A potent combination of the novel PI3K inhibitor, GDC-0941, with imatinib in gastrointestinal stromal tumor xenografts: long-lasting responses after treatment withdrawal

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Running title: In vivo efficacy of PI3K inhibitor in GIST

Keywords: GDC-0941, gastrointestinal stromal tumors, PI3K

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Grants support and potential conflicts of interest: This work is supported by research grants from: the Fonds voor Wetenschappelijk Onderzoek Vlanderen (FWO grant # G. 0510.06 - P. Schöffski), NIH GI SPORE (1P50CA12703-05 - J. Fletcher), and Life Raft Group (M. Debiec-Rychter and J. Fletcher). P. Schöffski received research grants from Genentech. Lori Friedman is employed by Genentech, whose drug was herein tested.

Abstract: 250 words

Main text: 5470 words

2 Figures, 3 Tables, 37 references

Supplementary materials: 5 Figures, 2 Tables
**Translational relevance:**

Regardless of the type of *KIT* mutation, the PI3K/AKT/PTEN pathway is substantial for tumor cell survival in imatinib-sensitive and -resistant GIST. The pharmacological inhibition of the PI3K pathway in combination with imatinib constitutes a promising strategy to overcome or delay resistance to standard treatment in GIST. *In vivo* data presented in this study show that GDC-0941, an orally bioavailable PI3K inhibitor, in combination with imatinib shows superior antitumor efficacy in comparison with standard treatment, resulting in sustained effects even after treatment withdrawal. Secondly, PTEN status emerges as a possible surrogate predictive biomarker of response in GIST treated with the combination of GDC-0941 and imatinib.
Abstract:

Introduction: Oncogenic signaling in gastrointestinal stromal tumors (GIST) is sustained via PI3K/AKT pathway. We used a panel of six GIST xenograft models to assess efficacy of GDC-0941 as single agent or in combination with imatinib (IMA).

Experimental design: Nude mice (n=136) were grafted bilaterally with human GIST carrying divers KIT mutations. Mice were orally dosed over four weeks, grouped as follows: A) control; B) GDC-0941; C) IMA and D) GDC+IMA treatments. Xenografts re-growth after treatment discontinuation was assessed in group C and D for additional four weeks. Tumor response was assessed by volume measurements, micro-PET imaging, histopathology and immunoblotting. Moreover genomic alterations in PTEN/PI3K/AKT pathway were evaluated.

Results: In all models, GDC-0941 caused tumor growth stabilization, inhibiting tumor cells proliferation but did not induce apoptosis. Under GDC+IMA, profound tumor regression, superior to either treatment alone, was observed. This effect was associated with the best histologic response, a nearly complete proliferation arrest and increased apoptosis. Tumor regrowth assays confirmed superior activity of GDC+IMA over IMA; in three out of six models tumor volume remained reduced and stable even after treatment discontinuation. A positive correlation between response to GDC+IMA and PTEN loss, both on gene and protein levels, was found.

Conclusion: GDC+IMA has significant antitumor efficacy in GIST xenografts, inducing more substantial tumor regression, apoptosis and durable effects than IMA. Notably, after treatment withdrawal, tumor regression was sustained in tumors exposed to GDC+IMA, which was not observed under IMA. Assessment of PTEN status may represent a useful predictive biomarker for patient selection.
Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the digestive system. About 95% of GISTs express the receptor tyrosine kinase KIT. About 80-85% of GIST carry somatic activating mutations either in the KIT or PDGFRA gene, being the causative events in GIST development (1). With the advent of imatinib (IMA), the clinical course of advanced, metastatic inoperable GIST has dramatically changed from an almost incurable disease with a dismal prognosis to a very treatable condition. This paradigmatic change is attributable not only to IMA but to other tyrosine kinase inhibitors (TKI) such as sunitinib, which collectively achieved significant clinical benefit in the vast majority of GIST patients with advanced disease (2,3). KIT/PDGFRA mutation type is a major determinant of response to IMA treatment (4,5). Despite the spectacular long-lasting responses to IMA, the majority of GIST patients develops resistance during the therapy and is then treated with sunitinib. Eventually, sunitinib also ceases to be effective, leaving GIST patients without an alternative approved treatment option (6,7).

Regardless of the type of KIT/PDGFRA mutation, the PI3K/AKT pathway is crucial for tumor cell survival of both IMA-sensitive and -resistant GIST (8,9). However, little is known about the modulation of the PI3K/AKT pathway in relation to genomic changes occurring during GIST progression (or selected during TKI therapy). Of note, progression towards malignancy in GIST is characterized by accumulation of secondary genetic events involving numerical and structural changes of chromosomes (2). In particular, the tumor suppressor gene PTEN (10q23.31) encodes for a protein known to be a negative regulator of the PI3K/AKT pathway. PTEN activities are not limited to the PI3K/AKT pathway, but also seem to be involved in the RAS/MAPK pathway and in the activity of the focal adhesion kinase (FAK), a crucial kinase involved in cell migration. Not surprisingly, somatic PTEN inactivating mutations or PTEN deletions are among the most common cancer related molecular changes in humans (10).

