Phosphoinositide 3-kinase inhibitors combined with imatinib in patient-derived xenograft models of gastrointestinal stromal tumours: rationale and efficacy

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Translational relevance

Advanced gastrointestinal stromal tumours (GISTs) are commonly associated with heterogeneous resistance against tyrosine kinase inhibitors (TKIs). The phosphoinositide 3-kinase (PI3K) signalling pathway is considered to be crucial for GIST tumour cell proliferation and survival. Therefore its inhibition can be relevant in overcoming the TKI resistance. In the current study we have demonstrated a significant anti-tumour effect of the three PI3K inhibitors: BEZ235, BKM120 (buparlisib) and BYL719, in GIST xenograft models. While the use of PI3K inhibitors as single agents has some limitations due to a complex signalling cross-talk and the presence of negative feedback loops, the combination with imatinib can potentially overcome these undesired effects on cell signalling. Apart from significantly enhanced efficacy of PI3K inhibitors combination in comparison with imatinib alone, we showed that the response to combination treatment depends on the KIT genotype and PI3K/PTEN genomic status.
Abstract:

**Introduction:** PI3K signalling pathway drives tumour cell proliferation and survival in gastrointestinal stromal tumour (GIST). We tested the *in vivo* efficacy of three PI3K inhibitors (PI3Kis) in patient-derived GIST xenograft models.

**Experimental design:** 168 nude mice were grafted with human GIST carrying diverse *KIT* genotypes and *PTEN* genomic status. Animals were dosed orally for two weeks as follows: control group (untreated); imatinib (IMA); PI3Ki (BKM120 - buparlisib, BEZ235 or BYL719) or combinations of IMA with a PI3Ki. Western blotting, histopathology and tumour volume evolution were used for the assessment of treatment efficacy. Furthermore, tumour regrowth was evaluated for three weeks after treatment cessation.

**Results:** PI3Ki monotherapy showed a significant anti-tumour effect, reflected in tumour volume reduction or stabilization, inhibitory effects on mitotic activity and PI3K signalling inhibition. The IMA+PI3Ki combination remarkably improved the efficacy of either single agent treatment with more pronounced tumour volume reduction and enhanced pro-apoptotic effects over either single agent. Response to IMA+PI3Ki was found to depend on the *KIT* genotype and specific model-related molecular characteristics.

**Conclusion:** IMA+PI3Ki has significant anti-tumour efficacy in GIST xenografts as compared to single agent treatment, resulting in more prominent tumour volume reduction and enhanced induction of apoptosis. Categorization of GIST based on *KIT* genotype and *PI3K/PTEN* genomic status combined with dose optimization is suggested for patient selection for clinical trials exploring such combinations.
Introduction

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour of the digestive tract and is the most common sarcoma in some regions of Europe (1, 2). In the vast majority (85-90%) of patients, GIST is driven by oncogenic KIT or PDGFRA mutations, which lead to constitutive activation of the tyrosine kinase activity of the encoded proteins (3). The majority of GISTs (75-80%) harbour KIT mutations, which are most frequently found in exon 11, encoding the juxtamembrane region of the protein. These mutations are typically in-frame deletions (e.g. p.W557_K558del), insertions, missense mutations or combinations of the above (4). Approximately 6% of GISTs carry mutations in KIT exon 9, which encodes the extracellular domain of the receptor. Less frequently (approximately 2%), GISTs harbour primary KIT exon 13 or exon 17 mutations, respectively encoding the ATP-binding site and activation-loop of the two receptor kinase domains (4). Around 20-25% of GISTs lack primary KIT mutations, of these GISTs about one third has PDGFRA mutations, resulting in PDGFRA kinase activation (4). PDGFRA mutations are mainly found in exon 18, most frequently presenting a p.D842V substitution in the activation loop of the kinase domain. PDGFRA mutations in exons 12 and 14, which respectively induce changes in the juxtamembrane domain and ATP-binding kinase domain I, are far less common (5). Surgical resection is the mainstay of treatment for non-metastatic, resectable GISTs, but unfortunately is not feasible in up to 30% of patients due to anatomic site, metastatic disease or tumour extent. Moreover, about 40% of patients will eventually relapse even after surgical resection (6). The approval of imatinib (IMA), the tyrosine kinase inhibitor (TKI), for the treatment of unresectable or metastatic GIST, together with sunitinib (approved for the treatment of patients failing IMA) and regorafenib (approved for the treatment of patients failing IMA and sunitinib) has revolutionized the therapeutic approach in GIST and dramatically improved the outcome of patients with advanced, metastatic disease. The response of GIST patients to TKIs depends on the underlying specific KIT/PDGFRA mutations in the tumour (7, 8). The emergence of heterogeneous resistance to TKIs, mainly through acquisition of secondary KIT/PDGFRA mutations, is one of the main problems in the treatment of GIST today (9, 10). After failure of all three approved lines of treatment, patients with advanced GIST are currently left without any registered treatment options. Therefore, novel therapeutic strategies are being developed and tested. One of the most promising approaches to combat resistance is inhibition of the PI3K pathway, which is downstream of KIT/PDGFRA. This pathway is deregulated in many human cancers, including GIST and other sarcomas (11-13). Results
obtained in vitro by Bauer et al. support the hypothesis that PI3K inhibition might be a valuable option in the clinical management of GIST patients (14). Recently our group showed anti-tumour effects of the combination of IMA with GDC-0941, an oral pan PI3K inhibitor (PI3Ki), in a panel of GIST xenograft models in vivo (15). In the current study we evaluated the efficacy of three other oral PI3Kis with different pharmacological properties in a variety of human GIST xenografts with variable molecular background. Buparlisib (BKM120; BKM) is a pan-PI3Ki, BEZ235 (BEZ) a dual pan PI3K/mTOR (mammalian target of rapamycin) inhibitor, and BYL719 (BYL) is a selective inhibitor of the PI3K catalytic p110α subunit (16-18). These inhibitors have been shown to potently inhibit tumour cell growth and induce apoptosis both in vitro and in vivo (19-21) in a number of preclinical studies.

