Preclinical Development

The Heat Shock Protein 90 Inhibitor IPI-504 Induces KIT Degradation, Tumor Shrinkage, and Cell Proliferation Arrest in Xenograft Models of Gastrointestinal Stromal Tumors

Giuseppe Floris, Maria Debiec-Rychter, Agnieszka Wozniak, Cristiana Stefan, Emmanuel Normant, Gavino Faa, Kathleen Machiels, Ulla Vanleeuw, Raf Sciot, and Patrick Schöffski

Abstract

The activity of the receptor tyrosine kinase KIT is crucial for gastrointestinal stromal tumor (GIST) growth and survival. Imatinib and sunitinib are very effective in advanced GIST, but have no curative potential. The observation that heat shock protein 90 (HSP90) inhibition results in KIT degradation prompted us to assess the efficacy of the HSP90 inhibitor retaspimycin hydrochloride (IPI-504) alone or in combination with imatinib or sunitinib in two GIST xenografts with distinctive KIT mutations. Nude mice were grafted with human GIST carrying KIT exon 13 (GIST-882; n = 59) or exon 11 (GIST-PSW; n = 44) mutations and dosed with imatinib (50 mg/kg twice daily), sunitinib (40 mg/kg once daily), IPI-504 (100 mg/kg 3 times per week), IPI-504 + imatinib, or IPI-504 + sunitinib. We evaluated tumor volume, proliferation and apoptosis, KIT expression and activation, as well as adverse events during treatment. Treatment with IPI-504 alone resulted in tumor regression, proliferation arrest, and induction of tumor necrosis. We documented downregulation of KIT and its signaling cascade in IPI-504–treated animals. Treatment effects were enhanced by combining IPI-504 with imatinib or sunitinib. On histologic examination, liver damage was frequently observed in animals exposed to combination treatments. In conclusion, IPI-504 shows consistent antitumor activity and induces KIT downregulation in GIST, as a single agent, and is more potent in combination with imatinib or sunitinib. The sequence of drug administration in the combination arms warrants further studies. Mol Cancer Ther; 10(10); 987–908. ©2011 AACR.

Introduction

Gastrointestinal stromal tumors (GIST) are the most common sarcomas of the digestive system (1). Approximately 95% of GISTs express the receptor tyrosine kinase KIT (2). Between 80% and 85% of GISTs carry somatic activating mutations in the KIT gene or in the platelet-derived growth factor receptor-α (PDGFRα) gene, which are causative events in GIST development (1, 2). Selective tyrosine kinase inhibitors (TKI), such as imatinib mesylate, have become standard palliative care for patients with advanced, inoperable disease (3, 4). However, imatinib shows only limited curative potential and is maintained indefinitely until emergence of intolerance or resistance (1, 5). Interestingly, response to imatinib is significantly dependent on the tumor genotype and not all patients respond equally to the standard imatinib dose (i.e., 400 mg/d; ref. 6). Thus, although the 400-mg schedule is sufficient to achieve a significant clinical benefit in GISTs harboring KIT exon 11 mutations, the tumors with mutation in KIT exon 9 might require a higher imatinib dose. Patients with GIST carrying mutations causing primary resistance to imatinib (such as PDGFRAp D842V) do not benefit from imatinib therapy. Therefore, imatinib treatment should be tailored to the type of KIT/PDGFRα mutation (6). GIST patients refractory or intolerant to imatinib are treated with another TKI, sunitinib malate (4). Eventually, sunitinib also ceases to be effective, leaving GIST patients without approved alternative treatment options. Resistance to TKIs is either preexisting or acquired through the clonal evolution of cells harboring secondary mutations that hamper TKI activity, or through KIT amplification (7, 8). The development of novel therapeutic strategies that are able to extend the...
therapeutic efficacy of TKI or to overcome the problem of intolerance or resistance to TKI is of utmost importance for GIST patients with advanced disease.

Heat shock protein 90 (HSP90) is one of the most abundant proteins in a cell (9). HSP90 regulates the conformation, function, and activation of a number of client proteins that are involved in the control of pivotal cellular functions, such as signaling, proliferation, and survival (10, 11). Many of these functions are deregulated in cancer; therefore, targeting HSP90 may be a powerful strategy for modulating the growth of certain malignancies (11). Several HSP90-inhibitors (HSP90-i) are currently being studied in phase I/phase II clinical trials in different malignancies.