Deregulation of the PTEN/PI3K/AKT pathway results in uncontrolled proliferation and cell survival of tumor cells (10). Clinically applicable approaches to counteract the effects of the deregulated pathway include a number of PI3K, AKT and mTOR inhibitors that are currently studied in early phase clinical trials (11). Previous in vitro evidence shows that inhibition of the PTEN/PI3K/AKT pathway by pan-PI3K inhibitor LY294002 translates into arrest of cell proliferation and induction of consistent tumor cells death in diverse IMA-sensitive and -resistant GIST cell lines (8). GDC-0941 is an orally bioavailable, potent and selective pan-
inhibitor of Class I PI3Ks and inhibits common mutant forms of the PI3K p110α subunit as effectively as wild type PI3K. In addition, GDC-0941 is a weak inhibitor of Classes II, III, and IV PI3K family members (including DNA-dependent protein kinase and mTOR). GDC-0941 is currently in clinical development in a number of solid tumors, showing promising results (12,13). In the present study we evaluated the efficacy of GDC-0941 in vivo, as single agent or in combination with IMA, using a panel of six GIST xenograft models carrying diverse KIT mutations and PTEN/PI3K/AKT pathway hyperactivation through different mechanisms.

**Materials and Methods**

**Cell lines, biopsy, and generation of mouse GIST xenografts**

GIST882 and GIST882Ly cell lines carrying the homozygous KIT exon 13 p.K642E mutation and the GIST48 cell line carrying double KIT mutations (KIT exon11 p.V560D and KIT exon17 p.D820A) were from Dr. J. Fletcher (Boston, MA, USA). GIST882Ly is a subline of GIST882 established by selective pressure, through continuous exposure to LY294002. GIST biopsies with either KIT exon 9 (GIST-BOE) or KIT exon 11 (GISt-PSW and GISt-DFR) mutations were obtained from patients radiologically progressing under IMA, and treated in the Department of General Medical Oncology, University Hospitals Leuven.

Female adult athymic NMRI nude mice were obtained from Janvier Laboratories, France. Heterotopic GIST xenografts were generated by subcutaneous bilateral injection of GIST882, GIST882Ly or GIST48 cells (5*10^6 cells per site, in culture medium), or by bilateral subcutaneous transplantation of fresh GIST biopsies, as previously described (14). The detailed characteristics of these models are presented in Table S1 (Supplementary materials). The number of mice used and xenograft’s passage information are presented in Table S2 (Supplementary materials).

**Drugs, experimental design and evaluation of response to treatment**

Imatinib mesylate was purchased from Sequoia Research Products and was dissolved in sterile water. GDC-0941 was kindly provided by Genentech and was dissolved in 0.5% methylcellulose/0.2% Tween 80 in a water bath (40°C). The solution was sonicated for 10 minutes prior administration.

All animal experiments were approved by the Ethics Committee of KU Leuven (Leuven, Belgium). A total of 136 mice bearing bilateral GIST tumors (average ~800 mm³) were
grouped as follows: group A - control mice (treated with sterile water p.o.); group B - GDC-0941 (75 mg/kg daily p.o.); group C - IMA (50 mg/kg twice daily p.o.); and group D - GDC+IMA combination (same doses and schedules as for single treatments). Detailed information on number of animals assigned to treatment groups is presented in Table S2 (Supplementary materials).

Treatment lasted four weeks, when animals were sacrificed and tumor samples were preserved for further analysis. In addition, for the IMA and GDC+IMA treatment groups, the tumor re-growth assessment was performed, i.e. four weeks of treatment was followed by another four weeks of observation after treatment discontinuation. In the aforementioned groups, half of animals were sacrificed at week four for efficacy assessment and the remaining half were euthanized at week eight. For GIST-PSW two separate experiments were performed; in the first, the treatment lasted 19 days (groups A-D), whereas in the second experiment re-growth in groups C and D was performed as described above. Tumor volume, body weight, histological assessment and Western blot analysis were implemented as previously described (14). The histological response (HR) was evaluated using Agaram et al. criteria, by assessing the magnitude of necrosis, myxoid degeneration and/or fibrosis on H&E staining, using the following grading system: grade 1 (0-10%), grade 2 (>10% and ≤ 50%), grade 3 (> 50% and ≤ 90%), and grade 4 (>90%) (15).

Micro positron emission tomography (micro-PET) studies

In GIST-PSW and GIST-DFR models the evaluation of response to treatment was complemented with FDG micro-PET scans, to study the glucose metabolism of the tumor under therapy, as previously described (16). Scans were performed at day 0 (baseline) and then week 4, 6 and 8. Images from PET scans were evaluated using the medical image data examiner Amide (Sourceforge, ver. 0.9.1). Values of FDG uptake were standardized according to the dose of FDG injected, body weight and specific scale factor to obtain the standardized uptake values (SUV) and the tumor/lesion glycolysis (TLG) values. The mean and the maximal SUV values were studied for each time point. TLG parameter describes the glucose uptake of a specific tumor in relation to volumetric changes; hence, it defines the metabolically active tumor mass. TLG was calculated using the following formula: SUV mean x micro-PET tumor volume.
**Mutational analysis, methylation status and fluorescence in situ hybridization (FISH)**

For molecular assessment DNA was isolated from frozen tumor fragments using QIAamp DNA Mini Kit (QIAGEN). *KIT* (ENSG00000157404) genotypes in our xenograft models were determined by mutational analysis as previously described (17). Mutation analysis for *PTEN* (ENST00000371953; exons 1-9), *PI3KCA* (ENST00000263967; exons 5, 6, 10 and 21) and *AKT* (ENSG00000142208; exon 4) genes were also performed. The primer sequences are available upon request. Moreover, DNA methylation status of *PTEN* promoter (SABIO CpG island ID:28526) was evaluated using EpiTect Methyl Profiler qPCR Assay (QIAGEN) according to manufacturer’s protocol.

