**Impact of oncogenic driver mutations on feedback between the PI3K and MEK pathways in cancer cells**

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| Citation       | Yuen, Hiu-Fung, Olga Abramczyk, Grant Montgomery, Ka-Kui Chan, Yu-Han Huang, Takehiko Sasazuki, Senji Shirasawa, Srivastava Gopesh, Kwok-Wah Chan, Dean Fennell, Pasi Janne, Mohamed El-Tanani, and James T. Murray. 2012. Impact of oncogenic driver mutations on feedback between the PI3K and MEK pathways in cancer cells. Bioscience Reports 32:413-422. |
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Impact of oncogenic driver mutations on feedback between the PI3K and MEK pathways in cancer cells

Hiu-Fung YUEN*†, Olga ABRAMCZYK‡, Grant MONTGOMERY*, Ka-Kui CHAN*, Yu-Han HUANG†, Takehiko SASAZUKI§, Senji SHIRASAWA§, Srivastava GOPESH∥, Kwok-Wah CHAN∥, Dean FENNELL¶, Pasi JANNE**, Mohamed El-TANANI*2 and James T. MURRAY*1,2

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Synopsis

Inhibition of the PI3K (phosphoinositide 3-kinase)/Akt/mTORC1 (mammalian target of rapamycin complex 1) and Ras/MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/ERK pathways for cancer therapy has been pursued for over a decade with limited success. Emerging data have indicated that only discrete subsets of cancer patients have favourable responses to these inhibitors. This is due to genetic mutations that confer drug insensitivity and compensatory mechanisms. Therefore understanding of the feedback mechanisms that occur with respect to specific genetic mutations may aid identification of novel biomarkers that predict patient response. In the present paper, we show that feedback between the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways is cell-line-specific and highly dependent on the activating mutation of K-Ras or overexpression c-Met. We found that cell lines exhibited differential signalling and apoptotic responses to PD184352, a specific MEK inhibitor, and PI103, a second-generation class I PI3K inhibitor. We reveal that feedback from the PI3K/Akt/mTORC1 to the Ras/MEK/ERK pathway is present in cancer cells harbouring either K-Ras activating mutations or amplification of c-Met but not the wild-type counterparts. Moreover, we demonstrate that inhibition of protein phosphatase activity by OA (okadaic acid) restored PI103-mediated feedback in wild-type cells. Together, our results demonstrate a novel mechanism for feedback between the PI3K/Akt/mTORC1 and the Ras/MEK/ERK pathways that only occurs in K-Ras mutant and c-Met amplified cells but not the isogenic wild-type counterparts. We conclude that monitoring K-Ras and c-Met status are important biomarkers for determining the efficacy of PI103 and other PI3K/Akt inhibitors in cancer therapy.

Key words: Akt, cancer cell, c-Met, K-Ras, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), phosphoinositide 3-kinase (PI3K).


INTRODUCTION

The PI3K (phosphoinositide 3-kinase)/Akt/mTORC1 (mammalian target of rapamycin complex 1) and MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/ERK pathways are the two major hyper-activated pathways that promote cancer progression through enhancing cell survival, metastasis and drug resistance. However, inhibition of either the PI3K/Akt/mTORC1 or MEK/ERK pathways for cancer therapy has been pursued for over a decade with limited success. Emerging data have indicated that only discrete subsets of cancer patients have favourable responses to these inhibitors. This is due to genetic mutations that confer drug insensitivity and compensatory mechanisms. Therefore understanding of the feedback mechanisms that occur with respect to specific genetic mutations may aid identification of novel biomarkers that predict patient response. In the present paper, we show that feedback between the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways is cell-line-specific and highly dependent on the activating mutation of K-Ras or overexpression c-Met. We found that cell lines exhibited differential signalling and apoptotic responses to PD184352, a specific MEK inhibitor, and PI103, a second-generation class I PI3K inhibitor. We reveal that feedback from the PI3K/Akt/mTORC1 to the Ras/MEK/ERK pathway is present in cancer cells harbouring either K-Ras activating mutations or amplification of c-Met but not the wild-type counterparts. Moreover, we demonstrate that inhibition of protein phosphatase activity by OA (okadaic acid) restored PI103-mediated feedback in wild-type cells. Together, our results demonstrate a novel mechanism for feedback between the PI3K/Akt/mTORC1 and the Ras/MEK/ERK pathways that only occurs in K-Ras mutant and c-Met amplified cells but not the isogenic wild-type cells through a mechanism that may involve inhibition of a specific endogenous phosphatase(s) activity. We conclude that monitoring K-Ras and c-Met status are important biomarkers for determining the efficacy of PI103 and other PI3K/Akt inhibitors in cancer therapy.

