIK3CA* mutation. Overall, our data suggest that alteration of the PI3K pathway is
326 one of the main mechanisms of primary resistance to MET TKIs in *METex14* NSCLC.

327 The most common PI3K-pathway alteration observed in our cohort was PTEN loss.
328 PTEN loss has been reported in 8-42.4% of NSCLC patients, but previous studies
329 have included different populations and used various thresholds for PTEN expression
330 (stained cells 0%²¹, <10%²², or <50% of all cancer cells²³). The frequency of PTEN
331 loss in oncogene-addicted NSCLC is poorly known. PTEN mutations have been
332 reported in 5% of EGFR-mutated NSCLC ²⁴, and homozygous deletion of PTEN has
333 been reported in 1 out of 24 EGFR-mutated NSCLC patients²⁵. The wide diversity of
334 genetic and epigenetic alterations affecting PTEN makes it hard to get a
335 comprehensive molecular profile of the *PTEN* gene. This explains why IHC has
336 emerged as an attractive and reliable technique for detecting PTEN loss. Like Soria
337 et al., we have chosen to define PTEN loss as the total absence of PTEN expression
338 (0% stained cells). Using this criterion, we found a 23% rate of PTEN loss. One
339 should note that as IHC misses PTEN mutations leading to loss of function without
340 loss of expression, the rate of true PTEN loss may be underestimated in our cohort.

341 The functional consequences of PTEN loss on responses to TKIs in oncogene-
342 addicted cancers are still largely unknown²⁶. In patients with EGFR-mutated NSCLC,
343 PTEN expression is associated with prolonged survival²⁷. In *METex14* NSCLC, the
344 impact of PTEN loss has never been addressed so far. Our results strongly suggest
345 that PTEN loss contributes to resistance to MET TKIs in *METex14* cancer cells
346 through sustained activation of the PI3K pathway.

347 *PIK3CA* mutations are found in 4% to 5% of NSCLC^{11,12,28} and can occur
348 concurrently with other oncogenic drivers, such as EGFR and KRAS²⁹. EGFR-
349 mutated NSCLC is the only oncogene-addicted NSCLC in which the impact of
350 *PIK3CA* mutations on responses to TKIs has been studied, and results remain
351 controversial. Preclinical data suggest that the *PIK3CA* mutation E545K decreases
352 sensitivity of EGFR-mutated cells to EGFR TKIs³⁰. Moreover, *PIK3CA* mutations
353 were initially proposed as an acquired mechanism of resistance to EGFR TKI in
354 EGFR-mutated NSCLC patients³¹ and were recently found in 3% of patients showing
355 disease progression after first-line osimertinib³². Yet recent findings indicate that the
356 *PIK3CA* mutation rate in EGFR-mutated NSCLC patients is similar whether the
357 patients have been treated with EGFR TKIs or not³³. This suggests a limited role of
358 *PIK3CA* mutations in resistance to EGFR TKIs. For patients with EGFR-mutated
359 NSCLC, Eng et al. report worse overall survival in the presence than in the absence
360 of a concurrent *PIK3CA* mutation, but these authors observed no significant
361 difference in objective response rate or time to progression in patients on EGFR TKIs
362³⁴. Our present findings suggest that alterations in the PI3K pathway may be
363 associated with resistance to MET TKIs, since all the *METex14* NSCLC patients
364 whose tumors harbored such an alteration showed disease progression as best
365 response on MET TKIs. The view that alterations of the PI3K pathway are involved in

366 resistance to MET TKIs is further validated by our results obtained with cell lines,
367 including a patient-derived cell line.

368 Besides PI3K-pathway alterations, other concurrent molecular alterations have been
369 described in *METex14* NSCLC, including *KRAS* mutation and amplification, *MDM2*
370 amplification, *TP53* mutation, and *EGFR* mutation. Frampton et al. and Awad et al.
371 respectively found a concurrent alteration in 100 out of 114 (88%) and 26 out of 28
372 (92%) *METex14* samples^{3,35}. This high frequency of concurrent molecular
373 aberrations may reflect the higher rate of smokers and elderly patients among
374 *METex14* NSCLC patients as compared to EGFR-mutated or ALK-rearranged
375 NSCLC patients. Interestingly, concurrent alterations may also impair the response to
376 MET TKIs. Recently, *KRAS* mutations were found in 4.4% of *METex14* NSCLC
377 patients, and most of these mutations were identified before any treatment with MET
378 TKI³⁶. Amplification of wild-type *KRAS* has also been described in *METex14* NSCLC
379 patients as a mechanism of primary and acquired resistance^{36,37}. Thus, extensive
380 molecular profiling of *METex14* tumors appears important in identifying patients who
381 may benefit from MET TKIs, and this profiling should include screening for PTEN loss
382 and *PIK3CA* mutations.

383 We find here that a combination of MET and PI3K inhibitors can inhibit the
384 proliferation of cells harboring a *METex14* mutation and a concurrent *PIK3CA*
385 mutation or PTEN loss. As *PIK3CA* mutations and PTEN loss lead to activation of the
386 PI3K pathway, these results suggest that PI3K pathway alterations cause resistance
387 to MET TKIs. Interestingly, Bahcall et al. have found that the same combination can
388 inhibit tumor growth in *METex14* tumors with concurrent wild-type *KRAS*
389 amplification³⁷. Taken together, these results strongly suggest that a combination of
390 MET and PI3K inhibitors might overcome several mechanisms of resistance to MET

391 TKIs in *METex14* NSCLC. So far in the the clinical setting, the use of panPI3K
392 inhibitors, either alone or in combination, has been hindered by safety issues ³⁸. A
393 phase I study evaluating a combination of capmatinib and buparlisib in adult patients
394 with recurrent glioblastoma was discontinued because of poor tolerance³⁹. Yet more
395 selective drugs have recently been developed and might offer a better safety profile
396 with promising activity. This would pave the way for future combinations.

397 In conclusion, although *METex14* mutations are associated with MET TKIs efficacy in
398 a number of patients, concurrent alterations can frequently occur and may impair the
399 response to MET inhibition. Our results suggest that PI3K-pathway alterations are
400 common and should be screened in order to identify patients most likely to benefit
401 from MET TKIs. Combining MET and PI3K inhibitors could overcome resistances due
402 to PI3K pathway alterations.

403

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412

413

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415

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555

556

557

558 **Figure legends**

559 **Figure 1. Alterations in the PI3K pathway are common in *METex14* NSCLC. (A)**

560 Baseline characteristics of and treatments received by the patients with an advanced
561 *METex14* non-small cell lung cancer. **(B)** Pie charts displaying the frequencies (%) of
562 alterations of the examined genes in the cohort (n=65) and PTEN expression within
563 each tumor sample tested by IHC (n=19). **(C)** Left panel (PTEN negative) shows
564 undetectable PTEN in cancer cells of tumor specimen from patient CHUL#11 ; Right
565 panel (PTEN positive) shows expression of PTEN in cancer cells of tumor specimen
566 from patient CHUL#15. Thin arrows indicate cancer cells and large arrows indicate
567 endothelial or immune cells (internal positive control). **(D)** Schematic presentation of
568 patients according to the response to MET TKI treatment and to the presence or
569 absence of a PI3K pathway alteration (n=17). Sub: substitution; Del : deletion; Indel :
570 insertion-deletion **(E)** Computed tomography scans of CHUL#11, showing multiple
571 lymph node metastases at baseline (upper left) and progression after two months of
572 MET TKI treatment (upper right). Scans of CHUL#18, showing emergence of bone
573 metastases after two months of MET TKI treatment (middle right panel). Lower
574 abdomen scans of CHUL#20, showing lymph node metastases at baseline (lower
575 left), which significantly increased after one month of MET TKI treatment (lower right
576 panel).

577

578 **Figure 2. Patient-CHUL#11-derived cell line with PTEN loss shows sustained**

579 **activation of the PI3K pathway and resistance to MET TKI. (A)** Melting curve
580 analysis of MET splicing isoforms and agarose gel electrophoresis of the amplicons
581 generated by fluorescence reverse-transcription PCR. cDNA from cell lines

582 expressing a characterized MET-exon-14-skipping alteration (Hs746T, H596) or a
583 wild-type MET (A549, PC9, GTL16) were used as controls. **(B)** Immunoblots for
584 ZORG cells versus PTEN-expressing cell lines. Tubulin was used as a loading
585 control. **(C)** ZORG cells were serum starved for 24 h and stimulated or not for 30 min
586 with 50 ng/ml HGF. Cells were harvested and subjected to western blotting with the
587 indicated antibodies. **(D)** The viability of ZORG cells in response to MET TKIs was
588 assessed by MTT assay after 72 h of treatment. The sensitive Hs746T cell line was
589 used as control. Results are presented as means \pm SEM of at least three
590 independent experiments. **(E)** Serum-starved ZORG and Hs746T cells were treated
591 for 30 min with each MET TKI at 1 μ M in the presence of HGF. Expression and
592 activation of MET and its related signaling pathways were investigated by western
593 blotting.