To assess *KIT* and *PTEN* copy number, a dual-color interphase FISH was performed on paraffin sections. Digoxigenin-labeled BAC’s RP11-568A2 DNA for *KIT* (4q12) and SpectrumGreen-labeled chromosome 4 centromeric probe (CEP4-SG, Abbott) or LSI PTEN(10q23)/CEP10 Dual Color Probe (Abbott) were used, respectively, in two independent experiments. Probe labeling, hybridization, and detection were carried out as previously described (17,18). The number of hybridization signals representing investigated genes and chromosome centromeres were individually recorded for at least 100 nuclei. From 0 to 1 CEP signals per nucleus in >60% of cells was defined as a whole chromosome loss. In addition, ratios of *KIT*/CEP4 or *PTEN*/CEP10 were calculated. A ratio of ≥2 was defined as specific gene amplification. Heterozygous/homozygous loss was considered if ratio was <0.6.

**Statistics**

The comparison between tumor volumes on day 0 (baseline) versus last day of experiment was performed using Wilcoxon’s matched paired test. The comparison between different treatment groups (histological assessments and micro-PET studies) was done by the Mann-Whitney U test (MW-U). Bonferroni’s correction was used for multiple testing. Statistically significant differences were defined as p<0.05. The STATISTICA software (Stat Soft, version 9.0) was used for all calculations.

**Results**

**Xenografts characterization**

The morphology of untreated GIST-PSW, GIST-BOE, GIST882 and GIST48 was identical to that previously described (14,18). GIST-DFR xenografts were composed of spindle cells; in
GIST882Ly a mixed population of monotonous spindle shaped and highly atypical and pleomorphic tumor cells was observed. By immunohistochemistry, GIST-related biomarkers KIT, ANO1, CD44 and ETV1 were expressed at variable degrees of intensity in all xenografts; CD34 was expressed in all models but one (GIST-BOE) (Supplementary Fig. S1).

Mutational analysis of the KIT gene confirmed the presence of mutations previously described in cell lines used for tumor induction (8). All xenografts derived from GIST biopsies showed the same KIT mutations as present in the original sample obtained from patients (Supplementary Table S1). Moreover, FISH analysis confirmed our previous KIT copy number results obtained from GIST-PSW, GIST882, GIST48 and GIST-BOE (14,18). In addition, in GIST-DFR we observed trisomy of chromosome 4 (as judged by ~60% of nuclei with three KIT and CEP4 signals), while a subpopulation of tumor cells (~25%) of GIST882Ly xenograft disclosed up to four copies of the chromosome 4. Nevertheless, KIT amplification was found in none of the xenografts.

Subsequently, we characterized the PTEN/PI3K/AKT signaling pathway. By FISH, we identified a heterogeneous spectrum of alterations affecting the PTEN gene. Namely, homozygous loss was observed in the GIST-PSW and GIST882Ly xenografts, while GIST48 was characterized by heterozygous PTEN loss (PTEN/CEP10=0.52). In GIST-DFR, polysomic chromosome 10 was found (~55% of nuclei with 3-4 CEP10 signals), which was associated with the loss of one PTEN allele in a subpopulation of cells (PTEN/CEP10=0.73), resulting in classification of this model into the group without PTEN loss. In the remaining models no copy number changes in the PTEN locus were observed by FISH. Mutational analysis of PTEN and methylation analysis of the promoter did not revealed further changes in the PTEN gene. Importantly, we confirmed the lack of PTEN expression in the GIST-PSW and GIST882Ly models at the protein level (Supplementary Fig. S2).

Of note, an additional mutation in exon 6 of PI3KCA gene (c.1093 G>A; p.E365K) was detected in GIST-PSW by direct sequencing. We confirmed the presence of this mutation in GIST-PSW tumors retrieved from different treatment groups and in the earlier grafts passages. This mutation has been described in endometrial carcinoma (COSM86044) (19). In the remaining models we did not find additional mutations in PIK3CA or AKT genes in any of exons tested.

Tumor volume assessment
We first treated 12 GIST-PSW mice, over 19 days. IMA led to objective responses [tumor regression to 17% (p<0.01, versus baseline)], confirming the high sensitivity of GIST-PSW to IMA. In contrast, GDC-0941 treatment resulted in a steady increase in tumor burden. Yet, the most remarkable tumor regression was observed under GDC+IMA treatment, which led to a decrease in tumor size to 6% of baseline volume (p<0.05). Importantly, statistical analysis confirmed that the GDC+IMA regimen was better than either treatment alone [p<0.01 in both comparisons with IMA and GDC-0941 (MW-U)] suggesting an additive effect (Table 1). Based on these results, we decided to assess the duration of response also after treatment discontinuation in IMA and GDC+IMA groups by performing a tumor re-growth assay in GIST-PSW and in five additional models (Supplementary Fig. S3A).

Overall, regardless of KIT genotype, at week four a 3.2-fold increase in tumor volume in control mice was recorded. Furthermore, treatment with GDC-0941 resulted in tumor burden stabilization in three out of five xenografts (namely GIST-BOE, GIST-882 and GIST-48), resulting overall in tumor growth delay rather than tumor regression (Table 1).

After four weeks under treatment with IMA, the tumor burden was reduced to 78% (p<0.001 versus baseline) when all models were considered together. Not surprisingly the KIT genotype had an impact on the response to IMA; namely, the best tumor regressions were observed in KIT exon 11 mutants (Table 1; Supplementary Fig. S3A).