HSP90 activity is regulated by a number of cofactors (co-chaperones) as well as conformational changes requiring ATP hydrolysis by the ATPase function of HSP90 (12). HSP90-i molecules act by competitively blocking HSP90 enzymatic activity, resulting in the degradation of its client proteins (13). The KIT receptor is one such HSP90 client protein. HSP90 inhibition in human cell lines of mastocytosis, a disease associated with somatic KIT point mutations, results in KIT inhibition and KIT downregulation (14). Similarly, in diverse GIST cell lines, HSP90 inhibition was proved to efficiently kill tumor cells (15). In addition, cell lines expressing GIST-related PDGFRA mutations showed high sensitivity to HSP90-i (16). These data provide the rationale to target HSP90 in GIST.

HSP90 inhibitors are natural or semisynthetic compounds. The natural antibiotics benzoquinone ansamycins are the best-characterized HSP90-i. Geldanamycin is the prototype molecule, but it has unacceptable toxicities (17). Two semisynthetic analogues known as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-demethylamoethylamino-17-demethoxygeldanamycin (17-DMAG) are less toxic; however, their pharmaceutical properties remain unsatisfactory (18–20). Retaspimycin hydrochloride (IPI-504; Infinity Pharmaceuticals), the hydroquinone hydrochloride salt of 17-AAG, is rapidly converted in vivo to 17-AAG and is 4,000-fold more soluble. It can be given intravenously and has a favorable pharmacologic profile, as shown in a number of clinical trials (21, 22).

We evaluated the efficacy of IPI-504, as single agent and in combination with either imatinib or sunitinib, in 2 xenograft models of GIST with distinct KIT mutations.

**Materials and Methods**

**Reagents and drugs**

Antibodies were either polyclonal (pc) or monoclonal (mc): p-Y719 KIT (pc), p-S473 AKT (pc), AKT (pc), p-T202/Y204 MAPK (pc), and p42/44 MAPK (pc) obtained from Cell Signaling. Antibodies to KIT (CD117; pc), HRP-polycyclonal rabbit antimouse immunoglobulins (lg), HRP-polycyclonal goat antirabbit lgs, the antirabbit Envision+ System—HRP-labeled polymer and 3-diaminobenzidine-tetrahydrochloride were purchased from DAKO (Glostrup, Denmark). Antibodies to HSP90 (pc), CDK4 (mc), HSP70 (pc), and NQO1 (pc) were from Stressgen (now Enzo Life), Invitrogen, and Santa Cruz, respectively. β-Actin (pc) and tubulin (mc) were purchased from Sigma-Aldrich. Western Lightning chemiluminescence reagent was from PerkinElmer. The Bradford protein assay and the polyvinylidene difluoride membranes were from BioRad. Electrophoresis was carried out on NuPAGE gels from Invitrogen. Imatinib was purchased from Sequoia Research Products and dissolved in water. Sunitinib was purchased from Selleck Chemicals and was dissolved in citrate buffer at pH 3.5. Retaspimycin (IPI-504) was provided by Infinity Pharmaceuticals.

**Mouse GIST xenograft models**

The GIST-882 (carrying KIT exon 13 mutation) and the GIST-PSW (carrying KIT exon 11 mutation) models were used to generate human GIST xenografts, as previously described (23). The GIST-882 cell line was a kind gift from Dr. Jonathan Fletcher (Department of Pathology, Brigham and Women’s Hospital, Boston, MA) in 2003. GIST-882 cells were tested and authenticated every 6 months in our laboratory by using standard karyotyping and mutational analysis. The original KIT primary mutation (KIT exon 13 K642E) of GIST-882 xenografts was confirmed by mutational analysis before each passage in the animals (23). Female, adult, athymic NMRI nude mice (36–42 g) were obtained from Janvier Laboratories. Human tumor xenografts were sequentially passed from mouse to mouse. For this study, we used 59 GIST-882 mice (fourth passage) and 44 GIST-PSW mice (seventh passage).