Key words: Akt, cancer cell, c-Met, K-Ras, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), phosphoinositide 3-kinase (PI3K).

pathways for treatment of cancers has shown increasingly limited success [1].

The development of anti-cancer drugs targeting MEK has been investigated for more than a decade [2] and MEK inhibition impairs colon tumour growth in vivo [3]. PD184352 (CI-1040) is an orally active highly selective and potent chemical inhibitor of MEK1/2 and was the first MEK inhibitor to enter clinical trials [4]. Tumours with higher pERK (phospho-ERK) expression are marginally more responsive to PD184352, although the overall anti-tumour activity of this drug is insufficient in multiple human cancers [5]. Promisingly, PD184352 was shown to inhibit pERK in pancreatic cancer and lead to stable disease with a median of 5.5 months in 28% of patients [6]. Overall, results from clinical trials indicate there are subsets of patients that are non-responsive to PD184352, thus patient stratification may prove effective in identifying PD184352 responders and extend the usefulness of this drug. K-Ras status and cellular background are important factors in determining sensitivity to PD184352. The K-Ras mutant C26 murine colon cell line is resistant to PD184352 [7], whereas thyroid cancer cells harbouring K-Ras and BRAF-activated mutations are more sensitive to PD184352 [8]. In addition, MEK inhibition results in feedback activation of the PI3K/Akt pathway in MDA (malondialdehyde) MB 231 breast cancer cells [9]. Finally, PD184352 also shows to produce synergistic therapeutic efficacy with other chemotherapeutic drugs, including taxol [10], sorafenib [11] and BMS-214662 [12].

The PI3K/Akt/mTORC1 pathway is a major focus for cancer therapy [13]. PI103 is a second generation inhibitor of class I PI3K with anti-tumour activity in a variety of human cancers [14–16]. PI103 also enhances tumour radiosensitivity [17] and chemosensitivity [18]. However, the use of PI103 is contentious, since combined use with sorafenib promotes tumour growth and survival in melanoma cells [19], but inhibits proliferation of hepatocellular carcinoma cells [20].

Combinatorial use of PI3K/Akt/mTORC1 and MEK/ERK pathway inhibitors synergistically induce apoptosis in MDA MB 231 and the hepatocellular carcinoma cell line Huh7 [9,20]. However, direct inhibition of PI3K also reportedly activates the HER2 receptor, thereby enhancing MEK/ERK signalling [21]. Given the lack of clarity with the use of these inhibitors, especially with the impact of genetic background on their effectiveness, a thorough understanding of the chemical–genetic interactions is required to improve the efficacy of therapies that target these pathways.

In the present study, we have investigated inhibition of the PI3K/Akt/mTORC1 and MEK/ERK pathways in a representative panel of breast, lung, prostate, oesophageal and colorectal cell lines with known genetic backgrounds. In particular, we assessed the molecular mechanisms of pathway feedback and cross-talk. We report that pathway interactions are cell line-specific, with cell lines having negative-feedback loops to either or both pathways. Furthermore, we found that K-Ras, c-Met and endogenous protein phosphatase activity are crucial in regulating feedback between the PI3K/Akt/mTORC1 pathway and the MEK/ERK pathway.

**MATERIALS AND METHODS**

**Cell culture**

MDA MB 231, MDA MB 157 and Hs578t (breast; A.T.C.C.) and A549 (lung; A.T.C.C.) cancer cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum). T47D (breast; A.T.C.C.), DU145 (prostate; A.T.C.C.), EC109 [22] (oesophageal; a gift from Professor S.W. Tsao, Department of Anatomy, University of Hong Kong, Hong Kong, China), and HCC827 and its c-Met amplified counterpart HCC827-GR5 [23] (lung; from Professor P.A. Janne, Dana-Farber Cancer Institute, Boston, MA, U.S.A.) cancer cell lines were maintained in RPMI 1640 supplemented with 10% FBS. HCT116 (active K-Ras mutant) and its wild-type isogenic counterpart Hkh-2 cells [24] (colon; from Professor T. Sasazuki, Department of Genetics, Medical Institute of Bioregulation, Kyushu University, Higashi, Japan, and Professor S. Shirasawa, Department of Pathology, International Medical Center of Japan, Tokyo, Japan) cancer cell lines were maintained in DMEM supplemented with 10% FBS and sodium pyruvate.