594

595 **Figure 3. PI3K inhibition restores sensitivity to MET TKIs in ZORG, a *METex14***
596 **cell line with PTEN loss. (A)** Proliferation of ZORG cells treated with a MET TKI
597 (capmatinib) at 0.5 or 1 μ M, a PI3K α/δ inhibitor at 0.5 or 1 μ M (GDC0941), or both
598 (each at 0.5 or 1 μ M) plus HGF at 20 ng/ml. IncuCyte® confluence measurements
599 were performed every 4 h for 196 h. Results are presented as means \pm SEM of three
600 independent experiments. Statistical significance was calculated with the Wilcoxon
601 test. **(B)** Dose-response curve of ZORG cells treated for 3 days with capmatinib,
602 GDC0941, or both and assessed by MTT assay. **(C)** ZORG cells were serum starved
603 for 24 h and treated for 30 min with DMSO, 1 μ M capmatinib, 1 μ M GDC0941, or
604 capmatinib plus GDC0941 (at 1 μ M each) and HGF at 50 ng/ml. Western blot
605 analyses with the indicated antibodies were performed on cell lysates. Tubulin was
606 used as loading control.

607

608 **Figure 4. Inhibition of PI3K restores sensitivity to a MET TKI in a *METex14* cell**
609 **line with a *PIK3CA* mutation**

610 **(A)** Viability curves of H596 cells treated with increasing doses of MET TKI. Viability
611 was assessed by MTT assay. IC₅₀ values were calculated with GraphPad Prism
612 version 5.01, as the means of three independent experiments **(B)** IncuCyte® analysis
613 of proliferation measured as confluence per well of H596 cells treated with DMSO,
614 capmatinib, GDC0941, or capmatinib plus GDC0941 (each at 0.5 or 1 μM) and HGF
615 at 20 ng/ml. Results are presented as means ± SEM of three independent
616 experiments. Statistical significance was calculated with the Wilcoxon test. **(C)** MET
617 expression and downstream signaling were investigated in H596 cell lines by western
618 blotting. For 30 min before lysis, serum-starved cells were treated with each TKI
619 alone and with both combined. **(D)** Dose-response curve of H596 cells treated for 3
620 days with capmatinib, GDC0941, or both and assessed by MTT assay.

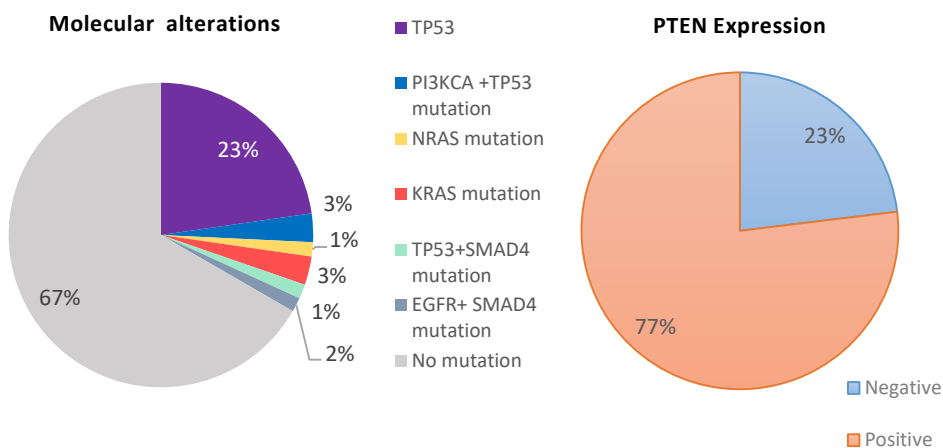
621 **Figure 5. *PIK3CA* mutations contribute to MET inhibitor resistance in *METex14***
622 **cells. (A)** IncuCyte® analysis of proliferation, measured as confluence per well, of
623 T47D parental cells versus T47DMETex14 cells treated with DMSO, capmatinib,
624 GDC0941, or capmatinib plus GDC0941 (at 0.5 or 1 μM each) and HGF at 20 ng/ml.
625 Results are presented as means ± SEM of three independent experiments. Statistical
626 significance was calculated with the Wilcoxon test. **(B)** MET expression and
627 downstream signaling were investigated in T47D and T47DMETex14 cell lines by
628 western blotting. For 30 min before lysis, serum-starved cells were treated with each
629 TKI alone and with both combined. **(C)** Dose-response curve of T47DMETex14 cells
630 treated for 3 days with capmatinib, GDC0941, or both and assessed by MTT assay.

Figure 1

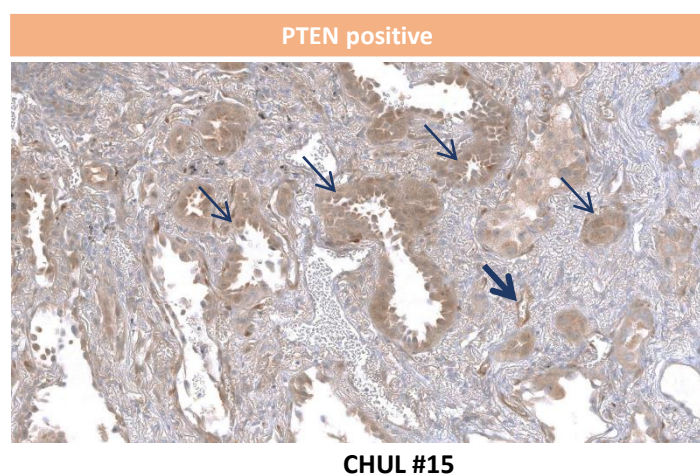
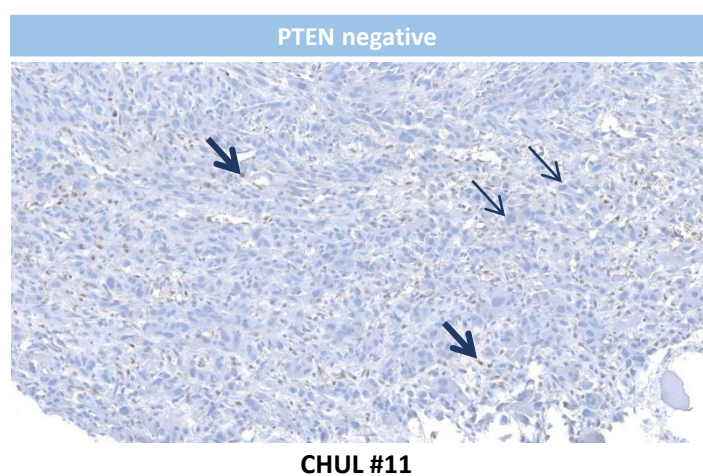
A

	n = 65
Age, median (years) (range)	75 (49-88)
Male	33 (51%)
Female	32 (49%)
Adenocarcinoma	53 (80%)
Pulmonary sarcomatoid carcinoma	3 (6%)
Pleomorphic carcinoma	1 (2%)
Other	8 (12%)
Stage at diagnosis	n=64
Stage I-III	21 (33%)
Stage IV	43 (67%)
Tabac status	n=64
Current Smokers	4 (6%)
Former Smokers	25 (39%)
Never Smokers	35 (55%)
Treatment received (advanced stage)	n=36
Chemotherapy	23 (64%)
MET TKI	17 (47%)
Immunotherapy	9 (25%)