Materials and Methods

GIST xenografts

GIST xenografts were established by bilateral, subcutaneous transplantation of human GIST tissues in female adult nu/nu NMRI (Janvier Laboratories) as described previously (15, 22-24). All but GIST48 xenografts were derived from biopsies obtained from patients treated in the Department of General Medical Oncology, University Hospitals Leuven, Belgium. The GIST48 model was derived from injection of the GIST48 cell line (gift from Dr. J.A. Fletcher, Boston, USA) in mice. All of these models have been extensively characterized and used for in vivo experiments (15, 22-24), and a summary of their most important characteristics can be found in Table 1. The mutational analysis for KIT and PIK3CA, as well as the fluorescence in situ hybridization (FISH) to assess PTEN (phosphatase and tensin homolog) copy number were performed as described previously (15). The xenografting of patient-derived mesenchymal tumour material has been approved by the Medical Ethics Committee, University Hospitals Leuven (S53483). All animal experiments were conducted in accordance with Belgian law and approved by the Ethics Committee for Laboratory Animals, KU Leuven, Leuven, Belgium.

Drugs and reagents

IMA, BEZ, BKM and BYL were provided by Novartis Pharmaceuticals Corporation. IMA was dissolved in sterile water, BEZ, BKM and BYL were dissolved in 0.5% methylcellulose (Sigma Aldrich, for BYL), supplemented with 0.05% (for BEZ) or 0.5% (for BKM) Tween
80 (Sigma Aldrich), followed by 30 minutes of sonication at 4°C. Additionally, sonication was applied to all solutions for 5-10 minutes prior the administration.

Western blotting (WB) and immunohistochemistry (IHC) were performed using the following primary antibodies: pKIT Y719, pAKT S473, AKT, α-tubulin, pMAPK T202/Y204, MAPK, PI3K p110α, phospho-Histone H3 (pHH3) (all from Cell Signaling Technologies); pKIT Y703 (Life Technologies); KIT (DAKO); β-actin and tubulin (Sigma Aldrich), cleaved PARP (Abcam), Ki67 (Thermo Scientific). For WB anti-rabbit or anti-mouse secondary antibodies, conjugated with horse radish peroxidase (HRP; DAKO) were applied, and specific bands were visualized using Western Lightning® Plus-ECL (PerkinElmer). For IHC, Signalstain® boost IHC detection reagent (Cell Signaling Technologies) and anti-rabbit or anti-mouse Envision+ system and 3’diaminobenzidine-tetrahydrochloride (DAB) (both from DAKO) were utilized.

Study design

For in vivo experiments 168 engrafted mice (314 tumours) were utilized. The average tumour volume was approximately 560 mm³ at the start of the experiment. Animals were grouped according to tumour size into different treatment groups: a control group (untreated), a group receiving standard treatment (IMA 50 mg/kg/BID, p.o.), groups receiving single agent PI3Ki and groups treated with a combination of IMA and PI3Ki. A detailed summary on treatment doses, schedules and number of animals in each experiment is given in Table 2.

Tumour volume and mouse body weight were assessed regularly as previously described (15, 22-24). Experiments were divided in two phases: two weeks treatment followed by three weeks of the observation to assess the effect of treatment discontinuation (regrowth experiment) in half of the mice; remaining animals were euthanized after two weeks of the treatment (Table 2). Tumour specimens in both phases were collected and fixed in 4% buffered formaldehyde or snap-frozen in liquid nitrogen for further histological and molecular analysis. For the analysis of the efficacy assessment, control tumours collected after two weeks of treatment and after the regrowth experiment were combined.

In a first stage of the study, BEZ and BYL were tested in two IMA-sensitive (UZLX-GIST3 and UZLX-GIST4) and in one IMA resistant (UZLX-GIST2, dose-dependent IMA resistance through KIT exon 9 mutation) models. In the second stage BKM was tested in UZLX-GIST2
and GIST48 (IMA resistance through a secondary KIT exon 17 mutation). A detailed description can be found in supplementary Table S1.

**Histological assessment**

Fixed tumour specimens embedded in paraffin were cut in 4 µm sections for haematoxylin and eosin (H&E) and immunostainings. Histologic response (HR) was graded by assessing the magnitude of necrosis, myxoid degeneration, and/or fibrosis on H&E staining: grade 1 (0%–10%), grade 2 (>10% and ≤50%), grade 3 (>50% and ≤90%), and grade 4 (>90%) as described previously (25, 26). Mitotic and apoptotic activity was assessed by counting mitotic figures and apoptotic cells in 10 high power fields (HPF, 400-fold magnification, 0.45mm field diameter) on H&E stained slides. IHC for pH3 and cleaved PARP as a measure for proliferative and apoptotic activity respectively, were assessed by counting positive cells in 10 HPF. Digital microscopic pictures were taken to calculate the Ki67 labelling index by calculating the average percentage of Ki67 stained tumour cells in 5 images taken at 400-fold magnification. Microscopy was done with an Olympus LH-30M microscope equipped with Color View digital camera and images were analysed utilizing Cell D imaging software (both from Olympus).

**Western blotting**

For WB, tumour lysates were prepared from snap-frozen tumour specimens as described previously (23). Levels of chemiluminescence were captured with the FUJI-LAS mini 3000 system (Fujifilm) and densitometry was performed using the AIDA software (Raytest) to semi-quantify the protein levels as previously described (15).