**Kinase inhibitors**

PI103 (Merck), a dual inhibitor of class I PI3K and mTORC1, was used at a concentration of 10 μM. PD184352, a specific MEK1/2 inhibitor, was a gift from Dr Rudi Marquez, University of Glasgow, Glasgow, U.K. and was used at a concentration of 2 μM. Rapamycin was obtained from EMD Biosciences and used at a concentration of 100 nM. OA (okadaic acid; Santa Cruz Biotechnology) was used at a concentration of 20 nM.

**Western blot analysis**

Western blot analysis was performed as previously described [25]. Anti-pAkt (phospho-Akt) (Ser473) (#4071), -Akt (#4691), -pERK1/2 (#4370), -ERK (#4685), -PARP [poly(ADP-ribose) polymerase] (#9544), -caspase 3 (#9662), -Bcl-XL (#2764) and -cyclin D1 (#2926) antibodies (Cell Signaling Technology) were used at a concentration of 1:1000, and the anti-actin antibody (A1978; Sigma) was used at a concentration of 1:10000.

**MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] cell survival assay**

MTT assay was performed as described previously [26]. Briefly, cells were seeded in a 96-well plate and allowed to grow for 24 h. Inhibitors were then added to the culture medium. At the indicated time point, cells were incubated with MTT (Sigma) at a concentration of 200 ng/ml for 4 h. The crystalized MTT was then dissolved in DMSO (Sigma) and the absorbance at 595 nm was recorded.
RESULTS

Prolonged inhibition of the MEK/ERK pathway by PD184352 up-regulates Akt phosphorylation

Previously, treatment of MDA MB 231 cells with PD184352 blocked pERK1/2 and enhanced pAkt, for up to 24 h [9]. In the present study, up-regulation of pAkt was observed following PD184352 treatment in MDA MB 231 at 24 h (Figure 1A) but further increased at 48 and 72 h. Conversely, pAkt did not increase substantially following treatment with PD184352 at 48 h, but was increased at 72 h in A549 lung cancer cell line (Figure 1A). In other breast cancer cell lines, including Hs578t and MDA MB 157, but not T47D cells, prolonged PD184352 treatment also increased pAkt (Figure 1B). These results suggest that inhibition of the MEK/ERK pathway feeds back on to the PI3K/Akt pathway, in a cell-line-specific manner.

Inhibition of PI3K/Akt/mTORC1 pathway up-regulates ERK1/2 phosphorylation

In both MDA MB 231 breast and A549 lung cancer cell lines, at 24, 48 and 72 h post-treatment, PI103 inhibited pAkt and increased pERK (Figures 1C and 1D). This was recapitulated with siRNA (small interfering RNA) targeting of Akt in MDA MB 231 cells, where reduced Akt expression also stimulated pERK (see Supplementary Figure S1 at http://www.bioscirep.org/bsr/032/bsr0320413add.htm).

Feedback between the PI3K/Akt and MEK/ERK pathways is cell-line-specific

To assess whether cross-talk is conserved in cancer cells of different origins, the effect of combining PD184352 with either PI103 or rapamycin was examined in breast (MDA MB 231, MDA MB 157 and Hs578t), lung (A549), prostate (DU145) and oesophageal (EC109) cancer cell lines. As expected, treatment with PD184352 inhibited pERK in all cell lines examined.
Inhibition of pERK up-regulated pAkt in MDA MB 231 and MDA MB 157 cells, but less so in Hs578t, A549 and DU145 cells (Figures 2A–2F). Surprisingly, in the oesophageal cancer cell line EC109, a significant down-regulation of pAkt was observed and was concomitant with decreased Akt protein expression.