With regard to combination treatment, we noted additive effect of the GDC+IMA in all models. Overall, we observed tumor burden reduction to 36% (p<0.001, versus baseline) after four weeks of GDC+IMA treatment. This response was significantly better than in any other group (p<0.01 versus IMA and GDC-0941) (Supplementary Fig. S3A).

No treatment-related side effects were observed in any treatment group, in any models used.

After treatment discontinuation, we observed immediate tumor re-growth of IMA-treated tumors to levels above baseline values (152% of baseline at week eight). The superior antitumor activity of GDC+IMA treatment was confirmed during treatment discontinuation. Even after 28 days of treatment cessation, the tumor burden was yet 73% of the baseline values (p<0.05), suggesting long-lasting volumetric effects of the combination regimen. A detailed description of the results obtained in each model is provided in Table 1 and Supplementary Fig. S3A.
As indicated by the molecular characterization, PTEN gene loss was frequently observed in our panel of xenografts. Thus, we used PTEN status both at the protein and at the genomic levels as a potential surrogate predictive marker of response. At the protein level we identified two groups: xenografts expressing PTEN (PTEN+: GIST-DFR, GIST-BOE, GIST882 and GIST48) and xenografts not expressing PTEN (PTEN-: GIST-PSW and GIST882Ly). Similarly, we used PTEN genomic status to divide mice into two groups, i.e. xenografts with PTEN retained (PTEN<sup>no loss</sup>, GIST-DFR, GIST-BOE and GIST882) and those with homozygous/heterozygous PTEN loss (PTEN<sup>loss</sup>, GIST-PSW, GIST882Ly and GIST48).

PTEN status affected substantially tumor volume responses to GDC-0941 single treatment. In particular, when compared to control tumors, GDC-0941 alone led to remarkable tumor growth delay only in PTEN- or PTEN<sup>loss</sup> mice. In contrast, under GDC+IMA, an additive effect of the two drugs was observed in PTEN- or PTEN<sup>loss</sup> mice, but was not observed in PTEN<sup>no loss</sup> animals. Most importantly, the tumor regression induced by GDC+IMA in PTEN- or PTEN<sup>loss</sup> groups was better than that recorded in the co-respective PTEN+ or PTEN<sup>no loss</sup> mice (14% or 12% of starting volume versus 43% or 55% respectively). This remarkable result was further confirmed during the tumor re-growth. Upon GDC+IMA discontinuation, we observed a steady re-growth of tumors only in PTEN+ or PTEN<sup>no loss</sup> mice, i.e. to 90% and 114% of baseline, respectively. Notably, in PTEN- or PTEN<sup>loss</sup> mice treated with GDC+IMA the tumor volume remained substantially small, even in the absence of treatment, being at week eight 21% (PTEN-) and 17% (PTEN<sup>loss</sup>) of baseline values (Supplementary Fig. S3B and C).

Given all above, these observations suggest that GIST remain mainly dependent on KIT. The loss of PTEN or the presence of PI3K mutations (only in GIST-PSW) does not seem to influence response to IMA. Nevertheless, loss of PTEN may represent a potential biomarker to select a subset of GISTs that could respond better to PI3K inhibitors or to the combination of IMA with a PI3K inhibitor.

**Micro-PET assessment**

In GIST-PSW and GIST-DFR models we assessed the dynamics of FDG uptake in relation to treatment, by studying the TLG values and the SUV values. In the GIST-PSW micro-PET studies have been performed two times, first in a short experiment over 19 days treatment and then in a tumor re-growth assay (four weeks treatment followed by four weeks treatment
discontinuation). Results obtained in IMA and GDC+IMA groups in the short experiment were similar to those obtained in the re-growth assay. In both xenograft models we observed a significant reduction of the TLG values after four weeks of IMA treatment (6.5% of the starting value in GIST-PSW and 20.1% in GIST-DFR in comparison to baseline, respectively; \( p < 0.05 \)), confirming their IMA sensitivity (Supplementary Fig. S4A and B). Under GDC-0941, TLG values were higher than baseline values, reflecting delayed tumor growth when compared to the TLG values recorded in control tumors (data not shown).

Combination treatment resulted in a significant reduction in TLG values in both xenografts. Interestingly, only in GIST-PSW mice statistical analysis indicated an enhanced therapeutic effect of the combination treatment in comparison to IMA single agent (2.3% and 6.5% of baseline TLG after four weeks of treatment, respectively; \( p < 0.05 \)). In the GIST-DFR mice the GDC+IMA treatment yielded the same level of reduction as IMA single agent (20.1% and 20.8% of TLG baseline, respectively) (Table 2 and Supplementary Fig. S4A and B).

As judged from micro-PET analysis during treatment discontinuation, TLG values of GIST-DFR xenografts remained ~50% of the starting values in both IMA (46.9%) and GDC+IMA (50.3%) groups, indicating an equal durable effect of the two treatment options in this model. On the other hand in GIST-PSW in the GDC+IMA group the glucose uptake of the tumors remained almost negligible and stable over additional four weeks after the treatment discontinuation, while in the IMA group we observed an immediate increase of TLG values to level almost equal to baseline values (73.2% of baseline). At week eight, the TLG values recorded in GDC+IMA treated GIST-PSW tumors was still 10% of the baseline values, indicating durable effects even after treatment discontinuation. The changes in SUV mean values were similar to the abovementioned TLG changes in both xenografts (Table 2 and Supplementary Fig. S4A and B).

