Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition

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Abstract: Combinations of MEK and PI3K inhibitors have shown promise in pre-clinical cancer models, leading to the initiation of clinical trials co-targeting these two key cancer signaling pathways. GDC-0973, a novel selective MEK inhibitor, and GDC-0941, a Class I PI3K inhibitor, are in early stage clinical trials as both single agents and in combination. The discovery of these selective inhibitors has allowed investigation into the precise effects of combining inhibitors of two major signaling branches downstream of RAS. Here, we investigated multiple biomarkers in the MAPK and PI3K pathway to search for points of convergence that explain the increased apoptosis seen in combination. Using washout studies in vitro and alternate dosing schedules in mice, we showed that intermittent inhibition of the PI3K and MAPK pathway is sufficient for efficacy in BRAF and KRAS mutant cancer cells. The combination of GDC-0973 with the PI3K inhibitor GDC-0941 resulted in combination efficacy in vitro and in vivo via induction of biomarkers associated with apoptosis, including Bcl-2 family pro-apoptotic regulators. Therefore, the data suggest that continuous exposure of MEK and PI3K inhibitors in combination is not required for efficacy in preclinical cancer models, and that sustained effects on downstream apoptosis biomarkers can be observed in response to intermittent dosing.

Introduction: The ERK/MAPK signaling cascade transduces multiple proliferation and differentiation signals within the cell via activation of the RAS GTPase and subsequent sequential activation of RAF, MEK, and ERK kinases. Aberrant regulation of this pathway contributes to many hallmarks of cancer cells, including uncontrolled proliferation, invasion, metastasis, angiogenesis, and evasion of apoptosis (1, 2). Inhibition of MEK (MAPK/ERK Kinase) is a promising strategy in the development of oncology therapeutics to control the growth of tumors that are dependent on aberrant ERK/MAPK pathway signaling (3-5). The ERK/MAPK pathway is upregulated in a large fraction of tumors, in part due to mutation of several components of the pathway. Oncogenic activating mutations have been identified in KRAS (22% of all cancers), NRAS (8%), HRAS (3%) and BRAF (20%) (COSMIC database; ref. 6). Activating BRAF
mutations, the majority of which are a single amino acid substitution (V600E) in the activation loop of the kinase (6, 7), are prevalent in malignant melanomas (66%) and are also found in colon cancer (15%) and thyroid papillary carcinoma (27%). Cancer cells transformed by BRAF V600E are highly sensitive to MEK1/2 inhibition (4, 8). In addition to RAS and RAF mutations, many tumors are activated by amplification or overexpression of upstream pathway components. Therefore, MEK1/2 inhibitors may have broad utility in tumors with MAPK pathway alterations.

PI3K–AKT pathway activation has been implicated in several types of cancer (9, 10). Activating and transforming mutations in the p110α subunit of PI3K are commonly found in multiple tumor types (11-13). In addition, the pathway is activated in numerous types of cancer by receptor tyrosine kinase signaling, RAS mutations, or the loss of the phosphatase PTEN (10).

Targeting either of these pathways individually can attenuate signaling and has been shown to be efficacious in animal models (14, 15). However, in some tumors, cell proliferation and survival are driven through multiple effector pathways, such as in tumors with concurrent activation of the RAS and PI3K pathways, as is seen in a subset of melanoma, lung and colorectal cancers. Targeting both of these pathways is significantly more efficacious in preclinical models than targeting either pathway alone (15, 16). Pathway feedback, resulting in activation of parallel pathways in response to targeted inhibitors, can also contribute to combination efficacy (15-18).

GDC-0973 is a novel small molecule inhibitor of MEK that is potent and highly selective. The results presented here show that when GDC-0973 is combined with the PI3K inhibitor GDC-0941 in cancer models, it results in combination efficacy in vitro and in vivo via induction of biomarkers associated with apoptosis, including Bcl-2 family pro-apoptotic regulators (19). These findings suggest that intermittent dosing regimens may be efficacious for combinations of MEK and PI3K inhibitors in the clinic, and that sustained exposure to inhibitors may not be required for maximal combination efficacy.
Materials and Methods:

Cell line single nucleotide polymorphism (SNP) fingerprinting: SNP genotypes were performed for all cell lines except 88MEL, DU-145.X1, and NCI-H520.X1. Cells were obtained from ATCC. Cell line identity was verified by high-throughput SNP genotyping using Illumina Golden gate multiplexed assays. SNPs were selected based on minor allele frequency and presence on commercial genotyping platforms. SNP profiles were compared to SNP calls from the Sanger database to confirm ancestry for the following cell lines: 22rv1, A2058, A375, A549, Calu-6, FaDu, HCT-116, Lovo, MiaPaCa-2, NCI-H2122, NCI-H441, NCI-460, and SKOV-3.

