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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Abstract

Purpose: 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 diverse KIT mutations. Mice were orally dosed over four weeks, grouped as follows: (A) control; (B) GDC-0941; (C) imatinib, and (D) GDC + IMA treatments. Xenografts regrowth after treatment discontinuation was assessed in groups C and D for an 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 cell 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 imatinib; in three 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 imatinib. Notably, after treatment withdrawal, tumor regression was sustained in tumors exposed to GDC + IMA, which was not observed under imatinib. 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% to 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 imatinib but to other tyrosine kinase inhibitors (TKI), such as sunitinib, which collectively achieved significant clinical benefit in the vast majority of patients with GIST with advanced disease (2, 3). KIT/ PDGFRA mutation type is a major determinant of response to imatinib treatment (4, 5). Despite the spectacular long-lasting responses to imatinib, the majority of patients with GIST develops resistance during the therapy and is then
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 gastrointestinal stromal tumors (GIST). The pharmacologic inhibition of the phosphoinositide 3-kinase (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. Second, PTEN status emerges as a possible surrogate predictive biomarker of response in GIST treated with the combination of GDC-0941 and imatinib.

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.A. Fletcher (Brigham and Women’s Hospital, Boston, MA). 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 imatinib, and treated in the Department of General Medical Oncology, University Hospitals Leuven (Leuven, Belgium).

Female adult athymic NMRI nude mice were obtained from Janvier Laboratories. Heterotopic GIST xenografts were generated by subcutaneous bilateral injection of GIST882, GIST882Ly, or GIST48 cells (5 × 10⁶ 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 Supplementary Table S1. The number of mice used and xenograft’s passage information are presented in Supplementary Table S2.

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 per os); group B—GDC-0941 (75 mg/kg daily per os); group C—imatinib (50 mg/kg twice daily per os); 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 Supplementary Table S2.

Treatment lasted 4 weeks, when animals were sacrificed and tumor samples were preserved for further analysis. In addition, for the imatinib and GDC+IMA treatment groups, the tumor regrowth assessment was conducted, that is, 4 weeks of treatment was followed by another 4 weeks of observation after treatment discontinuation. In the aforementioned groups, half of animals were sacrificed at
signals representing investigated genes and chromosome previously described (17, 18). The number of hybridization labeling, hybridization, and detection were carried out as LSI chromosome 4 centromeric probe (CEP4-SG, Abbott) or KIT 568A2 DNA for interphase FISH was conducted on paraffin sections. Digoxigenin-labeled deoxy-D-glucose (FDG) micro positron emission tomography (micro-PET) scans, to study the glucose metabolism of the tumor under therapy, as previously described (16). Scans were conducted at day 0 (baseline) and then at 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 × micro-PET tumor volume.

Micro positron emission tomography studies

In GIST-PSW and GIST-DFR models, the evaluation of response to treatment was complemented with [18F]fluoro-2-deoxy-D-glucose (FDG) micro positron emission tomography (micro-PET) scans, to study the glucose metabolism of the tumor under therapy, as previously described (16). Tumor volume, body weight, histologic assessment, and Western blot analysis were implemented as previously described (14). The histologic response was evaluated using Agaram and colleagues criteria, by assessing the magnitude of necrosis, myxoid degeneration, and/or fibrosis on hematoxylin and eosin (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%; ref. 15).

Mutational analysis, methylation status, and 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 conducted. The primer sequences are available upon request. Moreover, DNA methylation status of PTEN promoter (SABIO CpG island ID:28526) was evaluated using EpiTect Methylation Profiler qPCR Assay (Qiagen) according to manufacturer’s protocol.

To assess KIT and PTEN copy number, a dual-color interphase FISH was conducted on paraffin sections. Digoxigenin-labeled bacterial artificial chromosome’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 2 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 more than 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 or more was defined as specific gene amplification. Heterozygous/homozygous loss was considered if ratio was less than 0.6.

Statistics

The comparison between tumor volumes on day 0 (baseline) versus last day of experiment was conducted using Wilcoxon’s matched paired test. The comparison between different treatment groups (histologic 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 (IHC), GIST-related biomarkers KIT, ANO1, CD44, and ET1 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 3 KIT and CEPI4 signals), whereas a subpopulation of tumor cells (~25%) of GIST882Ly xenograft disclosed up to 4 copies of the chromosome 4. Nevertheless, KIT amplification was found in none of the xenografts.