**Histopathology**

We assessed HR by evaluating the magnitude of necrosis, myxoid degeneration or fibrosis induced by the treatment in tumor tissues on H&E staining (15). We observed minimal HR (grade 1-2) in the vast majority of the tumor under IMA treatment (36 out of 44 specimens) in all but one models tested. In GIST-DFR the HR under IMA was grade 3-4 in all tumors analyzed. Interestingly, this result was consistent with the changes recorded with the TLG values by micro-PET analysis. Namely, on H&E staining the tumor tissues of IMA-treated
GIST-DFR xenografts were significantly replaced by myxoid degeneration (hallmark of IMA response in GIST), which corresponds to an amorphous matrix almost devoid of cells and hence a metabolically inactive tissue.

Overall, the GDC-0941 treatment yielded grade 2-3 HR in about 1/3 of the specimen analyzed, suggesting some level of antitumor activity. However, the interpretation of these results was uncertain in GIST-DFR, GIST882 and GIST882Ly xenografts, since areas of spontaneous necrosis of the same magnitude were encountered also in a minority of control tumors of these models. In contrast, grade 3-4 HR was identified in the vast majority of tumors treated with GDC+IMA (30 out of 46 specimens). Remarkably, five out of six tumors showed grade 4 HR in the GIST882Ly model. On the other hand, none of the treatments yielded more than grade 2 HR in GIST-BOE grafts (Fig. 1).

Subsequently, we have evaluated the proliferative and apoptotic activity in tumors collected at the end of treatment. Overall, regardless of the model, control tumors showed high mitotic activity (mean 31.9 mitoses/10HPF, 95% CI= 27.9-35.9).

After four weeks under IMA, significant reduction of mitotic activity in all but one model (GIST-BOE) was observed. Generally, as indicated by mitotic count, the proliferative activity was 3-fold reduced as compared to control tumors. Under IMA the apoptotic activity was significantly induced only in GIST-PSW and GIST-DFR tumors, while in others we recorded either a slight increase or no significant change in comparison to control tumors.

On the whole, the activity of GDC-0941 was limited to a significant anti-proliferative effect, while the apoptotic activity remained substantially unchanged in all models. The combination GDC+IMA was better than either treatment alone. Overall, it yielded 10.1-fold reduction in mitotic activity and 3.5-fold increase in apoptotic activity. Apoptosis was significantly stimulated to higher extent than single agent treatments in three models, namely GIST-PSW, GIST-DFR and GIST48. Ki67- and CC3-immunostaining showed comparable results to mitotic and apoptotic count in all models (Table 3).

Of note, the mitotic and apoptotic counts evaluated at week eight, i.e. four weeks after treatment withdrawal, returned to the level observed in control tumors, suggesting that durable stabilization of tumor volume under combination regimen might be due rather to more efficacious induction of necrotic and stromal changes than complete eradication of active tumor cells.
This remarkable result of the combination treatment was also confirmed when the xenograft models were subdivided into two groups according to their PTEN status at the protein level and at the genomic level. In particular, under GDC+IMA treatment, most of tumors lacking the PTEN protein (PTEN−) or with homozygous/heterozygous loss of PTEN gene (PTEN<sup>loss</sup>) showed grade 3 or 4 HR. Conversely, grade 1 HR was present in 1/3 of tumors analyzed in the PTEN+ and PTEN<sup>no loss</sup> groups. The anti-proliferative and pro-apoptotic activities of the GDC+IMA were better in the PTEN− and PTEN<sup>loss</sup> groups as compared to the PTEN+ and PTEN<sup>no loss</sup> groups. However, PTEN status did not correlate with anti-proliferative and/or pro-apoptotic activities of IMA or GDC-0941, when administered as single agents (Table 3).

**Assessment of the oncogenic signaling in response to treatment**

As indicated by Western blot, KIT protein and its main intermediates were activated in all GIST xenografts. Activation of KIT was more pronounced in GIST-PSW, GIST-DFR, GIST-BOE and GIST48, while in the other two models this feature was less apparent, possibly suggesting a lower dependency on KIT. The PI3K 110 kD subunit was equally expressed in all models (Supplementary Fig. S5).

Consistent inhibition of both p-KIT<sup>Y703</sup> and p-KIT<sup>Y719</sup> isoforms under IMA in the GIST xenografts carrying KIT exon 11 mutations (at least 60% reduction for both isoforms) was observed (Fig. 1, Supplementary Fig. S5). However, while p-AKT and p-ERK were consistently inhibited in GIST-DFR, the activation of these KIT intermediates was still detectable in GIST-PSW and GIST48. In KIT exon 13 mutated GIST882 and GIST882Ly models, minimal KIT inhibition under IMA treatment (≤ 25% inhibition) resulted in a strong inhibition of p-ERK in both models (80% and 70% inhibition, respectively). In contrast, while p-AKT was strongly inhibited in GIST882, only 15% inhibition was observed in GIST882Ly. These findings most likely result from the hyperactivation of PI3K/AKT signaling pathway in the tumors carrying PTEN/PI3K alterations (GIST-PSW, GIST48 and GIST882Ly), which might require a higher IMA dose for equivalent inhibition. In GIST-BOE, IMA treatment caused only a minimal p-KIT and p-AKT inhibition (~ 40% and 10% reduction, respectively) as expected for KIT exon 9 mutant, while a more consistent inhibition of p-ERK was observed (90% reduction) (Fig. 2, Supplementary Fig. S5).