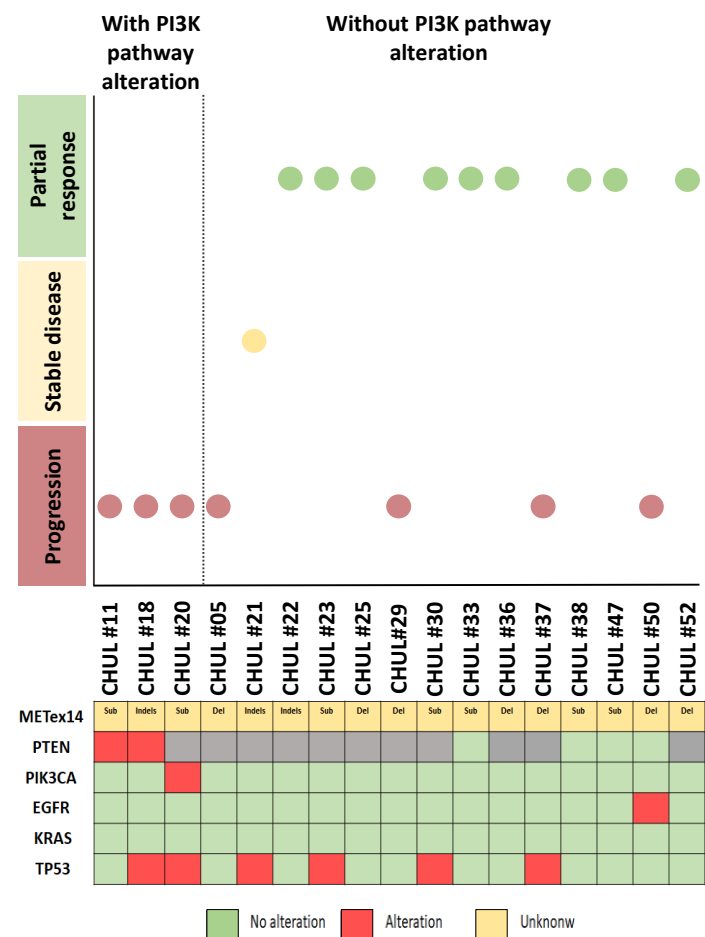
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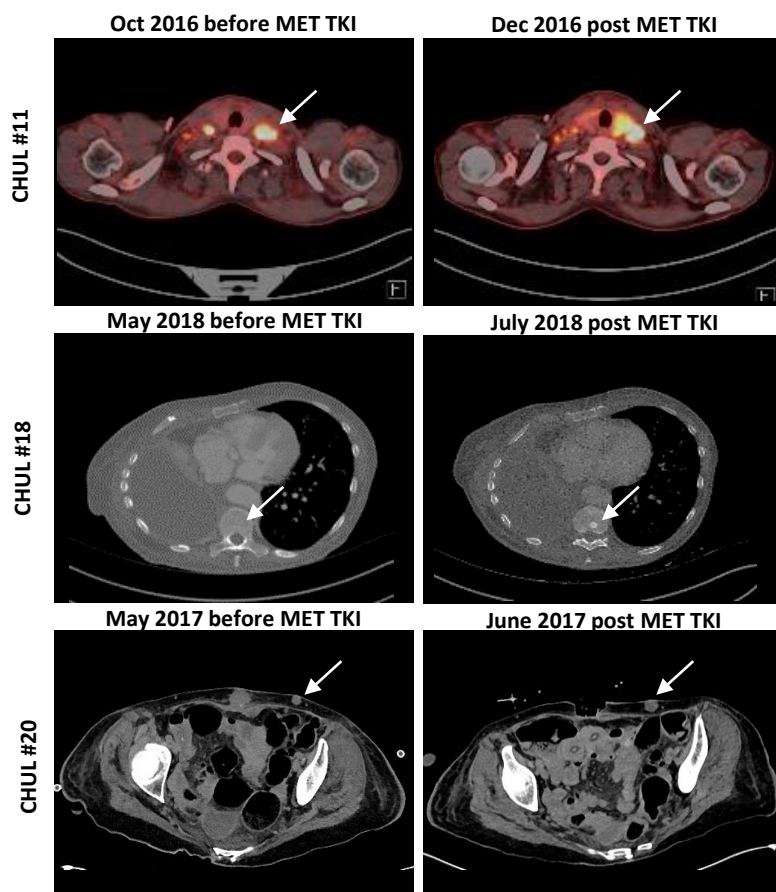
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