Subsequently, we characterized the PTEN/PI3K/ATK 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, whereas 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 1 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

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FISH. Mutational analysis of PTEN and methylation analysis of the promoter did not reveal further changes in the PTEN gene. Importantly, we confirmed the lack of PTEN expression in the GIST-PSW and GIST82Ly models at the protein level (Supplementary Fig. S2).

Of note, an additional mutation in exon 6 of PIK3CA 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; ref. 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. Imatinib led to objective responses [tumor regression to 17% (P < 0.01, vs. baseline)], confirming the high sensitivity of GIST-PSW to imatinib. 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 imatinib and GDC-0941 (MW-U)] suggesting an additive effect (Table 1). On the basis of these results, we decided to assess the duration of response also after treatment discontinuation in imatinib and GDC+IMA groups by conducting a tumor regrowth assay in GIST-PSW and in 5 additional models (Supplementary Fig. S3A).

Overall, regardless of KIT genotype, at week 4 a 3.2-fold increase in tumor volume in control mice was recorded. Furthermore, treatment with GDC-0941 resulted in tumor burden stabilization in 3 of 5 xenografts (namely GIST-BOE, GIST-882, and GIST-48), resulting overall in tumor growth delay rather than tumor regression (Table 1).

After 4 weeks under treatment with imatinib, the tumor burden was reduced to 78% (P < 0.001 vs. baseline) when all models were considered together. Not surprisingly the KIT genotype had an impact on the response to imatinib; 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, vs. baseline) after 4 weeks of GDC+IMA treatment. This response was significantly better than in any other group (P < 0.01 vs. imatinib 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 regrowth of imatinib-treated tumors to levels above baseline values (152% of baseline at week 8). 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

Table 1. Relative tumor volume assessment in GIST models after treatment (at week 4) and after regrowth (at week 8)

<table>
<thead>
<tr>
<th>Xenograft model</th>
<th>Control</th>
<th>Imatinib</th>
<th>GDC-0941</th>
<th>GDC+IMA</th>
<th>Imatinib</th>
<th>GDC+IMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>At week 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>All models</td>
<td>324.0 (240–409)</td>
<td>78 (62–94)</td>
<td>207 (139–279)</td>
<td>36 (26–45)</td>
<td>152 (107–197)</td>
<td>73 (53–93)</td>
</tr>
<tr>
<td>GIST-DFR</td>
<td>494 (193–794)</td>
<td>44 (35–53)</td>
<td>528 (149–908)</td>
<td>73 (30–115)</td>
<td>76 (9–142)</td>
<td>101 (41–160)</td>
</tr>
<tr>
<td>GIST-BOE</td>
<td>173 (137–209)</td>
<td>146 (121–170)</td>
<td>80 (90–126)</td>
<td>77 (58–96)</td>
<td>335 (192–477)</td>
<td>147 (128–166)</td>
</tr>
<tr>
<td>GIST82</td>
<td>232 (99–364)</td>
<td>86 (47–125)</td>
<td>102 (37–167)</td>
<td>16 (11–21)</td>
<td>151 (95–208)</td>
<td>90 (53–127)</td>
</tr>
<tr>
<td>GIST48</td>
<td>140 (76–205)</td>
<td>32 (25–38)</td>
<td>81 (51–111)</td>
<td>9 (6–14)</td>
<td>82 (50–113)</td>
<td>13 (4–30)</td>
</tr>
<tr>
<td>GIST-PSW</td>
<td>235 (246–451)</td>
<td>17 (10–24)</td>
<td>257 (168–346)</td>
<td>6 (2–15)</td>
<td>98 (70–125)</td>
<td>15 (4–33)</td>
</tr>
<tr>
<td>GIST82Ly</td>
<td>680 (137–1,223)</td>
<td>145 (75–216)</td>
<td>224 (120–328)</td>
<td>22 (19–25)</td>
<td>95 (–97–287)</td>
<td>25 (3–48)</td>
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<tr>
<td>PTEN (IHC status)</td>
<td></td>
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<tr>
<td>PTEN−</td>
<td>473 (281–664)</td>
<td>72 (32–112)</td>
<td>241 (184–297)</td>
<td>14 (8–19)</td>
<td>97 (63–130)</td>
<td>21 (9–33)</td>
</tr>
<tr>
<td>PTEN status by FISH</td>
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<tr>
<td>PTEN−loss</td>
<td>362 (221–503)</td>
<td>56 (32–80)</td>
<td>177 (126–227)</td>
<td>12 (8–15)</td>
<td>90 (69–111)</td>
<td>17 (9–26)</td>
</tr>
<tr>
<td>PTEN+no loss</td>
<td>285 (185–384)</td>
<td>99 (79–119)</td>
<td>239 (105–374)</td>
<td>55 (39–70)</td>
<td>208 (131–286)</td>
<td>114 (93–135)</td>
</tr>
</tbody>
</table>