The activity of KIT was unaffected in all models by GDC-0941 alone. Notably, a remarkable inhibition of p-AKT was observed in all but the GIST-PSW model (about 70% reduction in
five out of six xenografts). In parallel, some degree of p-ERK inhibition in GIST-DFR, GIST48, GIST-BOE and GIST882 xenografts were observed, while in GIST882Ly and GIST-PSW the p-ERK was up-regulated or unchanged, respectively.

Importantly, GDC+IMA yielded a more consistent inhibition of KIT signaling pathway, especially in models carrying mutations that are less sensitive to IMA. In GIST48, the combination treatment was the only one to result in complete p-AKT inhibition (96% reduction). Similarly in GIST-BOE, the combination arm caused a more consistent inhibition of p-AKT than any single agent treatment (83% reduction). Of note, GDC+IMA resulted in ERK inactivation in all xenograft models except GIST-PSW, which featured p-ERK hyperactivation (over 150%). The latter could be related to the accumulation of activated macrophages that were observed on the histological level exclusively in GIST-PSW tumors treated with GDC+IMA, or to the release of a negative signaling loop involving the RAS/MAPK pathway (Fig. 2, Supplementary Fig. S5) (20,21).

As expected, four weeks after treatment discontinuation the activation levels for KIT and its signaling intermediates reverted to levels found in tumors (data not shown).

Importantly, based on immunohistochemistry and immunoblotting PTEN expression did not change after four and eight weeks of experiment in all models. In GIST48 we also performed FISH analysis of PTEN, which confirmed the unchanged copy number status throughout whole experiment.

Discussion

The PI3K/AKT pathway is a major contributor to proliferation and survival in IMA-sensitive and IMA-resistant GIST (8,9). Using a panel of diverse GIST xenograft models carrying common KIT mutations, we showed for the first time the in vivo efficacy of the PI3K inhibitor GDC-0941, used as a single agent or in combination with IMA.

In the present study GDC-0941 caused either tumor growth delay or tumor burden stabilization, resulting in consistent reduction of the proliferative activity. However, despite a significant inhibition of the AKT activation, we did not observe substantial in vivo pro-apoptotic activity in these models. Bauer et al. described similar results in GIST882 cells treated with the PI3K inhibitor, LY294002, in vitro (8). Moreover, in genetically manipulated IMA-sensitive GIST cell lines in which AKT is constitutively activated, the survival of GIST
cells does not depend only on AKT activity (22). Thus the absence of consistent apoptotic activity in GIST treated with GDC-0941 is not surprising and underscores the dominant role of KIT in regulating GIST proliferation and survival (22).

The combination GDC+IMA treatment proved to be very effective in all xenografts tested here. This regimen reduced tumor burden better than IMA or GDC-0941 alone in the majority of models and dramatically enhanced anti-proliferative activity in all xenografts. Furthermore, in 65% of tumors treated with a combination regimen we observed high degree of HR (grade 3-4), and in 30% of the tumors the fraction of vital tumor cells was reduced to <10%. These astonishing effects may account for the outstanding long-lasting effect observed in the tumor re-growth experiments. Most remarkably, in three of the xenografts tested (GIST-PSW, GIST48 and GIST882Ly) the tumor volume remained stable and considerably small even after 28 days of treatment discontinuation. Our results are consistent with previous in vitro results, which demonstrated an additive anti-proliferative effect with the combination of LY294002 and low doses of IMA, in IMA-sensitive and -resistant GIST cell lines (8).

Generally, the pro-apoptotic activity of the GDC+IMA treatment exceeded that for either treatment alone, mainly in GIST xenografts carrying KIT exon 11 mutations. Two scenarios may explain this finding. Firstly, KIT-independent mechanisms may influence pro-apoptotic responses to therapies. For example, the differential expression of anti-apoptotic proteins such as BCL2 in GIST of distinct anatomic sites could hamper pro-apoptotic activity of antitumor targeted agents (23). For example, gastric GIST have less BCL2 expression compared to small intestine GIST. Secondly, KIT may have a dominant role in controlling cell survival in this setting. Thus, if the KIT receptor is not completely inhibited due to an oncogenic mutation with reduced sensitivity to IMA (e.g. KIT exon 9 or KIT exon 13), then apoptosis might not be substantially induced (22,24). Therefore, the additive pro-apoptotic effect of GDC+IMA observed in the GIST xenograft models carrying KIT exon 11 mutations may be related to their intrinsically higher IMA sensitivity. These observations suggest that the combination of PI3K inhibitors with higher doses of IMA may further stimulate apoptosis in GIST with non-exon 11 primary KIT mutations.

An additional observation of our study was the possibility for a molecular stratification according to PTEN/PI3K/AKT pathway alterations in advanced GIST. The GIST-PSW model is derived from a patient with advanced disease, who developed resistance to standard treatments. While we were not able to identify secondary KIT mutations in the original tumor,
we found the coexistence of a point mutation in the PI3K gene and homozygous deletion of the region encoding PTEN in chromosome 10 in the xenograft derived from that patient. Importantly, we were able to confirm the presence of these molecular events also in early passages of the xenograft suggesting their presence in the original tumor. A point mutation in the PI3K gene was recently described in one clinical case of a high risk, untreated primary GIST carrying a KIT exon 11 mutation (25). The PTEN and PI3K copy number changes/mutations are not present or rare in primary GIST, as suggested by recent study (26), but the incidence of these events in GIST under TKI therapy might be significant. The coexistence of genetic events other than KIT/PDGFRA mutations occurring in the PI3K pathway in GIST may correlate with tumor progression rather than primary resistance to therapy, since the GIST-PSW xenograft model preserved high sensitivity to IMA treatment. Interestingly, also in other cancer types the presence of mutations in the PTEN/PI3K/AKT pathway has been associated with worse prognosis and enhanced invasiveness (10,14,27,28). Nevertheless, this observation should be confirmed in a larger cohort of tumors to better understand the incidence and the role of PTEN/PI3K mutations in GIST.