Cell viability assays. GDC-0941 and GDC-0973 were obtained from the chemistry department at Genentech, Inc. (South San Francisco, CA). Cell viability and synergy assays were performed as previously described (18, 20). To ascertain the role of Bim in apoptosis mediated by GDC-0941 and GDC-0973, short interfering RNA (siRNA) oligonucleotides were obtained from Dharmacon RNAi Technologies (Chicago, IL). The quantity of cytoplasmic histone-associated DNA fragments was quantified using the Cell Death Detection ELISA Plus kit from Roche (Mannheim, Germany) according to the manufacturer’s instructions.

Immunoblotting. To prepare extracts, cells were washed once with cold PBS and lysed in 1X Cell Extraction Buffer (Biosource, Carlsbad, CA) as described by the manufacturer. For frozen tumor samples, tumors were pulverized on dry ice using a small Bessman tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA) and prepared as described by the manufacturer.

Antibodies to cleaved PARP, pAKT, pERK, pS6, Bim and cyclin D1 were obtained from Cell Signaling (Danvers, MA). The β-actin and GAPDH antibodies were obtained from Sigma (St. Louis, MO). Specific antigen-antibody interaction was detected with a HRP-conjugated secondary antibody IgG using ECL detection.
reagents (Amersham Biosciences, Pittsburgh, PA). The pERK and tERK band intensities were quantified using the LI-COR Odyssey® immunoblotting system (Lincoln, NE).

Pharmacodynamic assays. Frozen tumors were pulverized on dry ice using a small Bessman tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA) and prepared according to the manufacturer. For subsequent Western blot analysis, proteins were resolved by 4-12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Corporation, Billerica, MA) and immunoblotting performed as described above.

Tumor and Body Weight Measurement. Tumor volumes were determined using digital calipers (Fred V. Fowler Company, Inc.) using the formula \( \frac{L \times W}{2} \). Tumor growth inhibition (%TGI) was calculated as the percentage of the area under the fitted curve (AUC) for the respective dose group per day in relation to the vehicle, such that \( \%TGI = 100 \times \frac{1}{1 - \left(\frac{AUC_{treatment/day}}{AUC_{vehicle/day}}\right)} \). Curve fitting was applied to Log2 transformed individual tumor volume data using a linear mixed-effects model using the R package nlme, version 3.1–97 in R v2.12.0.

Results:

GDC-0973 and GDC-0941 are selective inhibitors of MEK and PI3K
GDC-0973 is a potent and highly selective inhibitor of MEK (Figure 1A), with a biochemical IC\(_{50}\) of 4.2 nM against MEK1. In biochemical assays, GDC-0973 demonstrated >100-fold selectivity for MEK when tested against a panel of >100 serine-threonine and tyrosine kinases (Supplementary Table 1).

GDC-0973 shows strong cellular potency in a broad panel of tumor types, particularly in BRAF or KRAS mutant cancer cell lines (Figure 1B; Supplementary
Table 2). In a panel of cancer cell lines, 80% of BRAF mutant lines (including V600E and non-V600E mutations) were sensitive to GDC-0973, 54% of lines carrying oncogenic mutations in KRAS or NRAS were sensitive, and 35% of the remaining lines were sensitive. As not all RAF and RAS mutant lines are sensitive to MEK inhibition, and not all WT lines are resistant, there are clearly additional genetic factors affecting sensitivity and resistance to MEK inhibition.

GDC-0941 is a potent inhibitor of Class I PI3K isoforms with biochemical IC₅₀s of 3-75 nM for the four Class I isoforms of PI3K (14). This compound demonstrates excellent selectivity against mammalian target of rapamycin (mTOR), DNA-PK, and a panel of >228 kinases (14). GDC-0941 shows strong inhibition of tumor cells in vitro and in vivo, especially those with activated PI3K pathway signaling (21). In this cell line panel, 63% of cancer cell lines with oncogenic mutations in PIK3CA were sensitive to GDC-0941 (Supplementary Table 2). A higher percentage of RAF and RAS mutant cell lines are resistant to GDC-0941 than RAF and RAS WT cell lines (Supplementary Figure 1).