**Statistical analysis**

Wilcoxon’s matched paired (WMP) test was used for tumour volume comparison between day 0 and at the end of every in vivo experiment. The Mann-Whitney U (MWU) test was used to compare histopathological results and tumour volume between different treatment groups. A value of p<0.05 was considered as statistically significant. STATISTICA 12.0 (StatSoft, Tulsa, USA) was used for all calculations.
Results

Tumour volume evolution

As expected, tumour volume reduction was seen in both KIT exon 11 mutants (KIT\textsuperscript{exon11}; Table 1) under IMA treatment after two weeks of treatment (26% of the starting volume in UZLX-GIST3 and 27% in UZLX-GIST4; p<0.005 for both, WMP) (Table 3 and supplementary Figure S1). Single agent PI3Ki treatment generally resulted in tumour growth delay in both models, and BYL even induced tumour volume stabilization in UZLX-GIST3. IMA+BEZ and IMA+BYL resulted in a superior tumour volume reduction compared to single agent IMA treatment in the UZLX-GIST4 model.

In the KIT exon 9 (KIT\textsuperscript{exon9}) UZLX-GIST2 model, IMA yielded tumour volume stabilization (115% after two weeks, p>0.05; WMP). Similar to IMA, BYL stabilized tumour volume (112% after two weeks), while both BEZ and BKM reduced tumour volume to respectively 77% and 74% (p<0.05, WMP). IMA+BKM and IMA+BYL, yielded a decrease in tumour burden to 58% and 66%, respectively (p<0.05; WMP); while IMA+BEZ was not superior to single agent BEZ treatment in reducing tumour volume. It has to be noted, that for the UZLX-GIST2 model a dose of 40mg/kg/QD was used for the single BEZ treatment, while 10mg/kg/QD was used in combination with IMA. This was due to the observation of severe adverse events (see below) under combination of 20 mg/kg/QD of BEZ with 50 mg/kg/BID IMA (results not shown).

Interestingly, in GIST48 (KIT\textsuperscript{exon11+17} mutant) the tumour burden was decreased by IMA to 52% as compared to baseline. Single agent treatment with BKM led to a comparable tumour volume decrease (reduction to 66%). Importantly, the IMA+BKM combination yielded a significantly better tumour volume reduction (to 14%) than either single treatment (p<0.005, MWU).

Of note, in the BEZ and BYL single agent treatment groups we observed mild body weight loss and dry-skin desquamation. In the BYL group, we also observed polyuria. While testing the IMA+BEZ combination in the first group of UZLX-GIST2 bearing mice, some animals showed severe body weight loss and dry-skin desquamation accompanied with fatigue, prompting us to euthanize them for ethical reasons on day 8 (results not included in this study). Additionally, IMA+BYL treated mice showed substantial body weight loss, moderate dry-skin desquamation, fatigue and more frequent urination. The onset of such undesirable
effects in the IMA+BYL group was delayed as compared to the IMA+BEZ cohort, and symptoms disappeared after treatment discontinuation. For these reasons we reduced the BEZ and BYL doses, both as single agents as well as in combination regimens in subsequent experiments and we retested IMA+BEZ with lowered BEZ dose in the UZLX-GIST2 model during the second stage of the study (Table 2).

**Histopathology**

**Histologic response**

All models retained the morphological features and showed the same KIT mutations as previously found in patients’ biopsies used to establish the xenograft models or in the original cell line.

Of all models tested, UZLX-GIST3 was most susceptible to induction of HR under the chosen experimental conditions. In this model, HR most often involved a replacement of viable tumour tissue by myxoid degeneration, a low cellular amorphous matrix, which resembles the typical response pattern of GIST to IMA in the clinic. IMA induced grade 2 or 3 HR in 87.5% of UZLX-GIST3 grafts. BEZ and BYL yielded grade 2 or 3 HR in 25% of tumours. Importantly, IMA+BEZ and IMA+BYL combinations induced even a higher level of response than any single agent treatment, yielding grade 4 in at least 50% of tumours (Figure 1A).

In UZLX-GIST4 and UZLX-GIST2 models HR was characterized mainly by necrosis and was less pronounced than in UZLX-GIST3 (Figure 1B and 1C). In UZLX-GIST2 histologic response in the majority of tumours was limited to grade 1 or 2 responses, while the interpretation of HR in the UZLX-GIST4 model was more uncertain as about 20% of control tumours showed spontaneous necrosis (Figure 1B and 1C). In GIST48, IMA and BKM induced grade 2 HR in 50% and 25% of tumours, respectively (Figure 1D). IMA+BKM induced grade 2 or higher HR in 40% of tumours.

**Mitotic and apoptotic activity**

In addition to HR, the mitotic and apoptotic activity was assessed in all untreated control tumours and treated tumours collected at the time of treatment discontinuation. When all xenograft models were considered, control tumours showed brisk mitotic activity with an average of 25 (in UZLX-GIST2), 26 (UZLX-GIST3), 37 (UZLX-GIST4) and 17 (GIST48) mitotic figures per 10 HPF.
As expected, mitotic activity was virtually absent under IMA treatment in both $KIT^{exon11}$ mutants as compared to control (p<0.005, MWU). Under IMA, the apoptotic activity was significantly induced only in the UZLX-GIST3 model (p<0.005, MWU) (Table 4). BEZ and BYL showed only limited effects on the mitotic and apoptotic activity in UZLX-GIST3 and -GIST4. Combination treatment with IMA, however, virtually silenced proliferative activity, similar to IMA alone. In addition, we found that IMA+BEZ and IMA+BYL were significantly better in inducing apoptosis as compared to either single agent treatment.