**Experimental design**

All animal experiments were approved by the ethical committee for laboratory animals of the Catholic University Leuven. A total of 103 mice bearing bilateral tumors of size approximately 600 mm³, on average, were assigned to 6 experimental groups as follows: (i) control mice receiving only sterile water (GIST-882, n = 14; GIST-PSW, n = 9); (ii) imatinib treatment (GIST-882, n = 12; GIST-PSW, n = 6); (iii) sunitinib treatment (GIST-PSW, n = 6); (iv) IPI-504 treatment (GIST-882, n = 18; GIST-PSW, n = 12); (v) IPI-504 + imatinib combination (GIST-882, n = 14; GIST-PSW, n = 6); and (vi) IPI-504 + sunitinib combination (GIST-PSW, n = 6). The group assignment of the animals was partly dependent on the graft’s growth potential and associated regression rate of the xenograft models, which is higher for GIST-PSW and lower for GIST-882. These resulted in different number of animals in specific sub-groups and nonsynchronous timing for experiments in both models. As an exploratory study, sunitinib was arbitrarily administered to animals carrying GIST-PSW, because this xenograft originated from...
Measurement (tumor volume) alternate days. The ellipsoid formula was used for tumor volume and body weight were assessed on all treatments; in GIST-882 the 2 drugs were given daily, and 100 mg/kg 3 times a week, respectively. The imatinib, sunitinib, and IPI-504 were administered by oral gavage at 50 mg/kg twice a day, 40 mg/kg once daily, and 100 mg/kg 3 times a week, respectively. The same doses of drugs were used for the combination-treatment groups; in GIST-882 the 2 drugs were given simultaneously, whereas in GIST-PSW there was a 2- to 4-hour interval between administrations of each drug. Notably, for both TKIs, dosing was used below the maximal tolerated dose to reduce the occurrence of side effects in animals. The treatment lasted 15 days, and tumor volume and body weight were assessed on alternate days. The ellipsoid formula was used for tumor measurement (tumor volume \( \times \pi/6 \)), and relative values to baseline expressed as percentage were used for each time point. Mice were humanely euthanized 2 hours after the last administration of compounds. Tumor pieces were fixed in 10% buffered formalin and, in parallel, snap frozen in liquid nitrogen.

**Evaluation of liver histology**

The 103 mice used in the efficacy studies were all necropsied after euthanization. Organs were collected from 43 randomly chosen animals to evaluate any effects associated with the different treatments. In addition, pilot studies to examine changes in liver histology associated with IPI-504 treatment were conducted with IPI-504 as a single agent or in combination with imatinib in 17 GIST-PSW mice. In these studies, IPI-504 was administered intraperitoneally (i.p.) at 150 mg/kg, 3 times per week \((n = 8)\); in the combination arm, the drugs were administered simultaneously \((n = 9)\). The imatinib dose was 50 mg/kg administered orally twice daily.

The histology of the livers collected was first screened on hematoxylin and eosin (H&E)-stained specimens. Subsequently, a self-made scoring system based on the functional anatomy of the liver acinus was created to score and grade the histologic changes observed (24). Histologic changes were interpreted as minimal (grade 1), mild (grade 2), moderate (grade 3), and severe (grade 4; Table 1).

**Histology**

Paraffin sections (5-um thick) were used for H&E staining and immunohistochemistry. Mitoses, apoptosis, and histologic response (HR) were assessed as previously described (23, 25). Microscopic examination was carried out with an Olympus LH-30M microscope; pictures were taken with an Olympus digital camera Color View, and analyses were done with the Olympus Cell D imaging software.

**Immunoblotting**

Tumor lysates were obtained from frozen tissues as previously described, and used for immunoblot analysis (23). Chemiluminescence was captured with the FUJI Mini-LAS3000-Plus imaging system. Subsequently, signals detected by chemiluminescence were semiquantitatively measured by densitometry using AIDA software (Raytest). The optical density of each band was assessed on digital pictures, and values were normalized against \(\beta\)-actin or tubulin. Finally, the relative values compared with nontreated tumors were calculated.