GDC-0973 has anti-tumor efficacy in BRAF and KRAS mutant human xenograft tumor models

GDC-0973 demonstrated activity in a number of human tumor xenograft models, particularly in models mutant for BRAF. Two dose-ranging efficacy studies in BRAF mutant and KRAS mutant human xenograft models are shown in Figure 2. In the A375.X1 BRAF<sup>V600E</sup> mutant melanoma xenograft model, treatment with doses of GDC-0973 above 3 mg/kg led to strong tumor growth inhibition (Fig 2A). In the NCI-H2122 KRAS<sup>G12C</sup> mutant NSCLC xenograft model, treatment with up to 5 mg/kg GDC-0973 led to moderate tumor growth inhibition and at 10 mg/kg approached tumor stasis (Fig 2B). GDC-0973 was tested in additional xenograft tumors with different genetic backgrounds and showed varying degrees of tumor growth inhibition across tumor types and genotypes (Table 1). There was a high
degree of sensitivity to GDC-0973 in BRAF\textsuperscript{V600E} mutant melanoma tumor models, with strong tumor inhibition often observed at low doses and regression at high doses. An exception was the A2058 BRAF mutant model, where the loss of PTEN may be a resistance factor (8). Aside from BRAF\textsuperscript{V600E} mutant models, there was no strict correlation of tumor inhibition with genotype. In particular, maximal tumor inhibition for KRAS mutant models ranged from 9-108% and tumor inhibition in KRAS WT models ranged from 21-96%, again suggesting that additional genetic factors contribute to the sensitivity to single agent MEK inhibition.

Pharmacodynamic (PD) studies were performed using the A375.X1 and NCI-H2122 xenograft models in which mice were treated with three daily doses of GDC-0973 at 1, 3, 5 or 10 mg/kg. Tumor samples were collected at 2, 8 and 24 hours post last dose and analyzed for phospho-ERK1/2 (pERK) and total ERK1/2 (tERK) levels. Band intensities were quantified and plotted relative to vehicle treated mice (time 0 hours). In the A375.X1 model, dose- and time-dependent suppression of pERK was observed, with the 10 mg/kg dose of GDC-0973 suppressing pERK 75% at 2, 8 and 24 hours (Figure 2C, Supplementary Figure 2). In the NCI-H2122 model, GDC-0973 inhibited pERK 83% at 8 hours and 73% at 24 hours (Figure 2D, Supplementary Figure 2). Lower doses of GDC-0973 demonstrated weaker suppression of pERK over time and dose-dependency consistent with the single agent anti-tumor activity of GDC-0973 in the A375.X1 and NCI-H2122 models, respectively.

GDC-0973 combined with the PI3K Inhibitor GDC-0941 causes apoptosis that is mediated in part by Bim

Continuous, simultaneous exposure to GDC-0973 and GDC-0941 resulted in dramatic loss of tumor cell viability relative to patient-matched normal cells in vitro (Figure 3A; Supplementary Figure 3). GDC-0941 and GDC-0973 were tested for their in vitro combination efficacy in a panel of melanoma cell lines using a 4-day CellTiterGlo viability assay. GDC-0941 and GDC-0973 act
cooperatively to inhibit the viability of all melanoma cell lines tested, 60% of which carry oncogenic mutations in BRAF (Supplementary Figure 4; Supplementary Table 2). GDC-0941 and GDC-0973 were also tested in a panel of NSCLC cell lines. Since the compounds showed efficacy as single agents in most of the NSCLC cell lines, synergy could be assessed in these lines using CalcuSyn, a program utilizing the Chou and Talalay combination index method of calculating the level of synergy (20). Strong synergy or synergy, as indicated by combination index values ≤ 0.3 or 0.3-0.7, respectively, was observed for 74% (n=35/47) of NSCLC cell lines, of which approximately half carry oncogenic mutations in KRAS (Suppl Table 2). The remaining 26% of lung cancer cell lines could not be evaluated for synergy because one of the compounds showed no efficacy as a single agent. There was no statistically significant genotype dependence of combination efficacy with respect to BRAF or KRAS mutation status in the cell lines tested.