In the UZLX-GIST2 model ($KIT^{exon9}$ mutant), IMA yielded a 1.5-fold reduction of mitotic activity (p<0.05, MWU). Remarkably, BEZ (11.7-fold) and BYL (2.2-fold) significantly reduced mitotic activity (p<0.005, MWU), while BKM did not induce a substantial decrease in mitotic activity. IMA+BYL (3.7-fold) and IMA+BKM (2.2-fold) induced a slightly more potent decrease in proliferative activity compared to either single treatment. Interestingly, BEZ as a single agent was more potent in decreasing mitotic activity than IMA+BEZ combination. This observation is most likely related to the higher dose (40mg/kg/QD) administered in the BEZ single agent experiment in the UZLX-GIST2 model as compared to the dose (10mg/kg QD) given in all other models as well as in the IMA+BEZ combination study in UZLX-GIST2. In UZLX-GIST2, neither treatment was able to induce an obvious pro-apoptotic affect.

In the GIST48 model IMA and BKM produced a similar decrease in mitotic activity (14.8- and 15.3-fold respectively) when compared to control (p<0.005, MWU). Importantly, IMA+BKM induced a more potent inhibition of mitotic activity than either IMA or BKM alone. Similar as in the $KIT^{exon11}$ mutants, combination treatment showed the best pro-apoptotic activity (3.3-fold increase as compared to the control, p<0.005, MWU). The results of mitotic and apoptotic activity, assessed on H&E, were confirmed using IHC markers in all models (Table 4).

**KIT and PI3K signalling evaluation**

WB was performed to assess the effects on KIT activation and signalling. The analysis showed expression and activation of KIT and known crucial signalling intermediates in untreated tumours from all GIST xenografts (Supplementary Figure S2). Furthermore, the PI3K p110α subunit was shown to be expressed in all our models, proving the presence of the main target for all PI3Ki (Supplementary Figure S2).
As expected, IMA inhibited (> 65%) pKIT Y719 and pKIT Y703 in both KITexon11 mutants (Figure 2A and 2B, and Supplementary Figure S2). Notably, IMA treatment inhibited pAKT more in UZLX-GIST3 than in UZLX-GIST4 model (92% and 32% reduction, respectively). Similarly, in the former model, BEZ and BYL decreased AKT activation by 95% and 72%, whereas there was no decrease in AKT activation under BEZ in UZLX-GIST4, and under BYL AKT activation was even increased as compared to controls. In UZLX-GIST3, the combinations of IMA+BYL and IMA+BEZ were similar to single IMA with respect to effects on AKT activation. By contrast, the combination of IMA with PI3Ki in UZLX-GIST4 led to improved efficacy in terms of inhibition of AKT activation. IMA+BEZ and IMA+BYL induced a decrease in AKT activation by 90% and 75% respectively, whereas IMA as a single agent yielded only a 32% reduction in AKT activation (Figure 2 and Supplementary Figure S2).

As expected, IMA induced a less pronounced inhibition of pKIT and pAKT in UZLX-GIST2 than in the KITexon11 mutants (37% and 35% reduction, respectively for both proteins). However, a substantial reduction in MAPK (84%) activation was observed (Figure 2C; Supplementary Figure S2). BEZ and BYL induced a more impressive decrease in pAKT (76% and 84% reduction) as compared to single IMA treatment, and BKM induced a complete inactivation of AKT (>99% reduction). Combination treatment did not result in a remarkable improved inhibition of AKT as compared to single agents.

Despite the secondary KITexon17 mutation, IMA induced a strong inhibition of both pKIT and its downstream intermediates in GIST48 (Figure 2D and Supplementary Figure S2). Importantly, under BKM and IMA+BKM activation of AKT was virtually absent, similar to what was observed in the UZLX-GIST2 model.

Of note, although the PI3Kis tested have no known direct activity against KIT and MAPK, the activation of these proteins showed inconsistencies and/or hyperactivation in our GIST xenograft models under PI3Kis and combination treatments. The most striking observation was a hyperactivation of MAPK in UZLX-GIST4 under PI3Kis, and even more prominent under combination treatments. These findings could be explained by complex cross-talk networks and release of negative feedback loops associated with the inhibition of PI3K signalling.
**Xenograft regrowth assessment**

Tumour regrowth after treatment discontinuation was observed in all models after treatment discontinuation irrespective of treatment. However, tumour regrowth rate as compared to IMA seemed delayed under IMA+BEZ and IMA+BYL in UZLX-GIST3 and under IMA+BYL in UZLX-GIST4 (Supplementary Table S2 and Figure S2). However, we did not observe any significant, long-lasting inhibition of mitosis or induction of apoptosis in either model irrespective of treatment regimen (Supplementary Table S2). Therefore, the delay in tumour regrowth is most likely related to the higher induction of histologic response under the combination regimens as compared to single agent observed after 2 weeks of treatment (Figure 1). In the UZLX-GIST2 model we observed an immediate tumour regrowth under IMA+BEZ 8 days after treatment discontinuation, therefore mice entered in xenograft regrowth in this stage of the study were euthanized at that time (data not shown).

**Discussion**

The PI3K/AKT signalling pathway is a crucial regulator of cell proliferation and survival in GIST (13, 14). For this reason we evaluated the efficacy of PI3K signalling inhibitors in animal models of this malignancy. In the current study we have shown anti-tumour effects of three PI3Ki (namely BEZ, BYL and BKM) with different pharmacological profiles. In combination with standard TKI treatment, we observed a remarkably improved efficacy as compared to single agent TKI treatment in GIST xenograft models with diverse KIT genotype and PTEN genomic status and a different sensitivity to the established TKIs.