NOTE: 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 4) or to imatinib-treated group (at week 8). *, P < 0.05; **, P < 0.005.

aIn GIST-PWS GDC-0941 efficacy was assessed only after 19 days.
GDC

Most importantly, the tumor regression induced by 
PTEN 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 2 groups: xenografts expressing 
PTEN loss: GIST-PSW, GIST882Ly, and GIST48) and xenografts not expressing 
PTEN loss mice, but was not observed in 
GDC-0941 single treatment. In particular, 
PTEN status affected substantially tumor volume responses to GDC-0941 single treatment. In particular, when compared with control tumors, GDC-0941 alone led to remarkable tumor growth delay only in 
PTEN loss mice. In contrast, under GDC+IMA, an additive effect of the 2 drugs was observed in 
PTEN loss mice, but was not observed in 
PTEN loss mice (14% or 12% of starting volume vs. 43% or 55%, respectively). This remarkable result was further confirmed during the tumor regrowth. Upon GDC+IMA discontinuation, we observed a steady regrowth of tumors only in 
PTEN loss mice, to 90% and 114% of baseline, respectively. Notably, in 
PTEN loss mice treated with GDC+IMA the tumor volume remained substantially small, even in the absence of treatment, being at week 8 21% (PTEN loss) and 17% (PTEN loss) of baseline values (Supplementary Fig. S3B and S3C).

Given all earlier, 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 imatinib. 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 
imatinib 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 study has been conducted 2 times, first in a short experiment over 19 days treatment and then in a tumor regrowth assay (4 weeks treatment followed by 4 weeks treatment discontinuation). Results obtained in imatinib and GDC+IMA groups in the short experiment were similar to those obtained in the regrowth assay. In both xenograft models, we observed a significant reduction of the TLG values after 4 weeks of imatinib treatment (6.5% of the starting value in 
GIST-PSW and 20.1% in 
GIST-DFR in comparison with baseline, respectively; P < 0.05), confirming their imatinib sensitivity (Supplementary Fig. S4A and S4B). Under GDC-0941, TLG values were higher than baseline values, reflecting delayed tumor growth when compared with 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 with imatinib single agent (2.3% and 6.5% of baseline TLG after 4 weeks of treatment, respectively; P < 0.05). In the GIST-DFR mice the GDC+IMA treatment yielded the same level of reduction as imatinib single agent (20.1% and 20.8% of TLG baseline, respectively; Table 2; Supplementary Fig. S4A and S4B).

As judged from micro-PET analysis during treatment discontinuation, TLG values of GIST-DFR xenografts remained approximately 50% of the starting values in both imatinib (46.9%) and GDC+IMA (50.3%) groups, indicating an equal durable effect of the 2 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 4 weeks after the treatment discontinuation, whereas in the imatinib group we observed an immediate increase of TLG values to level almost equal to baseline values (73.2% of baseline). At week 8, 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 above-mentioned TLG changes in both xenografts (Table 2 and Supplementary Fig. S4A and S4B).

**Histopathology**

We assessed histologic response 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 histologic response (grade 1–2) in the vast majority of the tumor under imatinib treatment (36 of 44 specimens) in all but one models tested. In GIST-DFR, the histologic response under imatinib was grade 3 to 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 imatinib-treated GIST-DFR xenografts were significantly replaced by myxoid degeneration (hallmark of imatinib 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 to 3 histologic response in about one third of the specimens analyzed, suggesting some level of antitumor activity. However, the interpretation of these results was uncertain in GIST-DFR, GIST882, and GIST882Ly xenografts, as areas of spontaneous necrosis of the same magnitude were encountered also in a minority of control tumors of these models. In contrast, grade 3 to 4 histologic response was identified in the vast majority of tumors treated with GDC+IMA (30 of 46 specimens). Remarkably, 5 of 6 tumors showed grade 4 histologic response in the GIST882Ly model. On the other
Table 2. Micro-PET results in GIST-PSW and GIST-DFR models