The effects of combined MEK and PI3K pathway inhibition on downstream pathway markers were evaluated in cell lines. Analysis of downstream pathway markers in the 888MEL (BRAF V600E) and A2058 (BRAFV600E) mutant melanoma cell lines showed that treatment with GDC-0973 decreased pERK while treatment with GDC-0941 decreased pAKT in these cell lines (Figure 3B). While GDC-0941 was able to decrease phosphorylation of ribosomal protein S6 (pS6) moderately on its own, pS6 showed greater decrease in response to the combination at both the S235/236 and S240/244 sites in multiple tumor cell lines (Figure 3B, Supplementary Figure 5A). The single agent and combination treatments were also assessed for their effect on apoptotic markers. Treatment with GDC-0973 and GDC-0941 resulted in a combinatorial increase of cellular mediators of apoptosis, such as cleaved poly [ADP-ribose] polymerase (PARP) and alternatively spliced isoforms of Bim (Figure 3B; ref. 19). The majority of cell lines tested showed a stronger response of Bim to GDC-0973 than to GDC-0941, whereas the increase in cleaved PARP was equally dependent on both compounds (Figure 3B, Supplementary Figure 5A), suggesting additional
apoptotic mechanisms are involved. Consistent with expectations based on the published literature (22, 23), combined GDC-0973 and GDC-0941 treatment increased Bim levels via several complementary mechanisms, including gene expression, BimEL phosphorylation, and Bim protein stabilization. Decreases of pS6 and Bim in tumors were also observed in vivo in response to the combination (Supplementary Figure 5B, C). To confirm the functional contribution of Bim in mediating apoptosis induced by MEK and PI3K inhibition, RNA interference studies were performed to ablate Bim in melanoma cells (Figure 3C; Supplementary Figure 6A, B). In the presence of increasing concentrations of GDC-0973 and GDC-0941, selective knockdown of Bim significantly attenuated cell death induced by MEK and PI3K blockade in A2058 melanoma cells (Figure 3C).

**GDC-0973 and GDC-0941 show improved efficacy in combination in BRAF and KRAS mutant xenograft models in vivo**

Similar to in vitro combination results, the combination of GDC-0973 and GDC-0941 showed increased efficacy compared to single agent treatment in multiple human xenograft tumor models (Figure 4, A-D). Four xenograft tumor models harboring BRAF or KRAS mutations with or without alterations in PTEN or PIK3CA were evaluated for response to GDC-0973 and GDC-0941 as single agents and in combination. For the A2058 (BRAFV600E, PTEN-), DLD-1 (KRASG13D, PIK3CAE545K), and NCI-H2122 (KRASG12C) models, doses of GDC-0973 and GDC-0941 were selected to produce moderate single agent activity in each model in order to visualize improvement of efficacy in the combination arms. For the A375 (BRAFV600E) model, all tested doses of GDC-0973 showed regression and all tested doses of GDC-0941 showed lack of tumor growth inhibition, however the combination of GDC-0973 and GDC-0941 led to a greater tumor response rate as GDC-0973 resulted in 70% (7/10) complete responses (CRs), whereas the combination resulted in 100% (10/10) CRs. As a result, upon cessation of treatment tumors were slower to grow back in the combination arm versus the GDC-0973 single agent arm (Figure 4A). In A2058
(Figure 4B), DLD-1 (Figure 4C) and NCI-H2122 (Figure 4D), combination of GDC-0973 and GDC-0941 resulted in stronger tumor suppression than either single agent alone in all models tested (Summarized in Supplementary Table 3). These results show that the combination activity observed in vitro translates into increased combined anti-tumor efficacy in vivo. Combination pharmacokinetic studies showed that there were no drug-drug interactions that impacted the exposure of either compound (24).