Conversely, treatment with PI103 resulted in inhibition of pAkt in all the cell lines examined, as expected. It also led to an increase in pERK in MDA MB 231, MDA MB 157, Hs578t, A549 and EC109 cells, but not in DU145 cells (Figures 2A–2F). Inhibition of mTORC1 can reportedly activate the PI3K/Akt and MEK/ERK pathways [27,30,31] in some cells, but inhibits the PI3K/Akt pathway in others [32]. Rapamycin treatment slightly increased pERK, without affecting pAkt in MDA MB 231 cells (Figure 2A). However, in MDA MB 157, A549 and EC109 cells, treatment with rapamycin resulted in increased pAkt, but pERK was unaffected (Figures 2B, 2D and 2F), further suggesting that class I PI3K inhibition is required to enhance pERK phosphorylation in these cell lines. In Hs578t cells, rapamycin treatment increased pAkt, while pERK was decreased (Figure 2C). Finally, in DU145 cells, rapamycin treatment had minimal effects on either pAkt or pERK (Figure 2E). Together, our results suggest that the PI3K/Akt and MEK/ERK negative-feedback loops are cell line/genetic-context-specific.

**Reduced cell survival following combined blockade of both pathways**

We next investigated whether inhibition of both of these pathways affected cell survival. In MDA MB 231 and A549 cells, in addition to decreased pERK and Bcl-X<sub>L</sub> expression, cyclin D1 expression was also decreased by PD184352 treatment and completely abolished when PD184352 was combined with PI103 (Figures 2G and 2H). Treatment with PD184352 and PI103, but not rapamycin, led to PARP and caspase 3 cleavage in both cell lines as early as 24 h (Figures 2G and 2H). However, a combined treatment of rapamycin and PD184352 in A549 cells resulted in a subtle increase in PARP cleavage, but not caspase 3, compared with treatment with either inhibitor alone (Figure 2H). These results suggest that inhibition of both PI3K and MEK is required for the observed synergistic activation of the apoptotic signalling pathway.

Cell survival was assessed by MTT assay to determine whether treatment of cells with PD184352 alone, or in combination with PI103 or rapamycin was inhibitory. PD184352 treatment reduced cell survival by 50% in MDA MB 231 cells (Figure 2I), but not A549 cells (Figure 2J). Rapamycin treatment did not affect survival of either MDA MB 231 or A549 cells, but when combined with PD184352, survival was slightly reduced compared with PD184352 alone, in both cell lines (Figures 2I and 2J). PI103 treatment severely impaired cell survival in MDA MB 231 and A549 cells by 60 and 80% respectively, and this was further reduced when combined with PD184352 treatment (Figures 2I and 2J). Combined treatment with PI3K and MEK inhibitors is more efficient at inhibiting cell survival and promoting apoptosis.

**K-Ras status affects feedback between the PI3K/Akt and MEK/ERK pathways**

K-Ras mutations drive hyper-activation of the MEK/ERK pathway to increase cell proliferation and are associated with drug resistance in most human cancers. Treatment of K-Ras-activating mutant HCT116 cells with PD184352, with or without serum, inhibited pERK and up-regulated pAkt at 24 and 48 h (Figures 3A–3C). Conversely, PI103 inhibited pAkt but also increased pERK (Figures 3A–3C, and Supplementary Figure S2 at http://www.bioscirep.org/bsr/032/bsr0320413add.htm). In Hkh-2 cells that are K-Ras wild-type treatment with PD184352 also down-regulated pERK and enhanced pAkt in the presence (Figures 3D and 3E) or absence (Figure 3F) of serum. However, in Hkh-2, irrespective of serum withdrawal, PI103 down-regulated pAkt while pERK was unaffected (Figures 3D–3F, and Supplementary Figure S2). Similar results were also observed in a second colorectal isogenic cell line pair, DLD1/DKO3, where DKO3 also showed no sensitivity to PI103, unlike DLD1 cells (see Supplementary Figure S3 at http://www.bioscirep.org/bsr/032/bsr0320413add.htm). Thus the K-Ras mutation is required for the activation of MEK/ERK pathway upon PI103-dependent inhibition of the PI3K/Akt pathway.