### Table 1. Semiquantitative scoring system for liver damage

<table>
<thead>
<tr>
<th>Lobular changes</th>
<th>Hepatocellular changes</th>
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<tbody>
<tr>
<td>Score for zone 1 (changes present in &gt;3 portal tracts)</td>
<td>Score for apoptotic bodies</td>
</tr>
<tr>
<td>None = 0</td>
<td>None = 0</td>
</tr>
<tr>
<td>Increased cellularity = 1</td>
<td>Scattered foci of spotty necrosis = 1</td>
</tr>
<tr>
<td>Increased cellularity with invasion of the limiting plate = 2</td>
<td>Prominent foci of spotty necrosis = 2</td>
</tr>
<tr>
<td>Bridging necrosis = 3</td>
<td>Bridging necrosis = 3</td>
</tr>
</tbody>
</table>

Note: Histologic changes were first evaluated in the zone 1 (periportal) and zone 3 (periterminal) of the Rappaport acinus (24); next, hepatic abnormalities were recorded. Each feature is scored according to the severity. The grade of liver damage was defined by the sum of each histologic change.

Each of the above features is summed to obtain the grade; normal liver = score 0.
Statistics

Comparisons between tumor volumes on day 0 versus day 14 were done with the Wilcoxon matched-pair test. Comparisons between groups were done using the Mann–Whitney U test, which was also used for assessing mitotic and apoptotic activity. Statistically significant differences were defined as values of $P < 0.05$, and the Bonferroni correction was used for multiple testing. The STATISTICA software (StatSoft) was used for calculations.

Results

Tumor volume assessment

The efficacy of IPI-504, as single agent and in combination with either imatinib or sunitinib, in GIST was evaluated using 2 mouse xenograft models with distinct KIT mutations (GIST-882 and GIST-PSW). The chemical structures of imatinib, sunitinib, and IPI-504 are shown in Figure 1A. Animals were treated for 15 days with control,
imatinib (50 mg/kg twice daily), sunitinib (40 mg/kg once daily; GIST-PSW only), IPI-504 (100 mg/kg 3 times per week; GIST-882 and GIST-PSW only), IPI-504 + imatinib, or IPI-504 + sunitinib (GIST-PSW only) as described in Materials and Methods. Of the 103 mice treated, 7 (6 GIST-882 and 1 GIST-PSW) were excluded from the evaluation of treatment response: 2 were euthanized before the end of the study for ethical reasons, 4 died due to mishandling, and 1 died unexpectedly before the end of the experiment.

By the end of the experiment, control tumors had doubled (GIST-882; Fig. 1B) or almost quadrupled their starting volume (GIST-PSW; Fig. 1C). Both GIST-882 and GIST-PSW xenografts were imatinib-sensitive, as previously observed (23). After 15 days of imatinib treatment, we observed a statistically significant reduction in GIST-882 tumor volume of about 15% from baseline (95% confidence interval (CI): 2–29; P < 0.05; Fig. 1B). In GIST-PSW tumors, imatinib reduced the tumor volume by 83% (95% CI: 75–98; P < 0.01), whereas sunitinib treatment resulted in a 66% reduction from baseline values (95% CI: 49–84; P < 0.01; Fig. 1C).

Administration of IPI-504 as a single agent resulted in reduced tumor volume by 69% (95% CI: 48–90; P < 0.001) and 84% (95% CI: 66–102; P < 0.01) of baseline values in GIST-882 and GIST-PSW, respectively (Fig. 1B and C). Interestingly, no statistically significant difference was observed between imatinib and IPI-504 treatments in the GIST-882 model, whereas in the GIST-PSW model, imatinib and sunitinib treatment resulted in significantly greater tumor regression than IPI-504 (P < 0.001 in both pair-wise comparisons).

The combination of IPI-504 + imatinib in GIST-882 had a greater effect than either treatment alone, yielding a 66% tumor regression (95% CI: 54–78; P < 0.001; Fig. 1B), and it showed additive antitumor activity in comparison with imatinib or IPI-504 (P < 0.001 in all pair-wise comparisons). Both combination treatments (IPI-504 + imatinib and IPI-504 + sunitinib) in GIST-PSW efficiently reduced the tumor burden (P < 0.01 in both groups), but the antitumor activity did not differ significantly from imatinib or sunitinib alone (at least P = 0.14; Fig. 1C).

Histopathology

Macroscopic and microscopic features of untreated tumors from control mice were identical to those described previously (23). KIT immunostaining showed the same pattern of expression in control tumors from both models: diffuse and cytoplasmic staining with scattered tumor cells displaying a Golgi-like pattern. The intensity of the staining was greater in the GIST-PSW model (Supplementary Fig. S1).