**Transient exposure to MEK and PI3K inhibitors results in apoptosis**

Given that exposure to GDC-0973 and GDC-0941 resulted in apoptosis, we hypothesized that transient inactivation of MEK and PI3K signaling could be sufficient to elicit anti-tumor efficacy. To examine this possibility, 888MEL cells were pre-treated with GDC-0973, GDC-0941, or both for 24 hours followed by washout of compounds and serial evaluation of downstream markers over time (Figure 5A). After treatment with single agent GDC-0941, the proximal pathway markers pAKT and pS6 were strongly inhibited but rapidly returned to baseline levels within 2 hours following washout. Treatment with single agent GDC-0973 resulted in a strong decrease of pERK that only partially rebounded after washout. Transient treatment with the combination had identical effects on pERK, pAKT, and pS6 as the single agents. However, there was a combined enhancement of Bim protein accumulation and cleaved PARP. Bim protein levels were modestly increased above baseline in response to single agent GDC-0973, and were maintained for 24 hours after compound washout. This increase was more pronounced in response to combination treatment. Cleaved PARP was modestly induced in response to single agent treatment with GDC-0973 or GDC-0941. It was more strongly increased in response to the combination, although it did not persist through 24 hours after washout. This is perhaps due to detachment of late apoptotic cells from the plates during repeated wash cycles.
To confirm the functional consequences of transient exposure to GDC-0973 and GDC-0941, cell death was assessed using a nucleosomal ELISA apoptosis assay. Transient treatment of 888MEL cells with either single agent GDC-0973 or GDC-0941 resulted in minimal to no apoptosis induction, whereas GDC-0973 plus GDC-0941 resulted in a 5-fold increase in apoptosis (Figure 5B). A comparable extent of apoptosis was observed for cells that were exposed to either a low concentration of the inhibitors for 24 hours or a high concentration for 8 hours. Effects of transient combination treatment on cell proliferation were also observed (Supplementary Figure 7 A,B). To confirm these results in vivo, mice bearing A2058 xenograft tumors were dosed daily with GDC-0941 and GDC-0973 for four days, and then tumors were excised and analyzed serially after the last dose. As expected, pS6 levels returned to baseline by 8 hours after the final dose (Supplementary Figure 7C). Taken together, these results show that continuous pathway suppression is not necessary to elicit sustained effects on proliferation and apoptosis.

Intermittent dosing of MEK and PI3K inhibitors is efficacious in vivo

The cell washout studies producing apoptosis suggested that continuous exposure to the combination of GDC-0973 and GDC-0941 is not required for efficacy. This was tested in the A375.X1, A2058, DLD-1 and NCI-H2122 xenograft tumor models by dosing both GDC-0973 and GDC-0941 on an every third day (Q3D) intermittent schedule (Figure 6A-D). Each tumor model varied in its sensitivity to the two compounds, and doses were chosen to produce modest single agent activity. When both drugs were dosed intermittently on an every third day regimen (Q3D) increased tumor growth inhibition was observed in response to the combination in all four models. While all the models showed some degree of improvement of efficacy in combination, tumor inhibition of the combination differed most from single agent activity in the DLD-1 colon cancer xenograft model, which carries mutations in both KRAS and PIK3CA.
Pharmacodynamic (PD) studies were performed in the A2058 xenograft model to assess the response of downstream markers to intermittent dosing with GDC-0973 and GDC-0941. Briefly, both GDC-0973 and GDC-941 were administered intermittently (Q3D) for a total of 2 doses at 15 mg/kg and 150 mg/kg, respectively. Tumors were harvested at 8 and 72 hours following the second dose and analyzed by Western Blot for various downstream pathway markers. At 8 hours post last dose, intermittent GDC-0973 treatment resulted in robust suppression of phospho-ERK, a mild decrease in Cyclin D1 levels, and an increase in BimS and BimL levels (Figure 6E). GDC-0941 treatment resulted in suppression of phospho-AKT with a modest decrease in phospho-S6 levels, as well as a slight increase in BimS and BimL levels (Figure 6E). Combination treatment resulted in a more pronounced decrease in Cyclin D1 levels at 8 hours as well as a stronger induction of BimS levels (Figure 6E). Mobility of the BIM S, L and EL isoforms was increased, consistent with a more stable and dephosphorylated form of BIM (Figure 3B; 22, 23). At 72 hours post last dose, pERK, pAKT and pS6 levels had returned to baseline levels in both single agent and combination treated tumors (Figure 6F). However, cyclin D1 levels remained lower and Bim levels remained slightly elevated in response to combination treatment. This is consistent with intermittent pathway knockdown resulting in more sustained effects on downstream markers of proliferation and apoptosis.