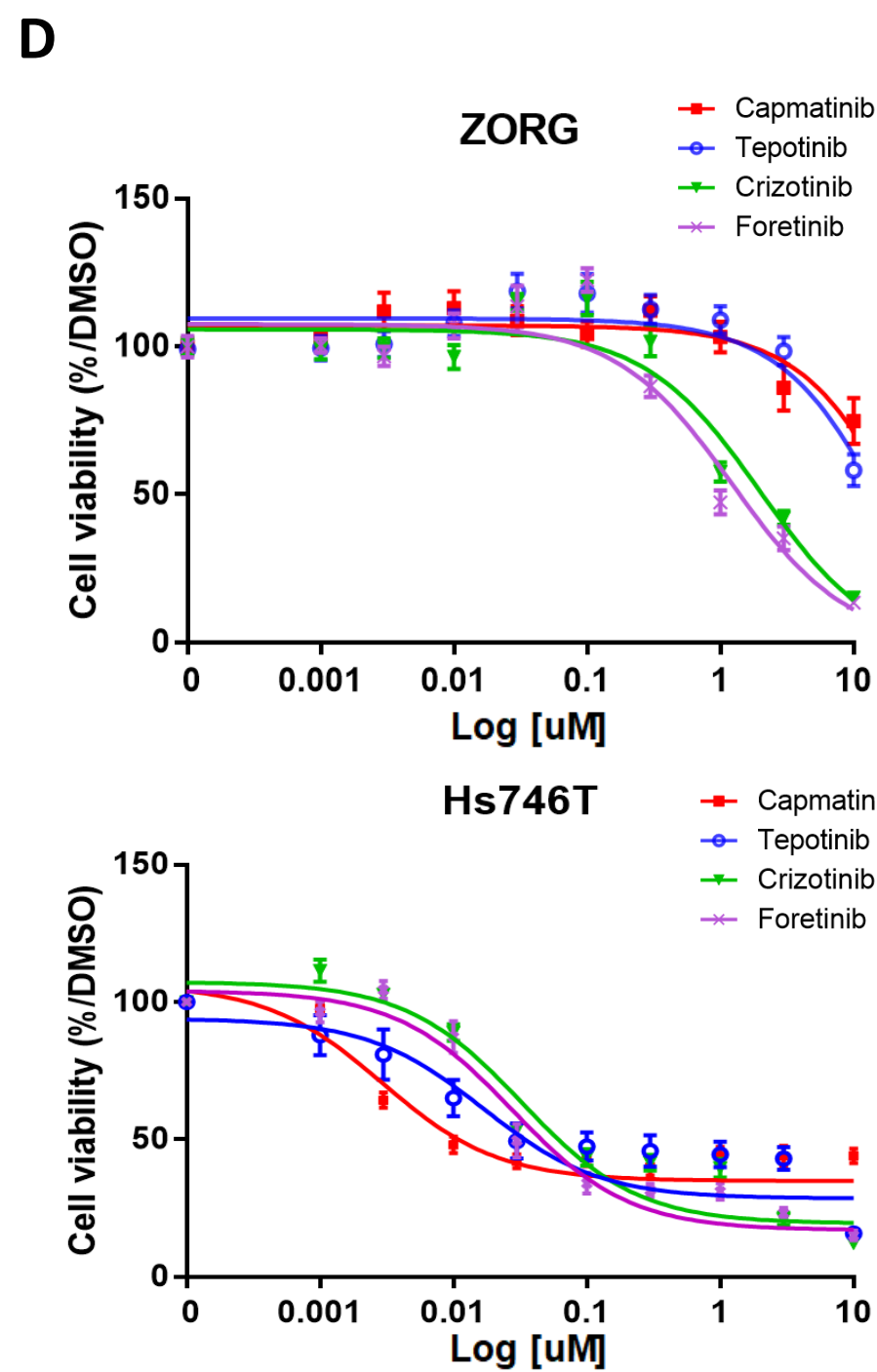
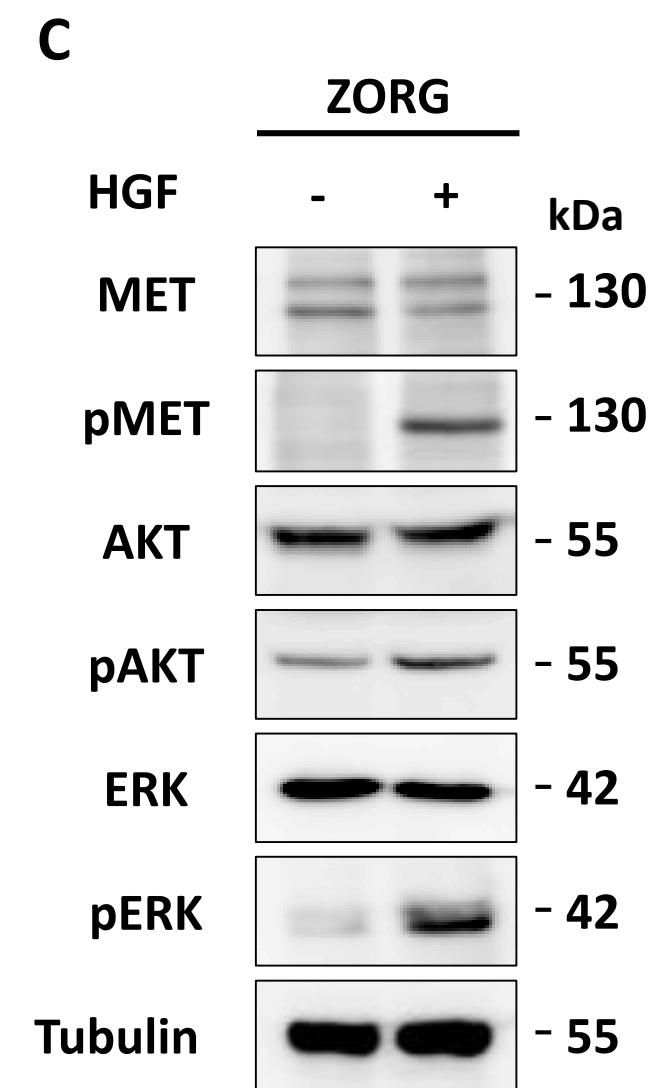
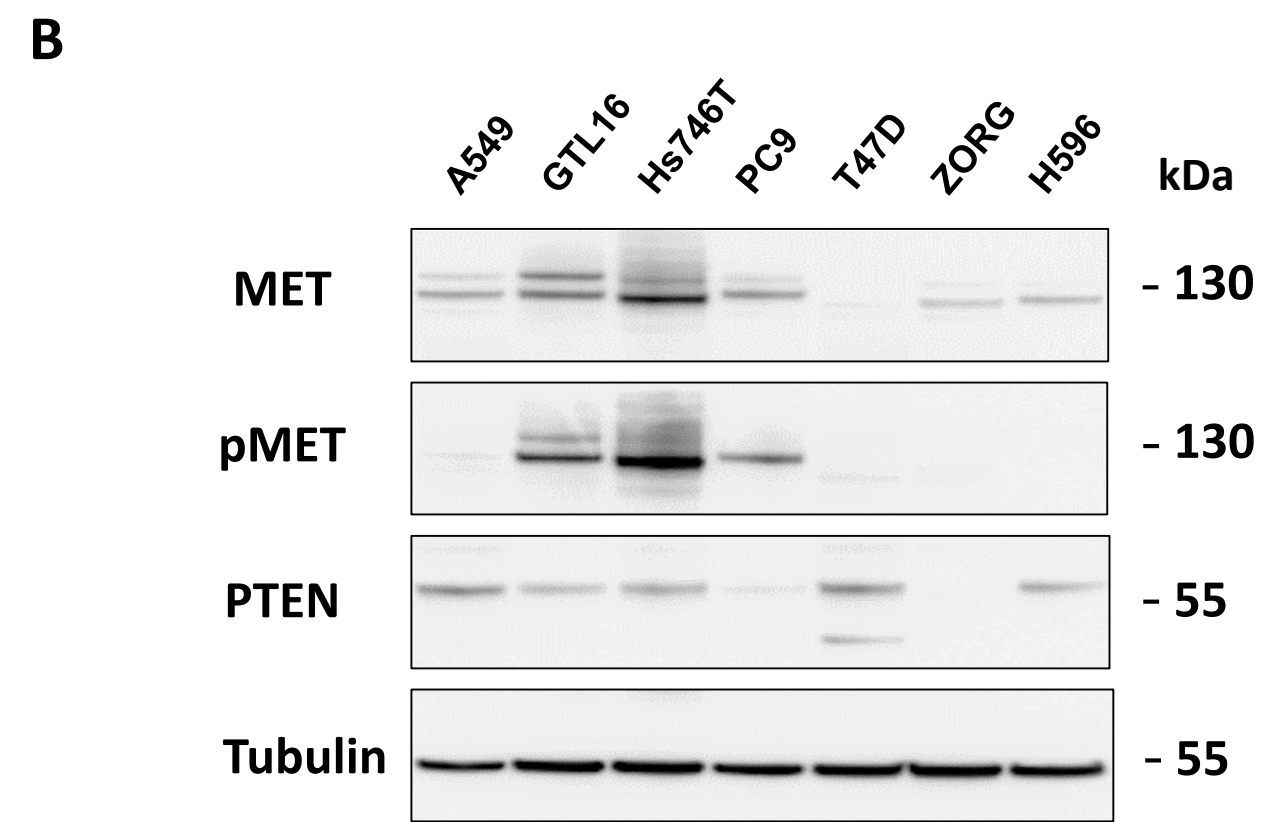
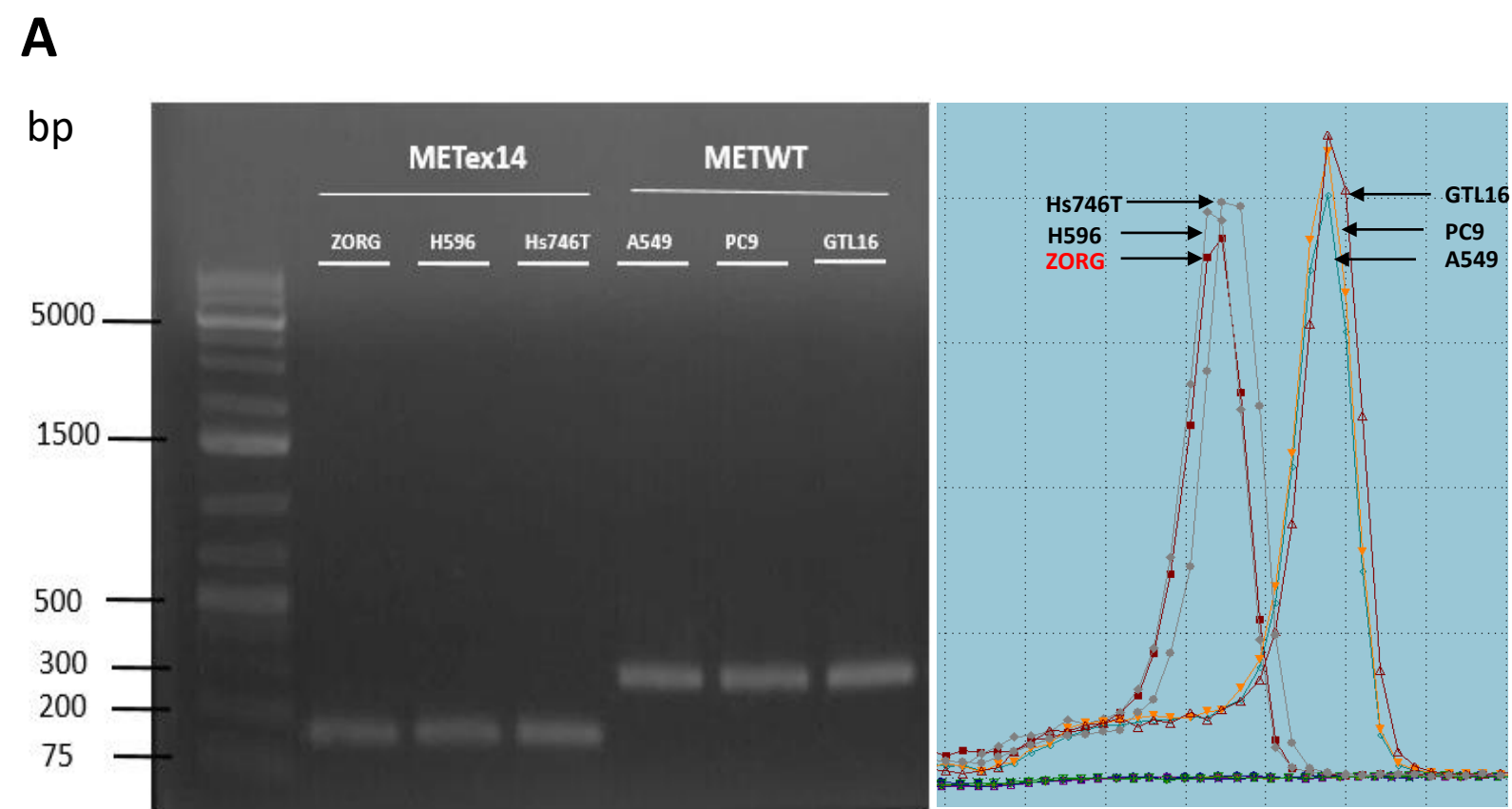