Genomic losses affecting the PTEN locus (10q23.31) were identified in three out of six xenograft models by FISH. We also confirmed the lack of PTEN protein expression in xenograft showing homozygous loss of PTEN gene (GIST-PSW and GIST882Ly). Chromosomal aberrations are frequently observed in GIST and are implicated in progression towards malignancy (2). Genome profiling studies carried out on primary GISTs reveal chromosome 10q loss in about 20-38% of patients (29,30). However, homozygous loss of PTEN was not observed, at least in IMA-naïve tumors. According to one study, about 40% of primary GIST have reduced levels of PTEN protein by immunohistochemistry, which was implicated as an independent prognostic marker (31). As of yet, the biological significance of aberrations involving the PTEN locus in GIST is largely unknown. It is not clear whether a dose-dependent effect in the levels of PTEN expression may contribute to malignancy also in GIST, as it is the case in transgenic models of breast carcinomas (28). Genomic alterations targeting the PTEN locus in GIST could be a consequence of the continuous treatment with TKI, resulting in selection of sub-clones of tumor cells with a preferential loss of PTEN. As observed in breast carcinomas and glioblastomas, PTEN loss-of-function could reduce the efficacy of TKI treatment in GIST (11,32,33). However, based on our results we cannot confirm this hypothesis, which would require a larger cohort of tumors.
Intriguingly, we observed a correlation between the genomic status of PTEN and response to treatment in the combination treatment group (i.e. GDC+IMA regimen). These studies suggest that patients whose GIST reveals at least a heterozygous deletion of the PTEN locus may have the highest benefit from combination of PI3K inhibitors with IMA. Thereby, we propose that PTEN status and possibly PI3K mutations could serve as a predictive biomarker for the selection of GIST patients who could respond the best to the combination of IMA and PI3K-inhibitors.

The dynamic evaluation of glucose metabolism in GIST by means of PET scans is regarded as a tool that relates well to IMA responses or resistance in GIST patients (34). Consistent with the observation in the clinic, we show that FDG uptake measured in GIST-xenograft bearing mice correlated well with response to IMA treatment. However, as observed in the GIST-DFR model the evaluation of the FDG uptake should not be limited to the study of SUV values, but should be complemented by the assessment of parameters that combine changes in the tumor tissue with the level of glycolysis, such as TLG values. Indeed, TLG values correlated well with histological changes observed in the tumor tissue and provided further information about the activity of the drugs over time. The systematic study of these parameters by PET scan could improve the radiological classification of responding or non-responding GIST under treatment with targeted agents in the clinic. Nevertheless, it must be acknowledged that FDG changes studied by PET do not measure directly the antitumoral effects of the drugs, but most likely reflect a relocation of glucose transporters in the cell membrane as response to treatment with targeted agents (19,35).

In conclusion, we provide the first evidence for the in vivo activity of the PI3K inhibitor GDC-0941 in GIST. GDC-0941 shows a significant anti-proliferative effect in highly proliferating GIST, and induces variable degrees of necrosis in tumor tissue. In combination with IMA the therapeutic activity of GDC-0941 is dramatically enhanced as shown by more prominent HR, induction of apoptosis and even more profound inhibition of cell proliferation. The therapeutic effects observed in the combination treatment provide more durable effects after treatment discontinuation. Assessment of the PTEN status at genomic and protein levels in GISTs and its relevance to TKI treatment (including PI3K inhibitors) warrants further study. However, the lack of PTEN expression is unlikely responsible for decreased sensitivity to standard treatment with IMA in GIST. Our results provide a very strong pre-clinical rationale for the use of the IMA+PI3K inhibitors combination in GIST patients in the clinic, and identify PTEN status as a potential predictive biomarker to select a subset of GIST.

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patients with higher sensitivity towards this type of therapeutic strategy. Of note, a first dose-finding clinical GIST trial, that is based on these and related observations and combines IMA with another PI3K inhibitor, has started recruiting patients (36).

Acknowledgements

We are grateful to Dr. Erna Dewil for the logistic support offered for the animal facility. We thank Lieve Ophalvens, Ulla Vanleeuw, Ann Van Santvoort and Vanessa Vanspauwen for their excellent technical assistance. Part of the results of the study have been presented at the annual meeting of the American Society of Clinical Oncology (Chicago, IL June 4th - June 9th 2010) (37).
References:


36. EUDRACT number 2011-002938-39; Clinical Trials.gov identifier NCT01468688

Figure Legend:

**Figure 1**
Histologic response (HR) evaluated in six models tested. HR was scored by assessing the magnitude of necrosis, myxoid degeneration and/or fibrosis on H&E staining using the following grading system: grade 1 (0-10%), grade 2 (>10% and ≤50%), grade 3 (>50% and ≤90%), and grade 4 (>90%).