Similar intermittent efficacy was observed using a different PI3K inhibitor, GDC-0980, when dosed daily (QD) or once a week (QWk) in combination with GDC-0973 (Supplementary Figure 8 A,B). Combination efficacy has also been published with a different MEK inhibitor, PD0325901, when dosed intermittently with GDC-0941 (15). Therefore, intermittent efficacy with MEK and PI3K inhibitors has observed with multiple inhibitors.

**Discussion:**

In the past several years there have been numerous studies published on combinations of targeted agents in preclinical cancer models, and more recently,
combinations of novel targeted agents have begun to be tested in the clinic (25, 26). For inhibitors of the PI3K and MEK pathways, there are currently over half a dozen Phase Ib combination trials in progress combining selective targeted agents against nodes in these pathways (27-30). There are many important questions to be addressed including which nodes in the pathways will be most efficacious and best tolerated in combination, identifying which patients will be most likely to benefit from a particular combination, and which doses and schedules will be optimal for efficacy and tolerability.

Dose and schedule selection can be affected by both the pharmacokinetic profile and in vivo mechanism of combination activity of the two agents. For the selective MEK inhibitor GDC-0973 and Class I PI3K inhibitor GDC-0941, the mechanism of combination activity was shown to be synergistic regulation of cap-dependent translation (pS6, p4E-BP1), cell cycle progression (cyclin D1, p27Kip1), and cellular apoptosis (Figures 3, 5; ref. 31). MEK/PI3K-dependent apoptosis was demonstrated by increased cleavage of PARP and mediated at least in part through increased levels of the proapoptotic Bim protein. Complete rescue of apoptosis was not observed in Bim loss-of-function experiments and this may be due to additional MEK and PI3K effectors that also contribute to survival signaling by these pathways (31-33).

Given that the effects on knockdown of downstream markers have typically been evaluated in cells under constant exposure to compound (34, 35), we tested how transient exposure to GDC-0973 plus GDC-0941 would affect the time course of downstream pathway inhibition and recovery. While the majority of pathway markers returned to baseline levels within two hours after removal of the compounds, Bim remained elevated up through 24 hours after compound removal, demonstrating a sustained elevation of apoptotic activity.

These findings translated in vivo, where pharmacodynamic data demonstrate that pS6 levels in tumors returned to baseline by 8-24 hours after dosing with
GDC-0973 + GDC-0941, indicating recovery of MAPK and PI3K pathway signaling at efficacious doses. Previous data has shown a similar time course for recovery of the pAKT signaling pathway after treatment with GDC-0941 (34). The fact that the Q3D schedule is efficacious suggests that continuous pathway knockdown is not required for tumor growth inhibition, as the compounds are cleared from the system and proximal pathway signaling returned to normal for >24 hours prior to the next dose.

The Q3D schedule used in these studies is a proof of concept that intermittent dosing can be efficacious, and is not suggestive that this is the optimal schedule to be used preclinically or clinically. Several factors will affect the optimal dose and schedule of a combination in the clinic (36). After gaining understanding of the mechanism of combination efficacy, the half-life of each of the compounds must be considered, which differ substantially between mice and humans. The schedule may also be affected by tolerability, thereby affecting the therapeutic index. The PK drivers of efficacy and toxicity may be different and are being assessed by MEK and PI3K pathway inhibitor combination trials, including GDC-0973 and GDC-0941. With efficacy in BRAF and KRAS mutant preclinical models achieved with both continuous and intermittent schedules, it generates multiple paths for clinical investigation.

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References
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Table 1. Summary of single agent activity of GDC-0973 in human xenograft tumors in mice by percent tumor growth inhibition (%TGI).

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Subscript numbers indicates that the value is average by that number of studies.
Figure Legends

Figure 1. GDC-0973 is a selective, potent MEK inhibitor with efficacy in BRAF and RAS mutant cell lines. (A) Chemical structure of GDC-0973. (B) GDC-0973 was tested in a panel of cell lines in 96-hour viability assays. Relative EC$_{50}$ values are plotted and cell lines are grouped by RAF and RAS mutation status. RAF mutant cell lines include those carrying V600E and other BRAF mutations. RAS mutant cell lines carry mutations in codons 12, 13, or 61 of KRAS or NRAS. All other lines are listed as WT. Specific mutations for each cell line are listed in Supplementary Table 2.