**K-Ras status affects cell apoptosis and survival**

Western blot analysis of PARP and caspase 3 cleavage showed that K-Ras wild-type Hkh-2 cells were more sensitive to PI103 than K-Ras mutant HCT116 cells (Figure 3). Thus sensitivity of the HCT116/Hkh-2 isogenic cells to treatment with PD184352, PI103 and both drugs together was assessed in a cell survival assay. Treatment with PD184352 alone reduced the percentage of surviving cells in both cell lines to a similar extent, and this was more obvious at 48 h (Figure 4). Similarly, PI103 also reduced cell survival but more potently in Hkh-2 than HCT116 cells and this was also more apparent at 48 h. Combined treatment with PI103 and PD184352 at 48 h also had a more profound effect on cell survival in Hkh-2 cells (Figure 4). Thus a K-Ras activating mutation confers resistance to PI103 and a combinatorial inhibition of both the PI3K/Akt and MEK/ERK pathways may enhance the apoptotic response in cancer cells harbouring a K-Ras activating mutation.

**c-Met amplification affects drug responses in HCC827 lung cancer cells**

The HCC827 lung cancer cell line and its gefitinib-resistant derivative GR5 cell line (amplified c-Met), were used to investigate the effect of c-Met amplification on drug sensitivity. Amplification of c-Met in HCC827 (GR5) cells caused up-regulation of pAkt and pERK (Figure 5A). Treatment with PD184352 suppressed pERK and up-regulated pAkt in both cell lines, irrespective of c-Met amplification (Figure 5A). However, PI103 treatment down-regulated pAkt, but did not up-regulate pERK in HCC827 cells, unlike GR5 cells (Figure 5A). Thus c-Met amplification sensitizes cells to MEK/ERK pathway activation following inhibition of the PI3K/Akt/mTORC1 pathway by PI103 and renders them...
Cross-talk between the PI3K/Akt and the MEK/ERK pathways

Figure 2  Differential sensitivities to drug-induced apoptosis in cancer cell lines

(A–F) Akt and ERK phosphorylation and expression of PARP in (A) MDA MB 231 (breast), (B) MDA MB 157 (breast), (C) Hs578t (breast), (D) A549 (lung), (E) DU145 (prostate) and (F) EC109 (oesophageal) cancer cell lines treated with PD184352 and PI103 alone or in combination. (G and H) Akt and ERK phosphorylation and expression of PARP, cleaved caspase 3, Bcl-XL and cyclin D1 in (G) MDA MB 231 and (H) A549 cells treated with various combinations of PD184352, PI103 and rapamycin. MTT cell survival assay for (I) MDA MB 231 and (J) A549 cancer cells treated with various combinations of PD184352, PI103 and rapamycin. Representative of three independent experiments, error bars are S.D.
Figure 3 K-Ras status affects response to PD184352 and PI103
Expression of PARP, pAkt, pERK, cleaved caspase 3 in K-Ras mutant HCT116 or wild-type Hkh-2 cells treated with PD184352 or PI103 for 24 (A and D) and 48 h (B and E) in the presence of serum or in serum-free conditions (C and F). Representative of three independent experiments.

Figure 4 K-Ras mutation desensitizes HCT116 cells to PI103
MTT cell survival assay in HCT116 and Hkh-2 cells treated with or without PD184352 and PI103 alone or in combination for 24 and 48 h. Representative of three independent experiments, error bars are S.D.

Inhibition of mTORC1 by rapamycin alone increased pERK in HCC827 cells, but was insufficient to up-regulate pERK in GR5 cells, compared with PI103 (Figure 5B). Combined use of both PD184352 and PI103 blocked both pathways and induced a synergistic induction of apoptosis in both cell lines and this synergistic effect was more potent in GR5 cells (Figure 5B). These results suggest that a combination treatment of PI3K and MEK inhibitors may give rise to a better killing effect in c-Met amplified cells, in which both negative-feedback loops are active.

OA restores PI103-dependent pERK activation in K-Ras wild-type Hkh-2 and c-Met wild-type HCC827 cancer cells
Drug-induced feedback activities can be controlled by either kinase or phosphatase activities. We assessed whether a phosphatase activity is participating in the feedback networks in the two pairs of isogenic cancer cell lines. In wild-type K-Ras harbouring Hkh-2 cells and c-Met normal HCC827 cells, PI103 treatment did not increase pERK, while PI103 did in their K-Ras mutant and c-Met amplified counterparts. PI103-induced pERK in HCT116 and GR5 cells was substantially increased in the presence of OA, although OA also increased basal pERK in both control and rapamycin-treated cells (Figures 6A and 6C). In Hkh-2 and HCC827 cells, OA also increased basal pERK in both control and rapamycin-treated cells, but in contrast, while
PI103 alone failed to induce pERK, co-incubation of PI103 with OA further increased pERK compared with control (Figures 6B and 6D). These results suggest that the intrinsic phosphatase activities may be required to suppress the PI3K/Akt to MEK/ERK feedback loop.