D



E





MET TKI	ZORG IC50 (μM)
Capmatinib	>10
Tepotinib	>10
Crizotinib	2.0 [1.45-2.75]
Foretinib	1.23 [0.83-1.82]

MET TKI	Hs746T IC50 (μM)
Capmatinib	0.003 [0.001-0.005]
Tepotinib	0.016 [0.01-0.03]
Crizotinib	0.032 [0.02-0.05]
Foretinib	0.027 [0.02-0.040]

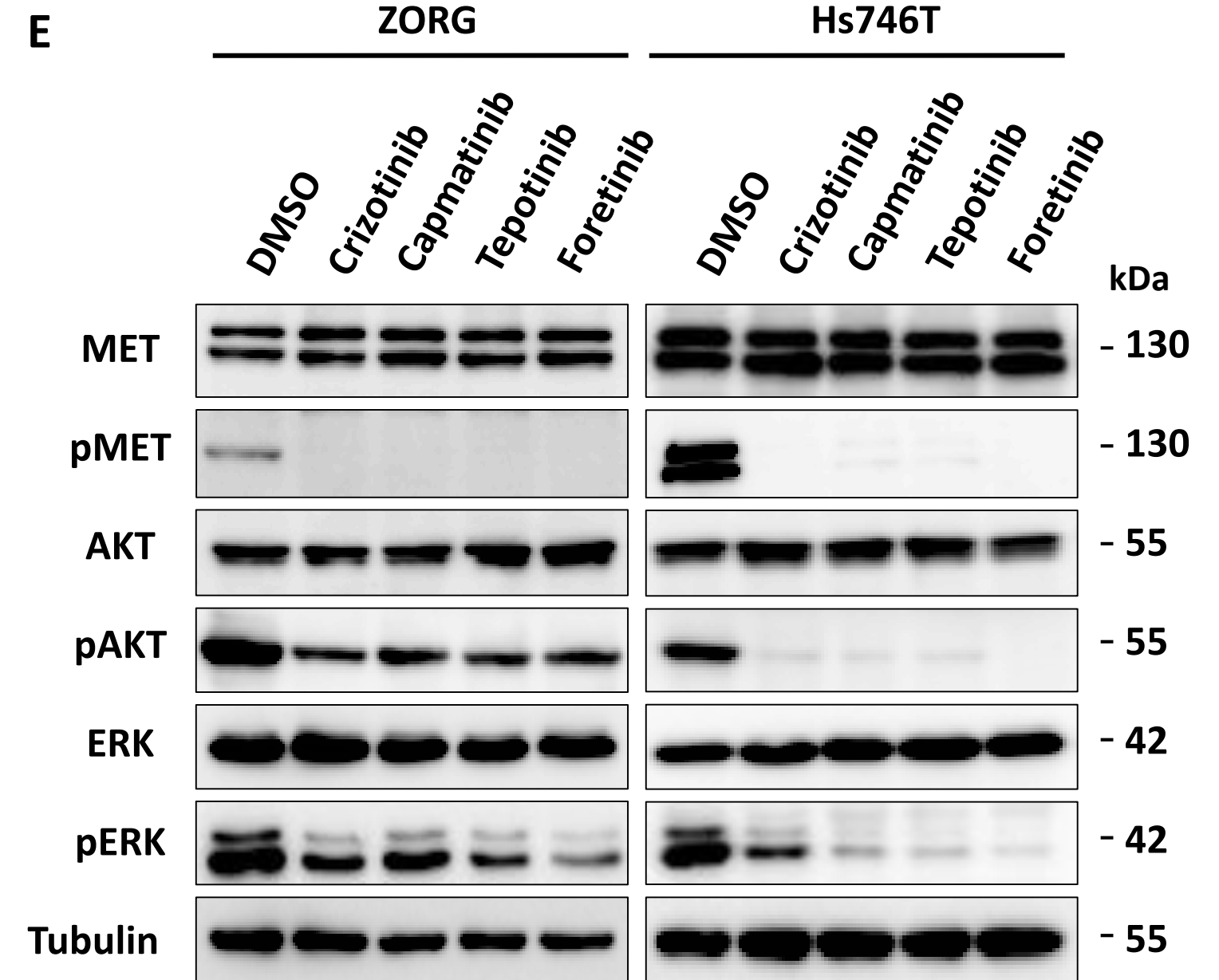
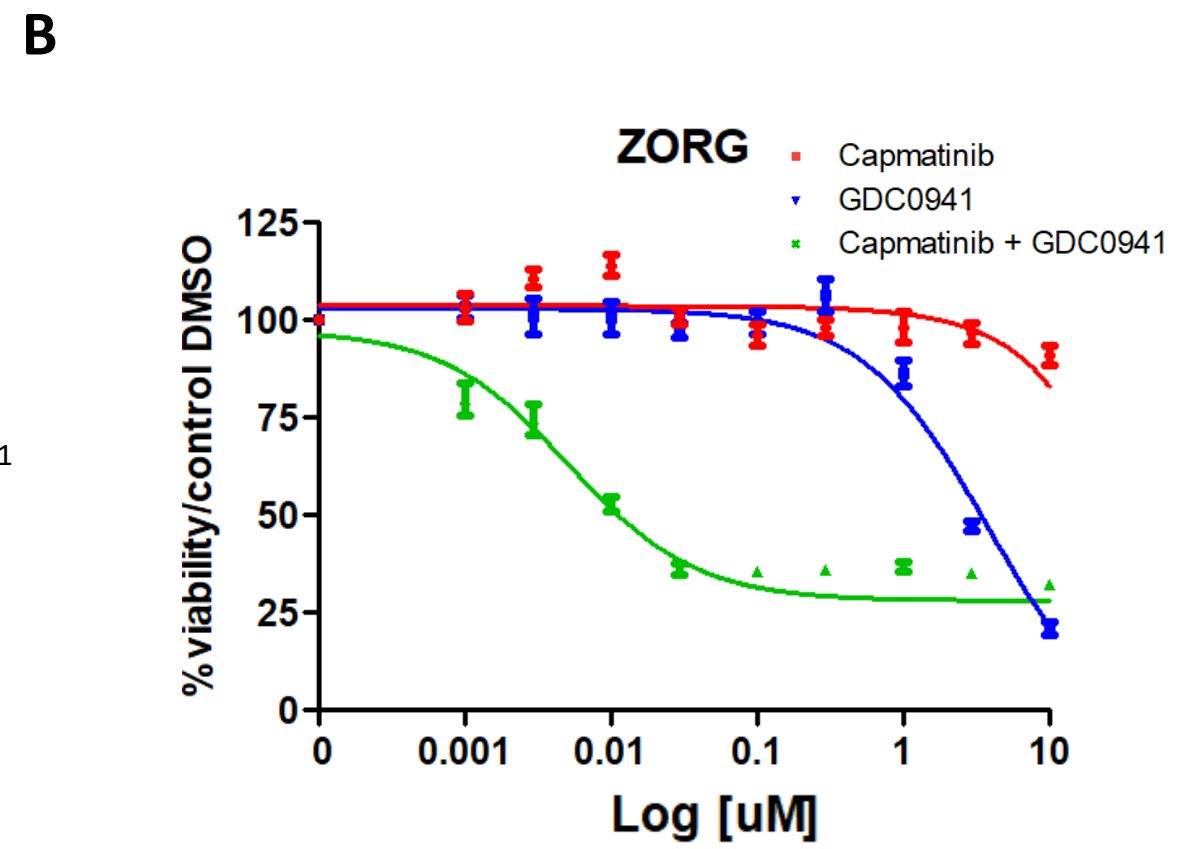
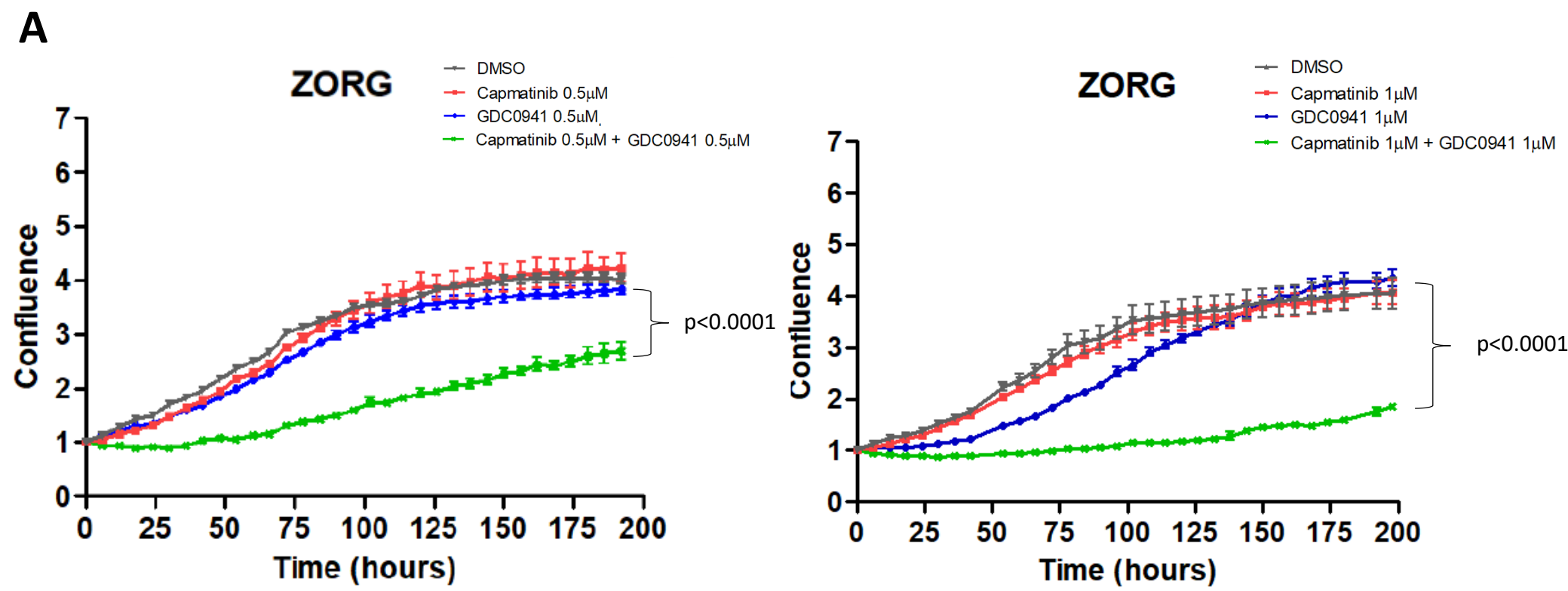


Figure 3



MET TKI	ZORG IC50 (μM)
Capmatinib	>10
GDC0941	2.09 [1.77-2.47]
Capmatinib + GDC0941	0.034 [0.02-0.04]