Figure 2. GDC-0973 single agent efficacy and PD in BRAF$^{V600E}$ and KRAS mutant tumor models. Dose-ranging efficacy studies were carried out in the (A) A375.X1 and (B) NCI-H2122 tumor xenograft models. GDC-0973 was dosed orally, daily (QD), for 21 consecutive days. Group mean tumor volumes and standard error of the mean (SEM) is shown. Animals taken off study are indicated with a slash mark. Percent body weight change is shown in the lower panels. Percent tumor growth inhibition (%TGI) is listed in the Supplementary Table 3. (A) In the A375.X1 model, 87% TGI was observed at 3 mg/kg, and 106% TGI was observed at 5 mg/kg. (B) In the NCI-H2122 model, 49% TGI was observed at 5 mg/kg, which approached stasis at 80% TGI at 10 mg/kg. Dose ranging PD studies were performed in the (C) A375.X1 and (D) NCI-H2122 models in which GDC-0973 was dosed at 1, 3, 5 and 10 mg/kg, QD x 3 days. Tumor phospho- and total-ERK-1/2 (pERK and tERK, respectively) was quantified by Western Blot as described in Materials and Methods and shown in Supplementary Figure 2.

Figure 3. Combination of GDC-0973 + GDC-0941 results in reduced viability, pathway inhibition, and increased apoptosis. (A) The 888MEL and A2058 BRAF mutant melanoma cell lines were treated with increasing concentrations of
GDC-0973 and GDC-0941 as single agents and in combination and assayed in a 96-hour viability assay. The highest drug concentrations corresponded to 4X EC50 doses for 888MEL (0.2 μM GDC-0973, 10 μM GDC-0941) and A2058 (10 μM GDC-0973, 10 μM GDC-0941) cells. (B) Top panels – Melanoma cells were treated with 1X EC50 concentration of MEK and PI3K inhibitors for 24 hours (888MEL: 0.05 μM GDC-0973, 2.5 μM GDC-0941; A2058: 2.5 μM GDC-0973, 2.5 μM GDC-0941) and protein lysates were analyzed by immunoblot. Bottom panels – RNA was extracted from the melanoma cells treated with 1X EC50 drug concentration for 16 hours and analyzed by Taqman gene expression assay. Bim RNA levels are normalized to GAPDH. (C) A2058 cells were treated with distinct siRNA oligonucleotide pools to Bim for 72 hours and subsequently treated for 24 hours with the indicated concentrations of GDC-0941 or GDC-0973 and analyzed by Cell Death Detection ELISA assay. Differences in cell death induction by GDC-0973 and GDC-0941 combination in the presence or absence of Bim were statistically significant (p < 0.05).

Figure 4. GDC-0973 and GDC-0941 combination results in tumor growth inhibition when dosed daily. In vivo combination efficacy studies were run in four tumor xenograft models: (A) A375 (BRAFV600E), (B) A2058 (BRAFV600E, PTEN’), (C) DLD-1 (K-RasG13D, p110αE545K), and (D) NCI-H2122 (K-RasG12C). Group mean tumor volumes and standard error of the mean (SEM) is shown. Animals taken off study are indicated with a slash mark. Vehicle (black open circles and lines), GDC-0973 (red triangles and lines), GDC-0941 (blue circles and lines), or a combination of the two drugs (green diamonds and lines) were dosed orally, daily (QD) for 21 consecutive days at the indicated doses labeled in the panels. Percent tumor growth inhibition (%TGI) is listed in the Supplementary Table 3. In the A375 study, mice treated with single agent GDC-0973 had a 70% complete response (CR) rate, whereas mice treated with the combination had a 100% CR rate.
Figure 5. Transient treatment of GDC-0973 + GDC-0941 results in apoptosis and prolonged accumulation of Bim. A. 888MEL cells were treated with 0.05 μM GDC-0973 and 5 μM GDC-0941 as single agents or in combination for 24 hours and then washed to remove the compounds. Lysates were made at subsequent time points after wash out (2 and 24 hours) and analyzed by immunoblot. B. 888MEL cells were treated with a 1XEC₅₀ concentration of GDC-0973 and/or GDC-0941 for 24 hours or a 4XEC₅₀ concentration for 4 hours prior to exchanging media to remove the compounds. Cells were analyzed at the 24 hours time point for apoptosis using the Cell Death Detection ELISA assay.