**DISCUSSION**

In the present study, we provide evidence for cell-line-specific feedback mechanisms that predict sensitivity to combined therapeutic targeting of the PI3K/Akt/mTORC1 and Ras/MEK/ERK signalling pathways. Using K-Ras active/wild-type isogenic cells and c-Met amplified cells, we reveal that activating K-Ras mutations and c-Met amplification facilitate enhanced activation of the Ras/MEK/ERK pathway, following inhibition of the PI3K/Akt/mTORC1 pathway. Activation of this feedback mechanism appears to be required for protection from apoptosis and hence cell survival in the presence of PI103 alone, since combined treatment with PI103 and PD184352 abrogates this cytoprotective effect. In addition, the importance of phosphatase(s) in this regulatory loop has also been demonstrated in the present study. Our results show that determination of both genetic...
background and whether these feedback mechanisms are active in cancers will inform treatment decisions. Mechanistically, we show that inhibition of the PI3K/Akt/mTORC1 pathway leads to activation of the Ras/MEK/ERK signalling cascade and this is dictated by K-Ras and c-Met status and protein phosphatase activity.

Active K-Ras harbouring HCT116 cells are more resistant to inhibition of PI3K/Akt/mTORC1 pathway than its wild-type K-Ras derivative Hkh-2 cells, which do not exhibit increased pERK following PI103 treatment. Similar results were observed in another isogenic cell line pair, DLD1/DKO3, where DKO3 also showed no sensitivity to PI103, unlike DLD1 cells (Supplementary Figure S3). HCT116 parental cells harbour a PIK3CA gain-of-function mutation, whereas DLD1 parental cells do not, suggesting that K-Ras mutations dictate sensitivity to PI103. Indeed, this is consistent with a mouse lung cancer model that is driven by mutant K-Ras; the tumour was not substantially responsive to a dual PI3K and mTORC1 inhibitor, NVP-BEZ235, but was synergistically inhibited by combined treatment of both NVP-BEZ235 and a MEK inhibitor, ARRY-142886 [33]. Thus we may have provided a molecular mechanism that explains the observation reported by Engelman et al. We propose that cancer cells harbouring active K-Ras mutations are able to activate ERK in response to PI3K/Akt/mTORC1 pathway inhibition. This negative-feedback loop is cytotoxic, thus combined inhibition of both pathways abrogates the negative-feedback loop, resulting in synergistic induction of apoptosis. Our results suggest that the efficacy of PI103 or other PI3K/Akt/mTORC1 pathway inhibitors would be greater in K-Ras wild-type patients.

Amplified c-Met signalling promotes cancer cell survival by activating both the PI3K/Akt/mTORC1 and the Ras/MEK/ERK pathways [34]. In a recent report, an HGF (human growth factor)-stimulated (c-Met agonist), gefitinib (EGFR (epidermal growth factor receptor) inhibitor) resistant, xenograft model for lung cancer was shown to be resistant to PI103 alone but was highly sensitive to combined treatment with PI103 and gefitinib [35]. Their results suggest that enhanced methionine receptor signalling following HGF treatment in lung cancer cells results in resistance to the PI3K/Akt/mTORC1 pathway by PI103. We show that c-Met amplification in GR5 cells generates a negative-feedback loop from the PI3K/Akt/mTORC1 pathway to the Ras/MEK/ERK pathway, which is absent in the parental isogenic HCC827 cells. Importantly, GR5 cells are also more resistant to inhibition of the PI3K/Akt/mTORC1 pathway compared with HCC827 cells. We further show that combined inhibition of these pathways, by PI103 and PD184352, synergistically induces the apoptotic pathway in GR5 cells. Our results, therefore suggest that c-Met amplification and signalling may be a biomarker for determining the sensitivity of lung cancers to PI3K/Akt/mTORC1 inhibitors and that a combinatorial inhibition of these pathways will be more effective in lung cancers with c-Met amplification.