In the present study, BEZ, a dual PI3K/mTOR inhibitor, caused tumour stabilization in both KIT\textsuperscript{exon11} mutants. In KIT\textsuperscript{exon9} mutants, it induced a more pronounced tumour growth delay and significant reduction in mitotic activity than IMA alone. AKT activation was strongly inhibited in KIT\textsuperscript{exon9} mutants, whereas in the KIT\textsuperscript{exon11} mutants AKT inhibition was only observed in the UZLX-GIST3 model, but not in the UZLX-GIST4 model. This observation could be explained by homozygous PTEN loss in the latter xenograft. Indeed, PTEN acts as a negative regulator of the PI3K/AKT signalling pathway (27). This hypothesis and our results are consistent with a study conducted by Quattrone and colleagues, in which the effect of PTEN silencing was evaluated in the IMA-sensitive GIST-T1 (KIT\textsuperscript{exon11} p.V560_Y579del) and IMA-resistant GIST430 (KIT\textsuperscript{exon11+13} p.V560_L576del+V654A) cell lines (28). Importantly, silencing of PTEN resulted in over-activation of AKT in both models in vitro. Moreover, AKT inhibition under BEZ treatment was less pronounced in PTEN silenced cells.
than in non-silenced. Additionally, *in vivo* studies performed by Floris *et al.* showed a more pronounced inhibitory effect of the pan PI3Ki GDC-0941 on AKT activation in KIT<sup>exon11</sup> mutant xenografts without PTEN loss, as compared to those with this specific genomic change (15). Of note, GDC-0941 is a pan PI3Ki, whereas BEZ235 is a dual PI3K-mTOR inhibitor. In addition, the UZLX-GIST4 model also harbours a mutation in exon 6 of the PI3KCA gene (c.1093 G>A; p.E365K). This mutation has been described before in endometrial carcinoma (COSM86044) (29, 30). Furthermore, Oda *et al.* have provided experimental evidence that this mutation can cause a PI3K gain-of-function phenotype (31).

In our study we also tested BKM, another pan PI3Ki, in the two IMA-resistant models, UZLX-GIST2 (dose dependent IMA-resistance through KIT<sup>exon9</sup> mutation) and GIST48 (IMA-resistance due to secondary KIT<sup>exon17</sup> mutation). BKM caused moderate tumour volume reduction and total AKT inhibition in both models, but the proliferative activity was inhibited only in the GIST48 model. This observation could be related to differences (e.g. molecular characteristics, drug clearance, etc.) between both models. Our findings are consistent with *in vivo* experiments with GDC-0941 mentioned above (15). In KIT<sup>exon9</sup> mutants, GDC-0941 induced tumour volume stabilization, a mild decrease in mitotic activity and more pronounced AKT inactivation than single agent IMA. In GIST48, effects of GDC-0941 were similar to our findings with BKM, although single agent BKM was more effective in terms of reduction in tumour volume and mitotic activity. Additionally, our results are also in line with work performed by Bauer and co-workers, who have observed inhibition of mitotic activity and AKT activation *in vitro* with the PI3Ki LY294002, a pan PI3Ki, in GIST48 (14). Despite the secondary KIT<sup>exon17</sup> mutation, in our experiments GIST48 showed a strong response to IMA in terms of reduction of tumour burden and mitotic activity. Moderate to strong responses to IMA have been previously described in GIST48, both *in vitro* and *in vivo*, and might be due to the heterozygous nature of the secondary KIT<sup>exon17</sup> mutation in GIST48 (14,15,23,32). Due to the lack of IMA resistance in the GIST48 model we decided not to further test PI3Kis in this model.

Beside BEZ and BKM, we also tested the efficacy of BYL, a specific inhibitor blocking the p110<sub>α</sub> catalytic domain of PI3K. BYL used alone induced a statistically significant tumour volume decrease only in UZLX-GIST3, while tumour burden increased in the UZLX-GIST2 and UZLX-GIST4 models. BYL induced a strong AKT inhibition in UZLX-GIST3 and UZLX-GIST2 models; in contrast, to UZLX-GIST4, where an elevation of AKT activation was observed.
Interestingly, as demonstrated by differential AKT inhibition, we have observed intra- and inter-model variability in the response of our xenograft panel to different PI3Kis. This variability is most likely explained by the distinct molecular characteristics of different GIST models and the complex nature of the PI3K signalling pathway. The PTEN/PI3K/AKT pathway is known to be associated with complex cross-talk networks (e.g. with the RAS and c-JUN N-terminal kinases pathways), and elaborate negative feedback loops modulating upstream signalling mediators, including receptor tyrosine kinases (RTKs) (33-35). Hence, the inconsistencies in MAPK and KIT activation under PI3Ki treatment observed in our studies and in other published experiments are most likely related to these complex networks of cross-talk and feedback loops. This is further illustrated by the differential effects on AKT activation by the diverse PI3Ki in our GIST models tested. Indeed, PI3K inhibition resulted in a strong inhibition of AKT phosphorylation in all models but UZLX-GIST4, which is characterized by homozygous PTEN loss and an additional PIK3CA mutation. As mentioned above, these molecular defects could be involved in the lack of AKT inhibition observed under BEZ and BYL in UZLX-GIST4. In addition, the difference in response could be explained by the different specificity of the tested inhibitors. Over-activation of AKT observed in xenografts treated with BYL, a specific PI3K p100α inhibitor, could be explained through release of negative feedback loops due to PI3K inhibition (33-35). In addition, class I PI3K are known to function as heterodimers of a catalytic subunit (p110α, β, δ, γ) and a regulatory subunit (p85α, p85β, p55γ, p101 or p84), and it has been shown that PI3K signalling in PTEN-deficient tumours seems to depend mainly on the p110β PI3K catalytic subunit for activation (36, 37). BEZ, as opposed to BYL, has activity against all PI3K p110 subunits and simultaneously inhibits mTOR, which could potentially inhibit the release of negative feedback loops (33-35). Certain PI3Ki characteristics, such as off-target effects, could also influence the response of GIST models with different molecular backgrounds.