We assessed the mitotic and apoptotic activity of the tumor cells. As indicated by mitotic count, both GIST-882 (on average 41.5 mitoses/10 hpf at 400× magnification; 95% CI: 33.4–49.7) and GIST-PSW (on average 41.2 mitoses/10 hpf; 95% CI: 33.6–48.8) were highly aggressive tumors (Fig. 2A and B). Regardless of the type of treatment, the mitotic activity was significantly reduced in both models (P < 0.0001 in all pair-wise comparisons with control; Mann–Whitney U test). On average, imatinib treatment reduced the mitotic index in GIST-882 by 4.8-fold. In GIST-PSW, imatinib and sunitinib treatment were equally efficient in reducing the mitotic index (P = 0.77), yielding a 17.9- and 24.2-fold reduction, respectively (Fig. 2A and B).

Administration of IPI-504 as a single agent reduced the mitotic activity of GIST-882 tumors by 3.3-fold, which was not significantly different from that in imatinib treatment (P = 0.43; Fig. 2A). IPI-504 treatment of GIST-PSW tumors significantly reduced the mitotic count in comparison to control (reduction by 5.1-fold; P < 0.0001; Fig. 2B), but its activity was less remarkable than that of imatinib or sunitinib alone.

In GIST-882 tumors, the IPI-504 + imatinib combination had better antimitotic effects than either single treatment (reduction by 23.7-fold; P < 0.0001). In GIST-PSW tumors, almost no mitoses were counted with IPI-504 + imatinib treatment; however, the antiproliferative activity did not differ significantly from that of imatinib or sunitinib alone (P = 0.08). Interestingly, the IPI-504 + sunitinib combination was better than any other GIST-PSW treatment (reduction by >300-fold; at least P < 0.001 in the comparisons; Fig. 2A and B).

None of the treatments induced significant apoptosis in the GIST-882 model (Fig. 2C). In the GIST-PSW model, a significant increase in apoptosis was observed only in the imatinib and sunitinib treatment groups yielding a 2.5- and 3-fold increase (P < 0.001), respectively. IPI-504 treatment of GIST-PSW tumors caused apoptotic activity to minimally increase, but it was not significantly different from the control (Fig. 2D). Both combination treatments further increased the apoptotic activity in GIST-PSW (up to 5.35-fold); however, only the combination of IPI-504 + imatinib was better than IPI-504 or imatinib single agents alone (P < 0.001; Fig. 2D).

Next, we assessed on H&E staining the grade of HR by using a previously published exploratory grading system (25). The HR was heterogeneous in both models and did not correlate with tumor shrinkage. In both models, imatinib treatment induced minimal HR in most tumors. Although imatinib was more efficient at decreasing the average tumor volume in GIST-PSW, higher degrees of HR were observed in GIST-882 xenografts (~10% of tumors with grade 3 HR; Fig. 2E). Sunitinib treatment led to grade 4 HR in one GIST-PSW tumor, histologically showing more evident effects than imatinib. Treatment with IPI-504 resulted mainly in tumor necrosis yielding grade 3 HR in 1 GIST-PSW tumor, and grade 2 HR in about half of the GIST-882 tumors, suggesting a prevalent cytotoxic effect (Fig. 2E). In tumors treated with the combination IPI-504 + imatinib and IPI-504 + sunitinib regimens, we observed higher degrees of HR than in the single treatment arms, suggesting a more efficient tumor cell death in the combination groups (Fig. 2E).
Evaluation of HSP90 inhibition in GIST

Following HSP90 inhibition, its client proteins are degraded (11, 12). The assessment of the level of such client proteins can provide indirect information about their degradation (11, 12). KIT, AKT, and CDK4 are HSP90 client proteins. Furthermore, the expression level of HSP70 is expected to increase following HSP90 inhibition (11, 12).

As shown by immunohistochemistry, KIT was still expressed regardless of the type of treatment in both models. When compared to control, the TKI did not affect the pattern and the intensity of KIT immunostaining in GIST-882 (only imatinib) and in GIST-PSW (imatinib and sunitinib). Interestingly, treatment with IPI-504 led to a different pattern of KIT immunostaining; the intensity became weaker and unevenly distributed, suggesting loss of KIT expression. However, the changes in KIT expression were more pronounced in GIST-882 than in GIST-PSW, most likely because of the lower basal level of KIT expression observed in GIST-882. With IPI-504 + imatinib treatment, the loss of KIT was even more remarkable in GIST-882 (Supplementary Fig. S2), whereas the differences in GIST-PSW were less pronounced (data not shown).