The Ras/MEK/ERK pathway is regulated by various phosphatases, including VHR (vaccinia H1-related) phosphatase [36], protein phosphatase 5 [37], MAPK phosphatase [38] and DUSP6 (dual-specific phosphatase 6) [39]. DUSP6 is also involved in cross-talk between mTORC1 and the Ras/MEK/ERK pathway [40]. In the present study, we show that the PP1 and PP2A/B phosphatase inhibitor, OA, restored the PI103-dependent increase in pERK in K-Ras wild-type and c-Met normal cells. This may be through accumulation of pAkt; however, PI103 acts on both PI3K, upstream of Akt and also on mTORC1 and mTORC2, while siRNA of Akt increased pERK, at least in MDA MB 231 cells. Thus accumulation of Akt phosphorylation, and therefore increased Akt activity, as a result of phosphatase inhibition is unlikely to cause increased pERK. Thus we propose that a protein phosphatase(s) activity is present or activated in K-Ras wild-type and c-Met normal cells preventing feedback from the PI3K/Akt/mTORC1 pathway to the Ras/MEK/ERK pathways. Conversely, this phosphatase activity is inhibited in K-Ras mutant or c-Met amplified cells, thereby preventing attenuation of ERK phosphorylation. Furthermore, identification of the phosphatase(s) activity that is involved may provide a useful biomarker that could distinguish PI3K/Akt/mTORC1-inhibitor-sensitive and-insensitive cancers and whether a combined inhibition of both pathways could be beneficial to the patients.

In conclusion, we report the importance of K-Ras mutation or c-Met amplification in controlling feedback from the PI3K/Akt/mTORC1 to Ras/MEK/ERK pathways. We have tested a number of cell lines of breast and colorectal origin and the phenomenon of cross-talk appears to be common, at least in the cell types we have examined. We argue that genetic subtyping of primary tumours for K-Ras mutation or c-Met amplifications will be valuable predictors of tumour response to PI3K/Akt/mTORC1 pathway inhibition. This approach will allow for patient stratification and tailored therapeutic choices, ultimately improving patient response rates and outcome.

**AUTHOR CONTRIBUTION**

Hiu-Fung Yuen did the majority of the experimentation and wrote the paper. Olga Abramczyk, Grant Montgomery and Ka-Kui Chan did additional experimental work. Yu-Han Huang, Takehiko Sasazuki, Senji Shirasawa, Srivastava Gopeh, Rkow-Wah Chan, Dean Fennell and Pasi Janne provided reagents and critical comments on the paper, Mohamed EI-Tanani and James Murray devised the hypothesis and experimental plan, and wrote the paper.

**FUNDING**

This study was supported by Cancer Research, U.K. (China Fellowship to H.-F.Y.) and Almac Discovery, U.K. (an exploratory grant to O.A.)

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Received 22 May 2012/29 May 2012; accepted 30 May 2012
Published as Immediate Publication 6 June 2012, doi 10.1042/BSR20120050
SUPPLEMENTARY ONLINE DATA

Impact of oncogenic driver mutations on feedback between the PI3K and MEK pathways in cancer cells

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Figure S1 Effect of knockdown of Akt by siAkt on total Akt and ERK phosphorylation
Akt was knocked down in MDA MB231 breast cancer cell line by siAkt (Cell Signaling Technology). Expression levels of total Akt, total and phosphorylated ERK and actin were examined by Western immunoblotting. Akt expression was almost completely abolished and ERK phosphorylation, but not the total ERK, was increased.

Figure S2 Effect of inhibitors on Akt and ERK1/2 phosphorylation
HCT116 and Hkh-2 cells were treated with various combinations of PI103, PD184352 and rapamycin. Phosphorylation of Akt and ERK was examined by Western immunoblotting. Increased ERK phosphorylation was observed following PI103 treatment in HCT116, but not Hkh-2, cells. Co-treatment with PI103 and PD184352 abolished Akt and ERK phosphorylation.
Figure S3 K-Ras status affects response to PD184352 and PI103 in a second colorectal cancer isogenic pair

Expression of PARP, pAkt, pERK, cleaved caspase 3 in K-Ras mutant DLD1 or wild-type DKO3 cells treated with PD184352 or PI103 for 24 h (A and D) and 48 h (B and E) in the presence of serum or in serum-free conditions (C and F). Representative of three independent experiments.