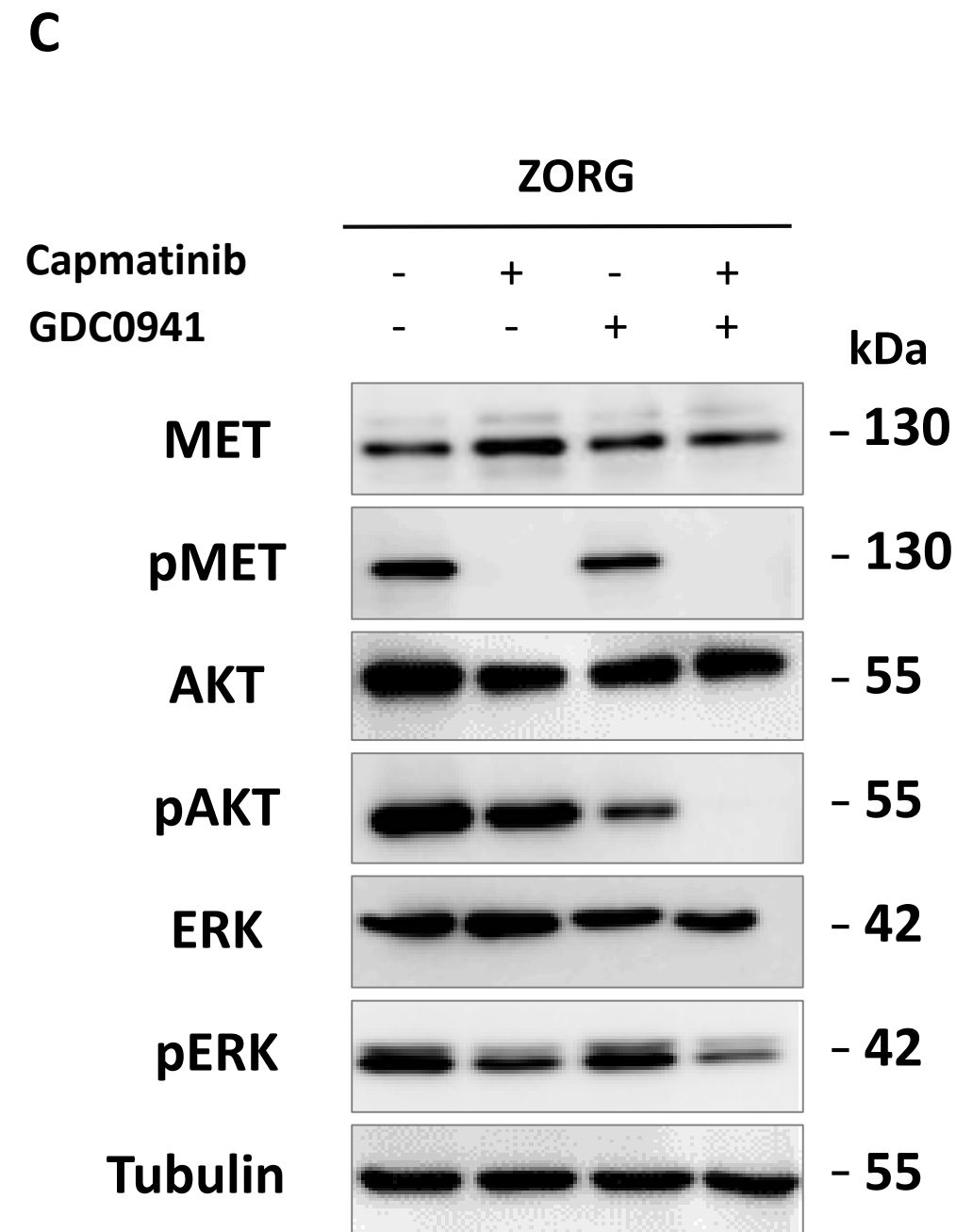
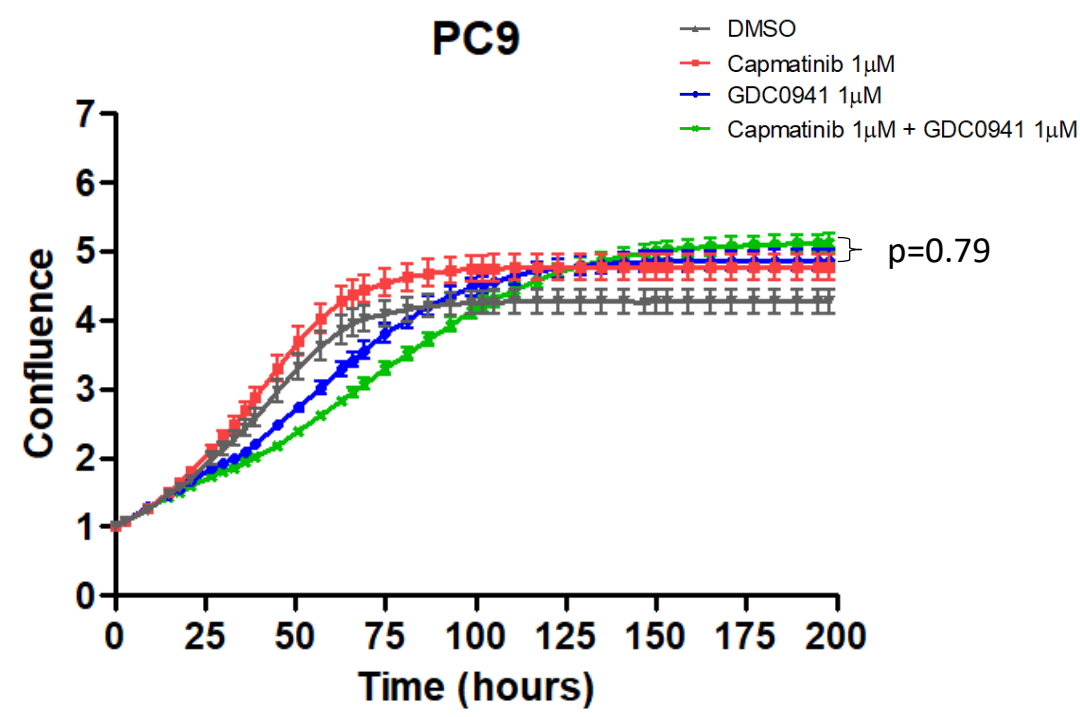
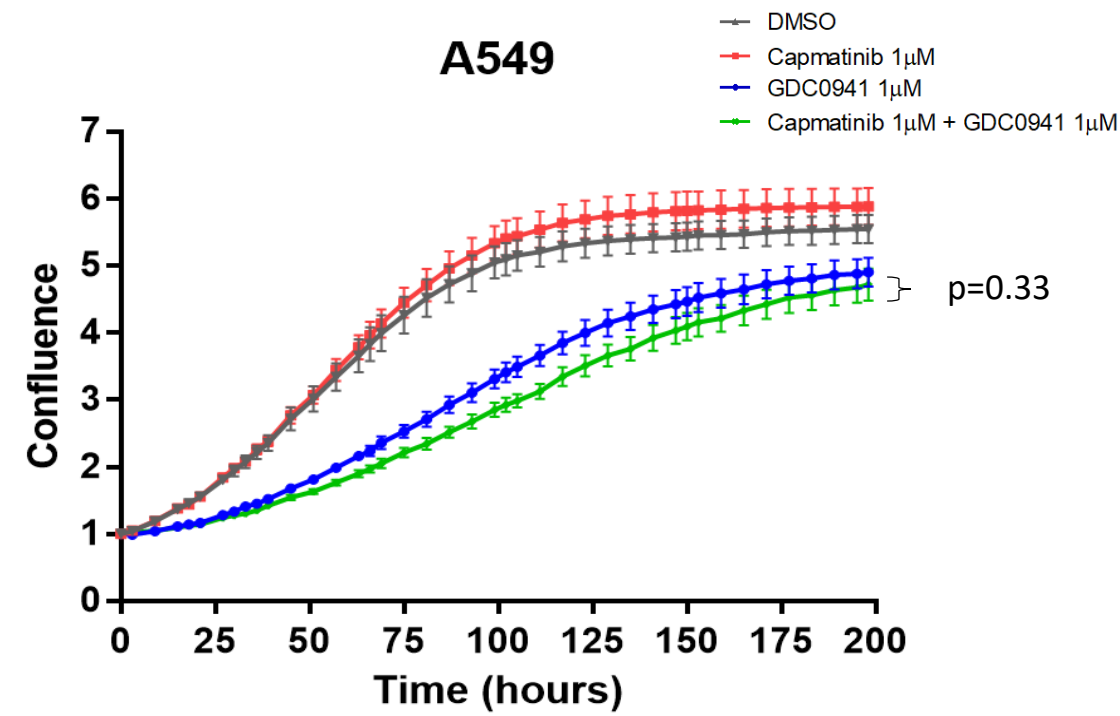