Next, we assessed the expression level of total KIT, AKT, and CDK4 by Western blot analysis and, semiquantitatively, by densitometry as described in Material and Methods (Fig. 3A–D). With imatinib treatment, KIT was partially downregulated in GIST-PSW, and remained almost unchanged in GIST-882 (Fig. 3A and C). Sunitinib
treatment reduced KIT expression more consistently than imatinib in GIST-PSW tumors (reduced by 75% in comparison with control; Fig. 3C and D).

In the IPI-504 group, KIT expression was reduced by 39% in GIST-882 and 31% in GIST-PSW when compared with control (Fig. 3). Notably, in GIST-882 the decrease in KIT expression was more evident because 2 of 7 cases analyzed showed almost complete loss of KIT expression (Fig. 3A).

With IPI-504 + imatinib treatment, KIT expression was nearly absent in GIST-882 tumors (90% reduction vs. control; Fig. 3A and B); however, the combination yielded only partial downregulation of KIT in GIST-PSW tumors (Fig. 3C and D). A more pronounced loss of KIT expression was observed following IPI-504 + sunitinib treatment in GIST-PSW tumors (55% reduction vs. control; Fig. 3C and D).

The level of AKT was visibly downregulated only in the GIST-882 tumors (57% decrease; Fig. 3A–D). The expression levels of CDK4 remained unchanged in all experimental groups regardless of the type of xenograft or type of treatment.

As expected, HSP70 was significantly upregulated in both xenografts in all IPI-504–treated tumors, both in the single treatment and in the combination arms (Fig. 3A–D).

HSP90 and the reductase DT-diaphorase/NQO1 were expressed in all tumors analyzed (data not shown). Notably, it has been suggested that the expression of NQO1 is important for determining the sensitivity of tumor cells to HSP90-i benzoquinone ansamycin derivatives (26). Taken together, these data indicate that IPI-504 is biologically active in GIST xenografts, and induces KIT and AKT degradation at variable degrees together with HSP70 upregulation. Interestingly, the GIST-882 model showed higher sensitivity than the GIST-PSW model in terms of KIT/AKT downregulation.

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**Figure 3.** Western blot and semiquantitative densitometry analysis. Whole tumor lysates were used to assess total expression and phosphorylation of KIT protein and its downstream signaling molecules AKT and MAPK. HSP70 was used as a biomarker to check activity and viability of IPI-504 in the tumors. Densitometry was measured as described in Materials and Methods; results are presented according to the formula: [(densitometry of treated tumor/densitometry of control tumor)/C0]. A, in GIST-882, KIT expression and inactivation were affected by IPI-504 exposure, but the combination IPI-504 + imatinib further enhanced these results. B, densitometry evaluation in GIST-882 shows HSP70 upregulation together with downregulation of KIT and AKT in IPI-504–treated tumors. C, IPI-504 and TKI exposure significantly decrease the activity of KIT and downstream intermediates in GIST-PSW. D, despite significant HSP70 upregulation, in GIST-PSW, only KIT showed some degree of downregulation following IPI-504 treatment.
Phosphorylation levels of KIT and its downstream molecules

The activation of KIT and its main downstream molecules by phosphorylation was assessed by Western blot analysis. As expected, both TKIs resulted in consistent inhibition of KIT and its signaling pathway in both models (Fig. 3A–D). In GIST-PSW tumors, although imatinib treatment reduced the phosphorylation level of KIT by 80%, AKT still showed some residual activity (~40% reduction; Fig. 3C and D). With sunitinib treatment, the GIST-PSW tumors showed a more homogeneous and consistent inhibition of KIT and its downstream signaling.

Overall, the activity of IPI-504 was similar to that observed with TKI treatment. Thus, the expression of phospho-KIT was reduced by 40% and 70% in the GIST-882 and the GIST-PSW models, respectively (Fig. 3A–D). Similarly, we observed substantial inhibition of AKT, whereas the inhibition of mitogen-activated protein kinase (MAPK) was more apparent in the GIST-PSW model (Fig. 3A–D).