BKM has been shown to induce changes in expression of genes involved in mitosis, and inhibition of microtubule dynamics (38). BEZ is also known to induce a number of off-target effects as was demonstrated by Kong et al. (39). They showed that BEZ does not solely target class I PI3K, but also potently inhibits class II and class III PI3K, whose functions are complex and not completely elucidated (36). These off-target effects could potentially affect the sensitivity to BEZ through reactivation loops.

It has been suggested that the combination of PI3Kis with RTK inhibitors or inhibitors of related active signalling pathways might prevent cross-activation of signalling pathways and
release of negative feedback loops (33-35). Indeed, combinations of PI3Ki with inhibitors of other pathways (e.g. RAF/MEK/MAPK) or RTK inhibitors have shown some synergistic effects in preclinical models (35, 40-42).

Our results provide strong evidence for the hypothesis that the combination of RTK inhibitors and PI3Kis could lead to improved efficacy over single agent PI3Ki and IMA. The most striking observation in our study was the prominent improved anti-tumour activity of combining IMA+PI3Ki over either single treatment. Certainly, combination treatment was more potent in reducing tumour volume than the administration of single agents, which was most pronounced in both ULZX-GIST4 (KIT\textsuperscript{exon11} mutant) and in GIST48 (KIT\textsuperscript{exon11+exon17} mutant). Moreover, the induction of apoptosis was much stronger under combination treatment than under single agent treatment regimens in all but the KIT\textsuperscript{exon9} mutant model. Additionally, a remarkable induction of HR characterized by myxoid degeneration was observed in UZLX-GIST3, which was more potent than under single agent IMA (grade 2 or higher in all treated tumours).

Our results are consistent with \textit{in vitro} studies, which have demonstrated improved anti-proliferative effects of combining PI3Ki with IMA in both IMA sensitive and resistant cell lines (14, 43). Our findings are also in line with experiments reported by our group (15), which have proven the superior efficacy of IMA+GDC-0941, over either single treatment. Similarly to our study, Floris \textit{et al.} observed a more potent reduction in tumour volume under combination treatment than under either single agent in general. GDC-0941+IMA also induced a high degree of HR (grade 3 or 4) in 65% of tumours. In the present study the amount of grade 3-4 HR was limited to 38%, 29% and 9% respectively for IMA+BYL, IMA+BEZ and IMA+BKM. We believe that this discordance in induction of HR is caused by a shorter duration of treatment (two weeks) in our experiments as compared to those performed by Floris and colleagues (4 weeks) (15). Additionally, since no statistically significant long-lasting effects on cell survival or proliferation were reported in our study or the one published by Floris \textit{et al.}, we believe that the lack of long-lasting effect on tumour regrowth in our experiments is caused by a lower degree of histologic response due to shorter treatment duration.

In both studies combination treatment was far more potent in inducing apoptosis than either single agent treatment in all but the KIT\textsuperscript{exon9} mutants. The lack of remarkable improved efficacy in KIT\textsuperscript{exon9} mutants could be attributed to the differential activation of signalling
molecules, discordances in gene and protein expression and IMA sensitivity between different KIT mutants. Duensing and coworkers have reported that specific KITexon9 mutants show lower levels of AKT and S6K activation than KITexon11 mutants (13). In addition, it has been shown that there are significant differences in gene and protein expression between KITexon9 and KITexon11 mutants (44, 45). These differences in signalling activation, and gene and protein expression could explain differences in response to treatment (e.g. effect on sensitivity to PI3K inhibition, reduced sensitivity to pro-apoptotic signals, etc.). Interestingly, the absence of enhanced efficacy upon combination of IMA and PI3Kis in KITexon9 could be due to the known reduced sensitivity to IMA in this subset of GIST. This hypothesis is supported by the fact that the two IMA sensitive KITexon11 and the GIST48 model all showed enhanced efficacy upon combination of IMA and PI3Kis. Therefore, we hypothesize that the response to a combination of a PI3Ki with IMA depends on the KIT genotype and sensitivity of the model to IMA-related KIT inhibition. Hence, similar to clinical observations where dose-escalation improved prognosis in KITexon9 mutants, dose-finding studies to optimize combination of IMA and PI3Ki could lead to an enhancement of efficacy (8).

Besides, the importance of KIT genotype in the response to combinations of IMA and PI3Kis, our findings support the importance of PTEN status for the sensitivity to PI3Ki. Indeed, the absence of AKT inactivation under single agent PI3Ki in UZLX-GIST4 was counteracted by combining IMA+PI3Ki, which led to improved efficacy over either single treatment. The same evidence comes from Quattrone and colleagues who have shown that PTEN silencing reduced the inhibitory effect of BEZ in GIST cell lines, which were reverted substantially by IMA+BEZ combination (28). Floris et al. showed that GDC-0941 had a more pronounced inhibitory effect on AKT in KITexon11 mutants without PTEN loss as compared to PTEN deficient KITexon11 mutants (15). However, to confirm this hypothesis, it would need to be validated in a clinical trial. Nevertheless, PTEN status will most likely not be the only important determinant of the response of GIST to PI3Ki. Several studies have described divergent gene expression profiles and activation of certain signalling pathways in different subsets of GIST patients (13, 44). Hence, variability in oncogenic signalling connected to KIT genotype and most likely altered after progression under standard treatment will further influence the response to PI3Ki. Our data provide justification for a more in depth molecular/genetic characterization of refractory GIST tissue compared to what is currently done in clinical routine. Interestingly, in two currently on-going clinical trials, testing the combination of IMA with either BYL or BKM in imatinib and sunitinib refractory patients,
KIT/PDGFRα/PIK3CA/PTEN mutational status and changes in downstream PI3K signalling markers will be evaluated in archival, pre-treatment or fresh tumour biopsies (when available) (46, 47). These clinical studies are better suited and will have more statistical power to provide us with a better understanding of the importance of PIK3CA, PTEN and KIT genotype in the response to PI3K inhibitors.