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>At week 4</th>
<th>At week 6</th>
<th>T.G.</th>
<th></th>
<th>PET vol</th>
<th>SUV mean</th>
<th>SUVmax</th>
<th>TLG</th>
<th></th>
<th>PET vol</th>
<th>SUV mean</th>
<th>SUVmax</th>
<th>TLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIST-PSW</td>
<td>12.6 (7.5)</td>
<td>17.7</td>
<td>105.5 (89.8–110.7)</td>
<td>77.9 (68.8–99.0)</td>
<td>20.8 (17.8–23.6)</td>
<td>186.0 (155.8–215.5)</td>
<td>117.5 (104.8–131.5)</td>
<td>69.9 (59.2–82.3)</td>
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<tr>
<td>GIST-DFR</td>
<td>25.0 (14.6)</td>
<td>46.7</td>
<td>105.5 (89.8–110.7)</td>
<td>77.9 (68.8–99.0)</td>
<td>20.8 (17.8–23.6)</td>
<td>186.0 (155.8–215.5)</td>
<td>117.5 (104.8–131.5)</td>
<td>69.9 (59.2–82.3)</td>
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</table>

Note: SUV and TLG values were obtained after micro-PET scans conducted at weeks 4, 6, and 8. The mean SUV values (SUV mean) and the maximal SUV values (SUV max) of the tumor masses were evaluated. TLG was calculated as follows: SUV mean x tumor volume as measured by micro-PET scan (PET vol). Results are shown as relative values, related to baseline. Statistical significance was calculated versus day 0 (baseline) with Wilcoxon’s matched paired test. *P < 0.05.

Assessment of the oncogenic signaling in response to treatment

As indicated by Western blot analysis, 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, whereas in the other 2 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-KITV737 and p-KITV719 isoforms under imatinib in the GIST xenografts carrying KIT exon 11 mutations (at least 60% reduction for both isoforms) was observed (Fig. 1 and 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 imatinib 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

Table 3. Evaluation of proliferative and apoptotic activity

<table>
<thead>
<tr>
<th>All groups</th>
<th>GIST-DFR</th>
<th>GIST-BOE</th>
<th>GIST822</th>
<th>GIST48</th>
<th>GIST-PSW</th>
<th>GIST822ly</th>
<th>PTEN (IHC status)</th>
<th>PTEN (status by FISH)</th>
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<tbody>
<tr>
<td>Mitosis (H&amp;E)</td>
<td></td>
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<tr>
<td>Imatinib</td>
<td>3.0**</td>
<td>89.0**</td>
<td>1.0</td>
<td>3.2**</td>
<td>7.0**</td>
<td>36.8**</td>
<td>2.1**</td>
<td>5.5**</td>
</tr>
<tr>
<td>GDC-0941</td>
<td>1.7**</td>
<td>2.2**</td>
<td>1.7**</td>
<td>1.9**</td>
<td>4.0**</td>
<td>1.6</td>
<td>1.2</td>
<td>1.3**</td>
</tr>
<tr>
<td>GDC+IMA</td>
<td>10.1**</td>
<td>59.3**</td>
<td>2.9**</td>
<td>16.8**</td>
<td>11.2**</td>
<td>331.0**</td>
<td>19.9**</td>
<td>47.8**</td>
</tr>
<tr>
<td>Apoptosis (H&amp;E)</td>
<td></td>
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<td>&gt;500*</td>
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**NOTE:** Results are shown as fold changes in comparison with 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. *, P < 0.05; **, P < 0.005.

*In GIST-PWS GDC-0941 efficacy was assessed only after 19 days.
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 imatinib dose for equivalent inhibition. In GIST-BOE, imatinib treatment caused only a minimal p-KIT and p-AKT inhibition (~40% and 10% reduction, respectively) as expected for KIT exon 9 mutant, whereas 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 5 of 6 xenografts). In parallel, some degree of p-ERK inhibition in GIST-DFR, GIST48, GIST-BOE and GIST882 xenografts were observed, whereas in GIST882Ly and GIST-PSW the p-ERK was upregulated 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 imatinib. 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 extracellular signal–regulated kinase (ERK) inactivation in all xenograft models except GIST-PSW, which featured p-ERK hyperactivation (more than 150%). The latter could be related to the accumulation of activated macrophages that were observed on the histologic 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; refs. 20, 21).