**Figure 2**
Densitometric assessment of active (phospho-) protein forms in KIT signaling pathway.
Floris and Wozniak et al., Figure 1

Histologic response (HR)

Number of tumors

Grade 1  Grade 2  Grade 3  Grade 4  Grade 1  Grade 2  Grade 3  Grade 4  Grade 1  Grade 2  Grade 3  Grade 4
IMA  GDC-0941  GDC+IMA

- GIST-DFR
- GIST-BOE
- GIST822
- GIST48
- GIST-PSW
- GIST822Ly
**Table 1**

Relative tumor volume assessment in GIST models after treatment (at week four) and after re-growth (at week eight). Arrows indicate whether relative tumor volume was less (arrow down) or more (arrow up) than the starting volume. Statistical significance was calculated using Mann-Whitney U test and related to the control treatment group (at week four) or to IMA-treated group (at week eight).

<table>
<thead>
<tr>
<th>Xenograft model</th>
<th>relative tumor volume (mean% [95% CI])</th>
<th>at week four</th>
<th>at week eight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>IMA</td>
<td>GDC-0941</td>
</tr>
<tr>
<td>all models</td>
<td>324.0 [240-409]</td>
<td>↑</td>
<td>78 [62-94]**</td>
</tr>
<tr>
<td>PTEN (IHC status)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PTEN status by FISH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance: * p<0.05; ** p<0.005; * in GIST-PWS GDC-0941 efficacy was assessed only after 19 days.
Table 2
Micro-PET results in GIST-PSW and GIST-DFR models. The standardized uptake values (SUV) and tumor lesion glycolysis (TLG) values were obtained after micro-PET scans performed at weeks four, six and eight. The mean SUV values (SUV mean) and the maximal SUV values (SUVmax) of the tumor masses were evaluated. TLG was calculated as follow: SUV mean x tumor volume as measured by micro-PET scan (µPET vol). Results are shown as relative values, related to baseline. Statistical significance was calculated versus day 0 (baseline) with Wilcoxon’s matched paired test.

<table>
<thead>
<tr>
<th>Treatment groups / models</th>
<th>Relative micro-PET values [% (95% CI)]</th>
<th>at week four</th>
<th>at week six</th>
<th>at week eight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ-PET vol</td>
<td>SUVmean</td>
<td>SUVmax</td>
<td>TLG</td>
</tr>
<tr>
<td>IMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIST-PSW</td>
<td>12.6</td>
<td>52.8</td>
<td>53.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>(7.5-17.7)*</td>
<td>(45.0-60.7)*</td>
<td>(45.2-59.9)*</td>
<td>(4.3-8.7)*</td>
</tr>
<tr>
<td>GIST-DFR</td>
<td>25.0</td>
<td>84.3</td>
<td>77.0</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>(14.6-35.4)*</td>
<td>(57.8-110.7)*</td>
<td>(56.8-97.1)*</td>
<td>(12.7-27.5)*</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>6.1</td>
<td>34.7</td>
<td>42.1</td>
<td>2.3</td>
</tr>
<tr>
<td>GIST-PSW</td>
<td>(-0.1-12.3)</td>
<td>(22.7-46.7)</td>
<td>(19.2-65.0)</td>
<td>(-0.3-4.9)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIST-DFR</td>
<td>20.1</td>
<td>105.5</td>
<td>86.5</td>
<td>20.8</td>
</tr>
<tr>
<td></td>
<td>(15.9-24.2)*</td>
<td>(89.8-121.2)*</td>
<td>(69.1-103.9)*</td>
<td>(17.6-24.0)*</td>
</tr>
</tbody>
</table>

Statistical significance: * p<0.05;
Table 3
Evaluation of proliferative and apoptotic activity. Results are shown as fold changes in comparison to control. Arrows indicate whether proliferation or apoptosis were less (arrow down) or more (arrow up) than respective control in different models. Statistical significance was calculated using Mann-Whitney U test.

<table>
<thead>
<tr>
<th></th>
<th>All groups</th>
<th>PTEN (IHC status</th>
<th>PTEN (status by FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GIST-DFR</td>
<td>GIST-BOE</td>
</tr>
<tr>
<td><strong>Mitosis (H&amp;E)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMA</td>
<td>↓ 3.0**</td>
<td>89.0**</td>
<td>1.0</td>
</tr>
<tr>
<td>GDC-0941</td>
<td>↓ 1.7**</td>
<td>2.2*</td>
<td>1.7*</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>↓ 10.1**</td>
<td>59.3**</td>
<td>2.9**</td>
</tr>
<tr>
<td><strong>Apoptosis (H&amp;E)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMA</td>
<td>↑ 1.8**</td>
<td>3.5**</td>
<td>1.1</td>
</tr>
<tr>
<td>GDC-0941</td>
<td>↑ 1.4</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>↑ 3.5**</td>
<td>6.1**</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Ki67</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMA</td>
<td>↓ 2.1**</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>GDC-0941</td>
<td>↓ 1.6**</td>
<td>1.4</td>
<td>2.2*</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>↓ 9.0**</td>
<td>5.8*</td>
<td>4.0**</td>
</tr>
<tr>
<td><strong>Cleaved caspase 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMA</td>
<td>↑ 1.4**</td>
<td>1.5</td>
<td>1.2*</td>
</tr>
<tr>
<td>GDC-0941</td>
<td>↑ 1.1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>↑ 2.2**</td>
<td>2.6**</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Statistical significance: * p<0.05; ** p<0.005; in GIST-PWS GDC-0941 efficacy was assessed only after 19 days.
A potent combination of the novel PI3K inhibitor, GDC-0941, with imatinib in gastrointestinal stromal tumor xenografts: long-lasting responses after treatment withdrawal

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Clin Cancer Res  Published OnlineFirst December 11, 2012.