Figure 4

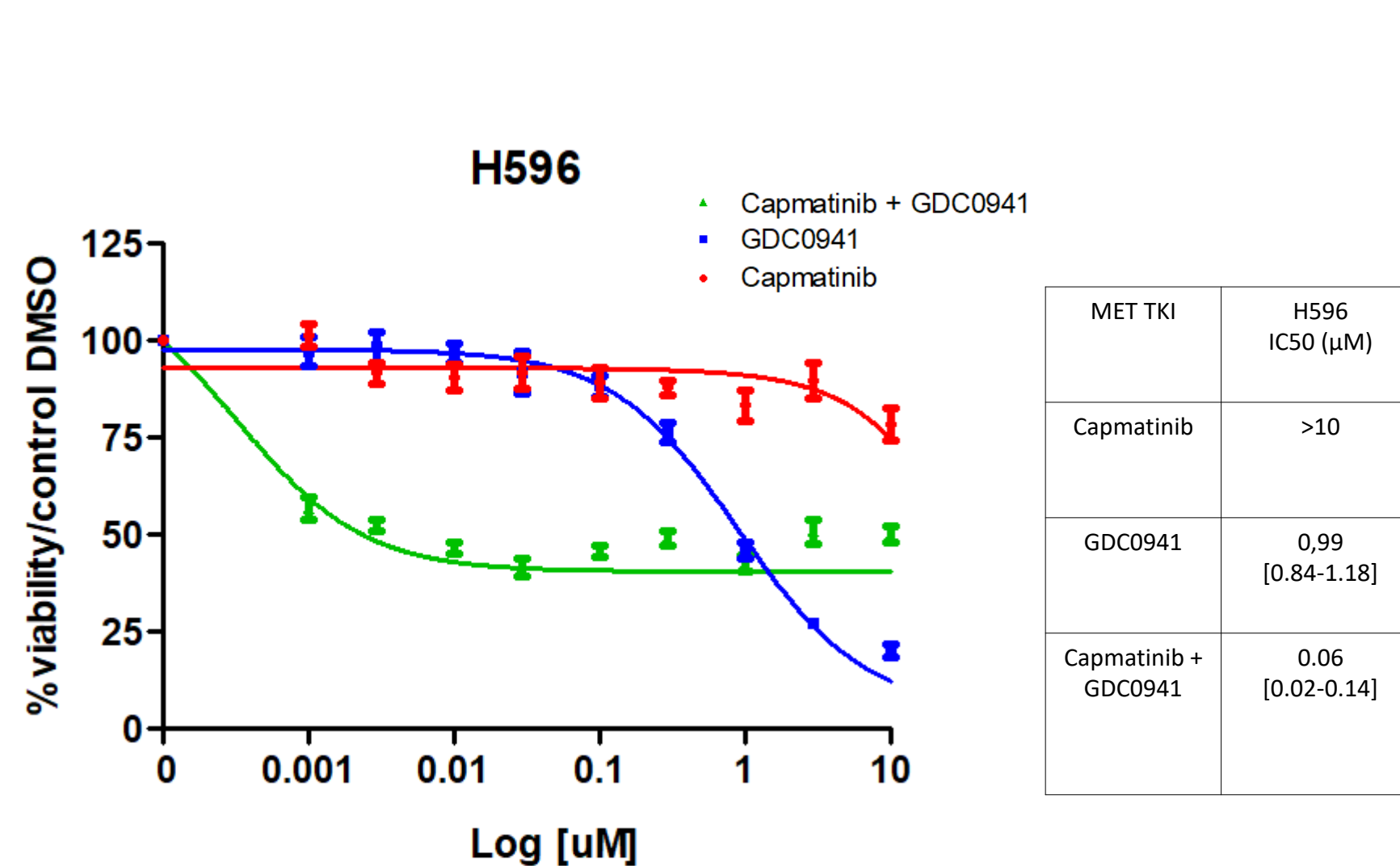
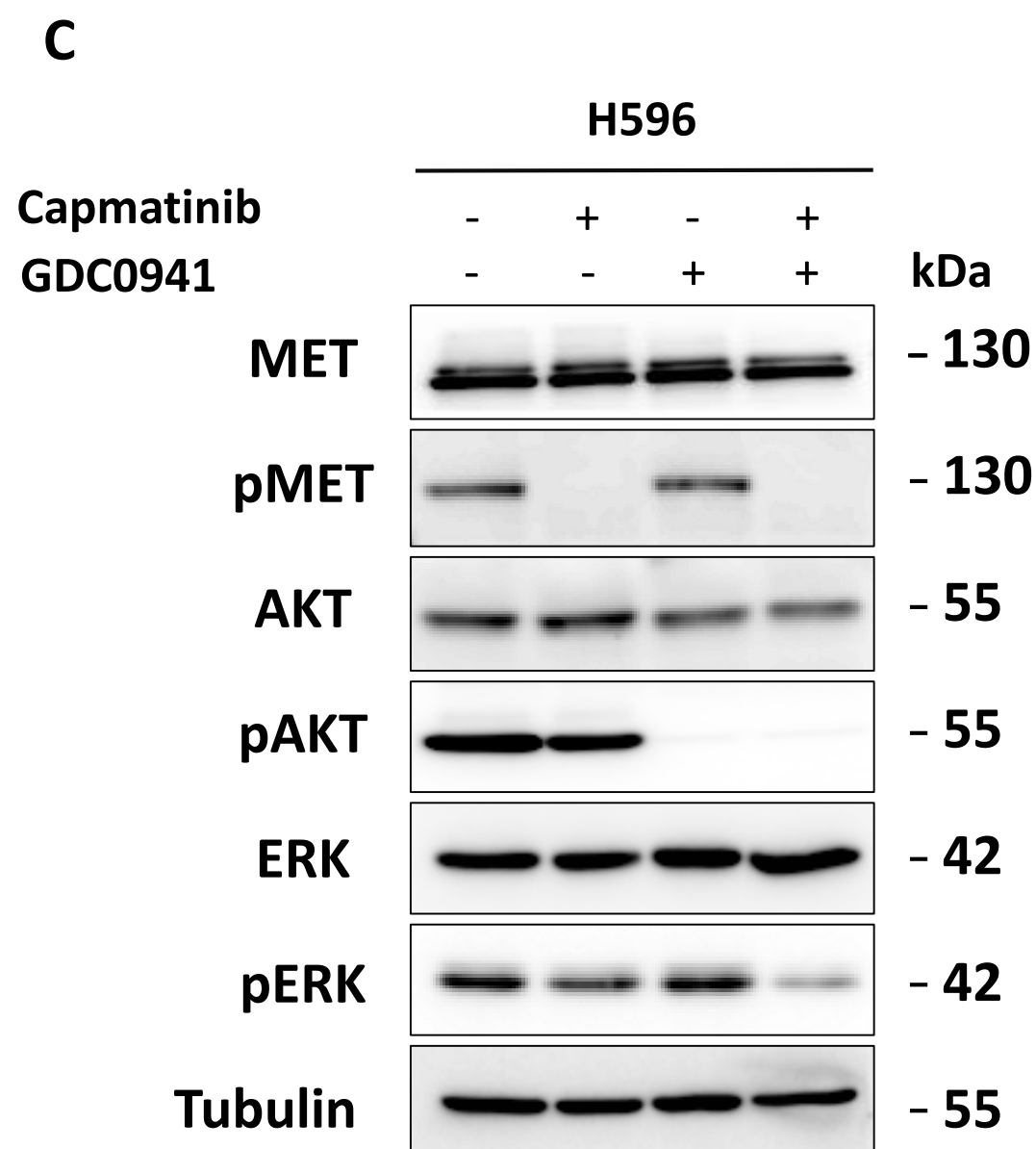
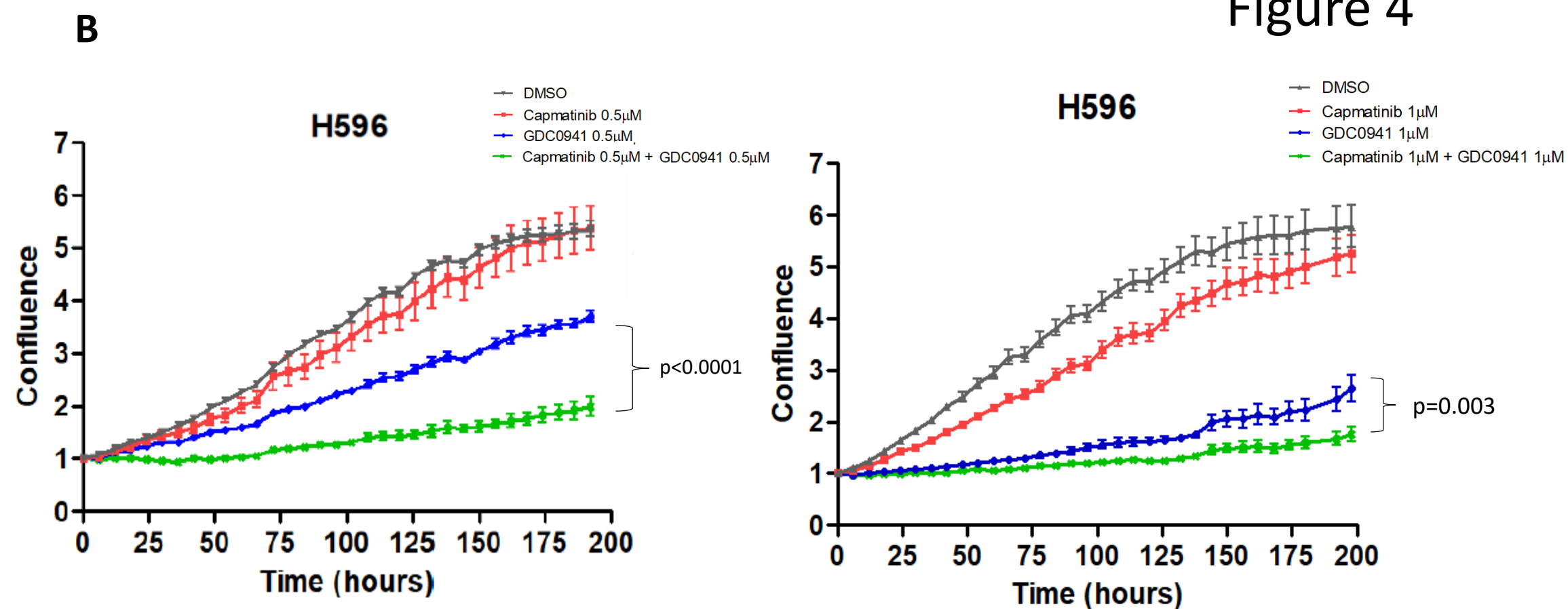