As expected, 4 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 IHC and immunoblotting PTEN expression did not change after 4 and 8 weeks of experiment in all models. In GIST48, we also conducted 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 imatinib-sensitive and imatinib-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 imatinib.

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 proapoptotic activity in these models. Bauer and colleagues described similar results in GIST882 cells treated with the PI3K inhibitor, LY294002, in vitro (8). Moreover, in genetically manipulated imatinib-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 imatinib or GDC-0941 alone in the majority of models and dramatically enhanced antiproliferative activity in all xenografts. Furthermore, in 65% of tumors treated with a combination regimen, we observed high degree of histologic response (grade 3–4), and in 30%
of the tumors the fraction of vital tumor cells was reduced to less than 10%. These astonishing effects may account for the outstanding long-lasting effect observed in the tumor regrowth experiments. Most remarkably, in 3 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 showed an additive antiproliferative effect with the combination of LY294002 and low doses of imatinib, in imatinib-sensitive and -resistant GIST cell lines (8).

Generally, the proapoptotic 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. First, KIT-independent mechanisms may influence proapoptotic responses to therapies. For example, the differential expression of antiapoptotic proteins such as BCL2 in GIST of distinct anatomic sites could hamper proapoptotic activity of antitumor-targeted agents (23). For example, gastric GIST have less BCL2 expression compared with small intestine GIST. Second, KIT may have a dominant role in controlling cell survival in this setting. Thus, if the KIT receptor is not completely inhibited because of an oncogenic mutation with reduced sensitivity to imatinib (e.g., KIT exon 9 or KIT exon 13), then apoptosis might not be substantially induced (22, 24). Therefore, the additive proapoptotic effect of GDC+IMA observed in the GIST xenograft models carrying KIT exon 11 mutations may be related to their intrinsically higher imatinib sensitivity. These observations suggest that the combination of PI3K inhibitors with higher doses of imatinib 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, as the GIST-PSW xenograft model preserved high sensitivity to imatinib 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 3 of 6 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 toward malignancy (2). Genome profiling studies carried out on primary GISTs reveal chromosome 10q loss in about 20% to 38% of patients (29, 30). However, homozygous loss of PTEN was not observed, at least in imatinib-naïve tumors. According to one study, about 40% of primary GIST have reduced levels of PTEN protein by IHC, which was implicated as an independent prognostic marker (31). As of yet, the biologic 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 subclones 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 imatinib. Thereby, we propose that PTEN status and possibly PI3K mutations could serve as a predictive biomarker for the selection of patients with GIST who could respond the best to the combination of imatinib 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 imatinib responses or resistance in patients with GIST (34). Consistent with the observation in the clinic, we show that FDG uptake measured in GIST-xenograft bearing mice correlated well with response to imatinib 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 histologic 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 radiologic classification of responding or nonresponding 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 antiproliferative effect in highly proliferating GIST, and induces variable degrees of necrosis in tumor tissue. In combination with imatinib, the therapeutic activity of GDC-0941 is dramatically enhanced as shown by more prominent histologic response, 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 imatinib in GIST. Our results provide a very strong preclinical rationale for the use of the IMA+PI3K inhibitors combination in patients with GIST in the clinic, and identify PTEN status as a potential predictive biomarker to select a subset of patients with GIST with higher sensitivity toward this type of therapeutic strategy. Of note, a first dose-finding clinical GIST trial, that is based on these and related observations and combines imatinib with another PI3K inhibitor, has started recruiting patients (36).

Disclosure of Potential Conflicts of Interest

A. Wozniak has honoraria from Speakers Bureau of Novartis. J.A. Fletcher has a commercial research grant from Deciphera and is a consultant/advisory board member of Novartis, Ariad, and Deciphera. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: G. Floris, J.A. Fletcher, M. Debiec-Rychter, P. Schöffski

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Floris, A. Wozniak, H. Li, I. Friedman, T. Van Looy, J. Wellsens, P. Vermaelen, M. Debiec-Rychter, P. Schöffski

Writing, reviewing, and/or revision of the manuscript: G. Floris, A. Wozniak, R. Sciot, C.M. Depraetere, M. Debiec-Rychter, P. Schöffski

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Floris, A. Wozniak, R. Sciot, M. Debiec-Rychter, P. Schöffski

Study supervision: G. Floris, A. Wozniak, R. Sciot, M. Debiec-Rychter, P. Schöffski

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References


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