Side effects observed in our study are most likely related to the concomitant inhibition of different signalling pathways and possible off-target effects of PI3Ki tested. Besides aforementioned off-target effects of BKM (mitotic index) and BEZ (class II and II PI3K), other off-target effects have been observed (38, 39). BEZ is known to inhibit DNA-dependent protein kinase, which is an important member of the DNA repair mechanism and could explain some of the adverse events observed under IMA+BEZ combination (39). Polyuria observed in the BYL and IMA+BYL treated groups could be explained through an effect of BYL on insulin-mediated glucose regulation (48).

In conclusion, we were able to show in vivo anti-tumour activity of three PI3Ki in GIST, providing supportive evidence of the use of this class of targeted agents in this malignancy. However, complex networks of cross-talk pathways and release of negative feedback loops are known to be associated with the use of PI3Ki. Overall, the combination of IMA+PI3Kis led to significantly enhanced efficacy over either single agent treatment, as observed by more pronounced tumour volume reduction, and greater pro-apoptotic effects than IMA single treatment. However, molecular categorization of GIST patients and dose-optimization should be considered in the further clinical development of such experimental combinations. Our results provide a convincing preclinical rationale for two on-going clinical trials in refractory GIST patients combining IMA+BYL or IMA+BKM (46, 47).

Acknowledgements

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Part of the results of the study has been presented at the Annual Meeting of the American Society of Clinical Oncology (June 1-5, 2012, Chicago, IL). J Clin Oncol 30, 2012 (suppl; abstr 10030).
References


43. Li F, Growney J, Battalagine L, Qiu S, Manley P, Monahan J. The effect combining the KIT inhibitor Imatinib with the PI3K inhibitor BKM120 or the dual PI3K/mTOR inhibitor BEZ235 on the proliferation of gastrointestinal stromal tumor cell lines [abstract]. Cancer Res 2012;72(8 Suppl):Abstract nr 2239.


Figures legend:

**Figure 1.** Assessment of histologic response of categorized per treatment in UZLX-GIST3 (A), UZLX-GIST4 (B), UZLX-GIST2 (C), and GIST48. Histologic response was graded by assessing the magnitude of necrosis, myxoid degeneration, and/or fibrosis on H&E staining: grade 1 (0%–10%), grade 2 (>10% and ≤50%), grade 3 (>50% and ≤90%), and grade 4 (>90%) (25, 26).*BEZ and BYL were not evaluated in GIST48, BKM was evaluated only in UZLX-GIST2 and GIST48.

**Figure 2.** KIT signaling assessment categorized by treatment in UZLX-GIST3 (A), UZLX-GIST4 (B), UZLX-GIST2 (C) and GIST48 (D). Western blotting pictures were used for densitometric analysis; bands were normalized for equal loading and total protein forms, and subsequently compared to control as previously described (15).
Table 1. Detailed description of xenograft models used in the study

<table>
<thead>
<tr>
<th>Model name</th>
<th>Origin</th>
<th>KIT mutational status</th>
<th>Imatinib sensitivity</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>UZLX-GIST2</td>
<td>Patient biopsy</td>
<td>KIT&lt;sup&gt;exon9&lt;/sup&gt; p.A502_Y503dup</td>
<td>Dose-dependent imatinib resistance</td>
<td></td>
</tr>
<tr>
<td>UZLX-GIST3</td>
<td>Patient biopsy</td>
<td>KIT&lt;sup&gt;exon11&lt;/sup&gt; p.W557_V559delinsF</td>
<td>Imatinib sensitive</td>
<td></td>
</tr>
<tr>
<td>GIST48</td>
<td>Patient-derived cell line</td>
<td>KIT&lt;sup&gt;exon11^+17&lt;/sup&gt; p.V560D and p.D820A</td>
<td>Imatinib resistant</td>
<td>Heterozygous PTEN loss</td>
</tr>
</tbody>
</table>
Table 2. Summary of efficacy experiments. Detailed description of the number of mice entered and the doses and schedules of drugs in all *in vivo* experiments.

<table>
<thead>
<tr>
<th>Xenograft model</th>
<th>Passage</th>
<th>Control</th>
<th>IMA</th>
<th>BEZ</th>
<th>BYL</th>
<th>BKM</th>
<th>IMA+BEZ</th>
<th>IMA+BYL</th>
<th>IMA+BKM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>dose</td>
<td>n</td>
<td>dose</td>
<td>n</td>
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<tr>
<td>UZLX-GIST3</td>
<td>11</td>
<td>7</td>
<td>(3)</td>
<td></td>
<td>8</td>
<td>(4)</td>
<td>10mg/kg</td>
<td>QD</td>
<td></td>
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<td>untreated</td>
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<td></td>
<td></td>
<td>8</td>
<td>(4)</td>
<td>50mg/kg</td>
<td>BID</td>
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</tr>
<tr>
<td>UZLX-GIST4</td>
<td>18</td>
<td>6</td>
<td>(3)</td>
<td></td>
<td>6</td>
<td>(3)</td>
<td>50mg/kg</td>
<td>BID</td>
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<tr>
<td></td>
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<td>untreated</td>
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<td></td>
<td></td>
<td>8</td>
<td>(4)</td>
<td>50mg/kg</td>
<td>BID</td>
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<td></td>
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</tr>
<tr>
<td>UZLX-GIST2*</td>
<td>10</td>
<td>7</td>
<td>(3)</td>
<td></td>
<td>7</td>
<td>(3)</td>
<td>40mg/kg</td>
<td>QD</td>
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<td></td>
<td></td>
<td>8</td>
<td>(4)</td>
<td>50mg/kg</td>
<td>BID</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>UZLX-GIST2*</td>
<td>14°</td>
<td>3</td>
<td>(2)</td>
<td></td>
<td>4</td>
<td>(2)</td>
<td>50mg/kg</td>
<td>BID</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>untreated</td>
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<tr>
<td></td>
<td></td>
<td>n/a</td>
<td></td>
<td></td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIST48</td>
<td>10</td>
<td>4</td>
<td>(3)</td>
<td></td>
<td>6</td>
<td>(3)</td>
<td>50mg/kg</td>
<td>BID</td>
<td></td>
</tr>
<tr>
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<td>untreated</td>
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<td></td>
<td></td>
<td>7</td>
<td>(3)</td>
<td>30mg/kg</td>
<td>QD</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* n number of mice entered in the experiment; between brackets the remaining number of mice that entered the xenograft regrowth experiment after treatment discontinuation, if applicable