Fig 6. GDC-0973 and GDC-0941 combination results in tumor growth inhibition when dosed intermittently. In vivo combination efficacy studies were run in four tumor xenograft models: (A) A375.X1 (BRAF^{V600E}), (B) A2058 (BRAF^{V600E}, PTEN⁻), (C) DLD-1 (K-Ras^{G13D}, p110α^{E545K}), and (D) NCI-H2122 (K-Ras^{G12C}). Group mean tumor volumes and standard error of the mean (SEM) is shown. Animals taken off study are indicated with a slash mark. Vehicle (black open circles and lines), GDC-0973 (red triangles and lines), GDC-0941 (blue circles and lines), or a combination of the two drugs (green diamonds and lines) were dosed orally, every third day (Q3D) for 21 consecutive days at the indicated doses labeled in the panels. Percent tumor growth inhibition (%TGI) is listed in Supplementary Table 3. Tumor PD studies were performed in the A2058 xenograft tumor model at 8 hours (E) and 72 hours (F) post last dose. Briefly, tumor-bearing mice were treated for a total of 2 doses of GDC-0973 and GDC-0941 at 15 mg/kg and 150 mg/kg, respectively, both given Q3D. Multiple downstream response markers were assessed by Western blot.
Figure 1

A

B

Cellular Viability - EC50 µM

RAF Mut  RAS Mut  WT
Figure 2

A375.X1 (BRAF<sup>V600E</sup>)

NCI-H2122 (KRAS<sup>G12C</sup>)

Vehicle
GDC-0973 (1 mg/kg, PO, QD)
GDC-0973 (3 mg/kg, PO, QD)
GDC-0973 (5 mg/kg, PO, QD)

Mean Tumor Volume (mm<sup>3</sup>)

Body Weight Change (%)

Day

Mean Tumor Volume (mm<sup>3</sup>)

Body Weight Change (%)

Day

GBC-0973 (1 mg/kg PO QD x3)
GBC-0973 (3 mg/kg PO QD x3)
GBC-0973 (5 mg/kg PO QD x3)
GBC-0973 (10 mg/kg PO QD x3)

PERK/ERK (% of control)

Time (hr)
Figure 3

A. 888MEL (BRAF^{V600E})

B. 888MEL and A2058

C. A2058 (BRAF^{V600E}, PTEN-)

Cell viability (normalized)

Drug concentration (μM)

Cell viability (normalized)

Drug concentration (μM)

Cell viability (normalized)

Drug concentration (μM)

Control siRNA

Bim siRNA pool

Drug treatment (EC_{50} value)

Drug treatment (EC_{50} value)

Drug treatment (EC_{50} value)
Figure 4

QD-QD Dosing
Vehicle
GDC-0973
GDC-0941
Combination

A375 (BRAF<sup>V600E</sup>)
3 + 100

A2058 (BRAF<sup>V600E</sup>, PTEN<sup>-</sup>)
6 + 30

DLD-1 (KRAS<sup>G13D</sup>, PIK3CA<sup>E545K</sup>)
5 + 100

NCI-H2122 (KRAS<sup>G12C</sup>)
5 + 75
Figure 5

A

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- p-AKT(T308)
- p-ERK1/2
- p-S6(S235/236)
- cl-PARP
- Bim
- Actin

B

NucELISA cell death (RLU)

- 1X EC_{50}, 24 h
- 4X EC_{50}, 4 h

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Figure 6

Q3D-Q3D Dosing
Vehicle
GDC-0973
GDC-0941
Combination

A375.X1 (BRAFV600E)

A2058 (BRAFV600E, PTEN+)

DLD-1 (KRASG12D, PIK3CAE545K)

NCI-H2122 (KRASG12C)

A2058 (BRAFV600E, PTEN+)

GDC-0973 -0941 Combo

2 hrs post-dose

E

Vehicle 0973 -0941 Combo

p-ERK1/2
ERK1/2
p-AKT(T308)
AKT
p-S6(S235/236)
S6
Cyclin D1
BimEL (23kD)

BimL (15kD)
BimS (12kD)
Actin
GAPDH

A2058 (BRAFV600E, PTEN+)

GDC-0973 -0941 Combo

72 hrs post-dose

F

Vehicle 0973 -0941 Combo

p-ERK1/2
ERK1/2
p-AKT(T308)
AKT
p-S6(S235/236)
S6
Cyclin D1
BimEL (23kD)

BimL (15kD)
BimS (12kD)
Actin
GAPDH
Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition

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