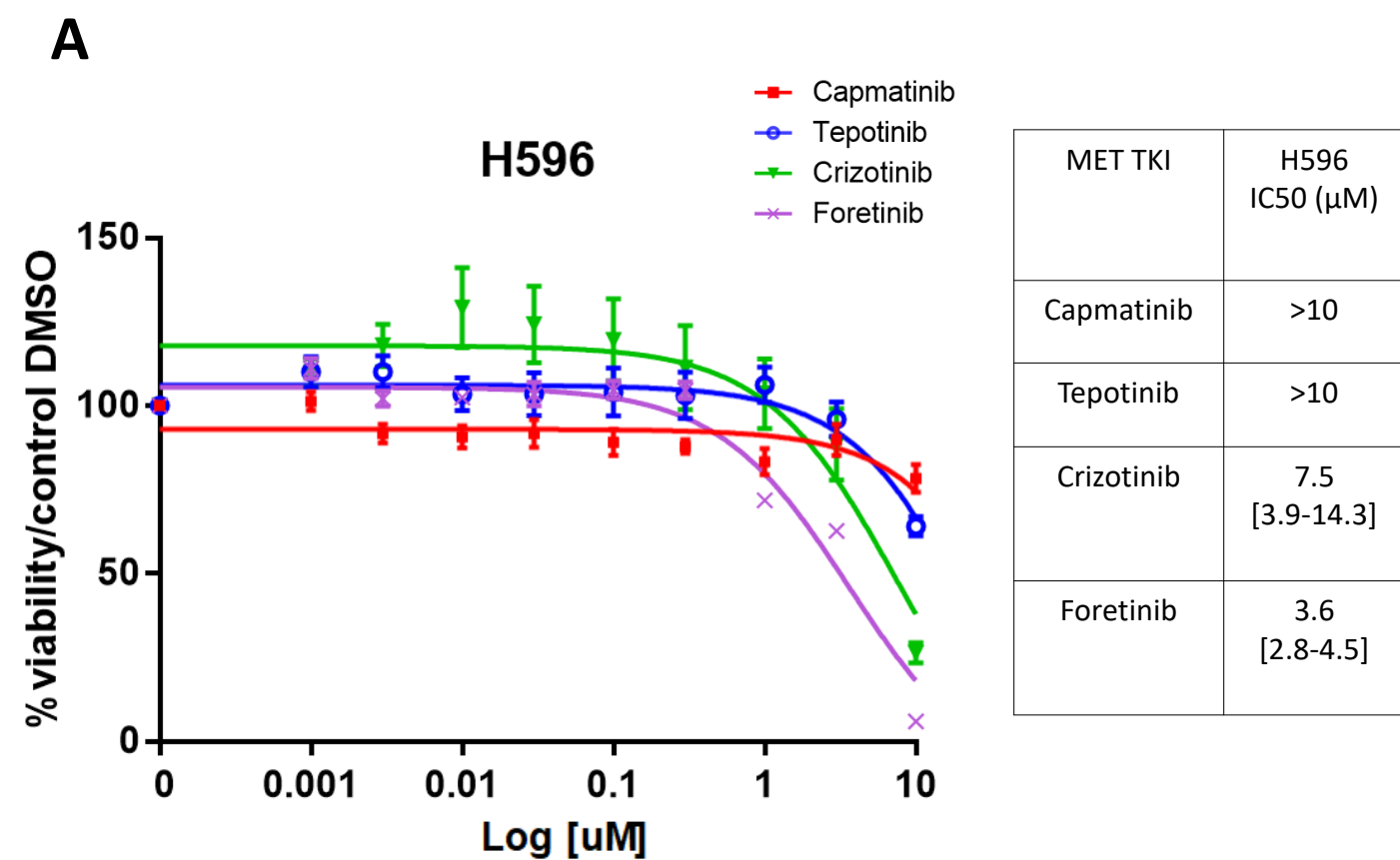
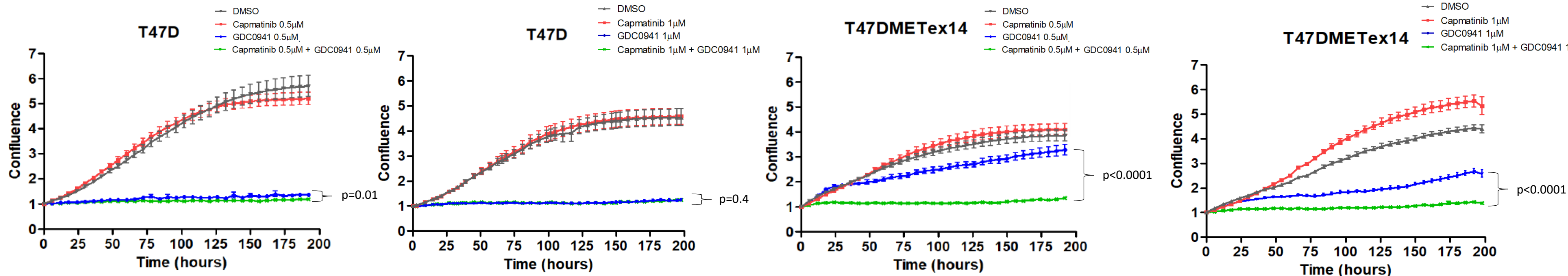
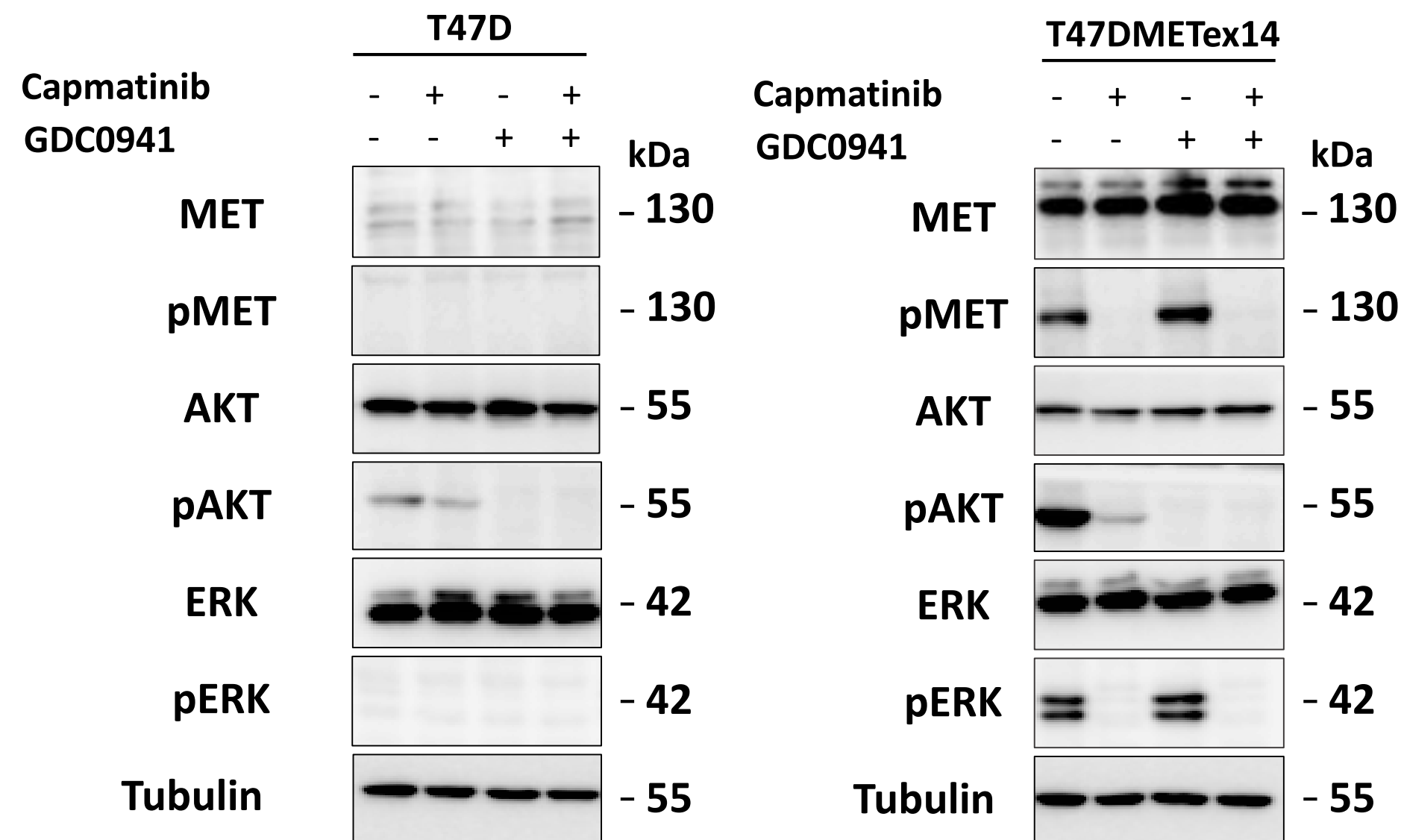


Figure 5

A



B



C