* Same dose and schedule as for single treatment

* UZLX-GIST2 was used in two stages of the study: firstly IMA, BYL, BEZ, IMA+BEZ (sacrificed early, results not shown), and IMA+BYL were tested; due to observed toxicities doses were lowered in all other experiments. Additionally, in a second stage of the study IMA, BKM, IMA+BKM were tested, and also IMA+BEZ was retested. Results for control and IMA treated tumours of two experiments for UZLX-GIST2 were combined for analysis.

* In the UZLX-GIST2 we observed an immediate regrowth of tumour volume under IMA+BEZ 8 days after treatment withdrawal, therefore mice that entered xenograft regrowth in this stage of the study were euthanized at that time

n/a - not applicable; QD - once a day; BID - twice a day; IMA - imatinib; BEZ - BEZ235; BYL - BYL719; BKM - BKM120
Table 3. Relative tumour volume assessment in GIST models after treatment (end of the 2nd week), data are presented as percentages (±SD) of the baseline values.

<table>
<thead>
<tr>
<th></th>
<th>KIT&lt;sup&gt;exon11&lt;/sup&gt;</th>
<th>KIT&lt;sup&gt;exon9&lt;/sup&gt;</th>
<th>KIT&lt;sup&gt;exon11+17&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UZLX-GIST3</td>
<td>UZLX-GIST4</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>217± 95</td>
<td>175± 71</td>
<td></td>
</tr>
<tr>
<td>IMA</td>
<td>26± 9**</td>
<td>27± 8**</td>
<td>115± 48**</td>
</tr>
<tr>
<td>BEZ</td>
<td>124± 49**</td>
<td>119± 35*</td>
<td>77± 20**</td>
</tr>
<tr>
<td>BYL</td>
<td>86± 26**</td>
<td>129± 30</td>
<td>112± 159**</td>
</tr>
<tr>
<td>BKM</td>
<td>n/a</td>
<td>n/a</td>
<td>74± 21**</td>
</tr>
<tr>
<td>IMA+BEZ</td>
<td>22± 8**</td>
<td>15± 6**</td>
<td>74± 21**</td>
</tr>
<tr>
<td>IMA+BYL</td>
<td>23± 13**</td>
<td>11± 5**</td>
<td>66± 16**</td>
</tr>
<tr>
<td>IMA+BKM</td>
<td>n/a</td>
<td>n/a</td>
<td>58± 26**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14± 5**</td>
</tr>
</tbody>
</table>

Mann–Whitney U test was performed for statistical analysis, comparing treatment arm with the control group (* p<0.05, **p<0.005)

SD - standard deviation; n/a – not applicable
Table 4. Histological assessment of proliferative and apoptotic activity, performed on tumours collected after two weeks of treatment.

<table>
<thead>
<tr>
<th>Xenograft model</th>
<th>KIT exon 11</th>
<th>KIT exon 9</th>
<th>KIT exon 11+17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H&amp;E pH3 Ki67</td>
<td>H&amp;E pH3 Ki67</td>
<td>H&amp;E pH3 Ki67</td>
</tr>
<tr>
<td>UZLX-GIST3</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
</tr>
<tr>
<td>UZLX-GIST4</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
</tr>
<tr>
<td>UZLX-GIST2</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
</tr>
<tr>
<td>GIST48</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
<td>↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**</td>
</tr>
</tbody>
</table>

- Mitotic and proliferative activity:
  - IMA: ↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**
  - BEZ: ↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**
  - BYL: ↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**
  - BKM: n/a n/a n/a n/a
  - IMA+BEZ: ↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**
  - IMA+BYL: ↓↓↓** ↓↓↓** ↓↓↓** ↓↓↓**
  - IMA+BKM: n/a n/a n/a n/a

- Apoptotic activity:
  - IMA: ↑7.4** ↑8.8**
  - BEZ: ↑1.9 ↑2.1
  - BYL: ↑1.2 =1.0
  - BKM: n/a n/a
  - IMA+BEZ: ↑18.9** ↑27.2**
  - IMA+BYL: ↑21.2** ↑29.7**
  - IMA+BKM: n/a n/a

Results are shown as fold changes in comparison with control, arrows indicated by increase (arrow up) or decrease (arrow down).

Mann-Whitney U test was performed for statistical analysis; comparing result from treatment arm with control group * p<0.05, ** p<0.005.

↓↓↓ - more than 100-fold decrease; n/a – not applicable; H&E - haematoxylin and eosin staining; pH3 - phospho-histone-H3 immunostaining; Cl-PARP - Cleaved PARP immunostaining.
Figure 2_Van Looy et al.

A. UZLX-GIST3

B. UZLX-GIST4

C. UZLX-GIST2

D. GIST48

- Imatinib
- BEZ235
- BYL719
- BKM120
- Imatinib+BEZ235
- Imatinib+BYL719
- Imatinib+BKM120
Clinical Cancer Research

Phosphoinositide 3-kinase inhibitors combined with imatinib in patient-derived xenograft models of gastrointestinal stromal tumours: rationale and efficacy

Thomas Van Looy, Agnieszka Wozniak, Giuseppe Floris, et al.

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