The combination of IPI-504 + imatinib further enhanced the level of inactivation of KIT in both xenograft models. In GIST-882 tumors the downstream molecules showed higher levels of inhibition with IPI-504 + imatinib treatment than with single-agent imatinib or IPI-504 treatment. In GIST-PSW tumors, only AKT was efficiently inhibited by the combination of the 2 drugs. The combination IPI-504 + sunitinib led to a significant inhibition of both KIT and downstream molecules in GIST-PSW (Fig. 3A–D).

Toxicology and adverse events

The drugs were generally well tolerated; no significant body weight loss was recorded. At necropsy, we did not observe gross changes in the majority of the mice used for the efficacy study. Regardless of the type of treatment, the heart, lungs, kidneys, spleen, and bowel showed no specific alterations. Livers collected from imatinib-treated mice were normal.

Of the 60 livers examined from the current and pilot studies (43 + 17), the histology in the majority of livers was in the none to minimal severity range; an occasional animal had a mild change (see Table 1 for scoring details and Table 2 for results). Treatment with orally administered IPI-504 3 times per week at 100 mg/kg resulted in liver histopathology that was nearly identical to the control group. Changing the dose and route of administration to 150 mg/kg IPI-504 administered 3 times weekly via i.p. injection resulted in an increase in the number of animals with moderate liver changes (n = 2).

When IPI-504 (at 150 mg/kg i.p. in the pilot studies or 100 mg/kg orally in the efficacy studies) was administered simultaneously with imatinib, there was an increase in the incidence and severity of histopathologic changes in the liver. When the administration was staggered by 2 to 4 hours (IPI-504 at 100 mg/kg orally), the liver histology resembled that in the vehicle control group. In addition, the histology of the liver following treatment with IPI-504 + sunitinib did not appear to be any different from that in vehicle control animals.

Examination of the livers collected from animals exposed to IPI-504 at 150 mg/kg i.p. or as part of the IPI-504 + imatinib simultaneous combination showed varying degrees of tissue damage. In particular, the histologic changes were localized around the terminal veins (centrilobular portion of the liver), whereas the portal tracts were unaffected. Frequent foci of spotty necrosis and apoptotic hepatocytes were observed (Fig. 4A–C). Changes around terminal veins of the subcapsular region of the liver were frequently more prominent (Fig. 4D). On histologic examination, the livers of these mice showed moderate to severe necrosis of the parenchyma between adjacent terminal veins (also known as bridging necrosis with terminal/terminal pattern), which left few unaffected hepatocytes around the portal tracts (Fig. 4E). In addition, wide areas of subcapsular necrosis of the left liver lobe were observed (Fig. 4F). Interestingly, these morphologic changes resemble those observed in hepatic ischemia–reperfusion injury, both in humans and in mice (27).

Discussion

The present study provides in vivo evidence that the HSP90-i IPI-504 is active against GIST tumors and induces KIT protein degradation. We used 2 GIST xenograft models with distinctive KIT mutations, originating either from the imatinib-sensitive GIST-882 cell line or from a biopsy collected from a patient with advanced disease (GIST-PSW). The animals were treated for 15 days with IPI-504, imatinib, or a combination of the 2 drugs. Animals with the GIST-PSW tumors were also treated with sunitinib, alone or in combination with IPI-504. In addition, we illustrated a compendium of histlogic changes observed in the liver of animals treated with IPI-504.

To date only a few laboratories have succeeded in the establishment of GIST xenografts, the majority of which are imatinib-sensitive models. In our study, GIST tumors carrying KIT single mutation, when grafted in mice, grew 2 or 3 times faster than those with KIT double mutations. Unfortunately, at the time of our experiments we had not yet succeed in the establishment of xenografts with imatinib-resistant mutation. Nonetheless, testing novel therapeutic strategies in imatinib-sensitive models may have 2 significant clinical implications. Firstly, it proves the efficacy of compounds that, as single agents, could potentially substitute for TKI in GIST patients who become TKI-intolerant. Secondly, combination treatments may increase the curative potential ensuring prolonged responses and eventually overcoming or delaying the resistance.

IPI-504 led to a substantial reduction in tumor burden in all xenografts. On histologic examination, additional signs of the efficacy of IPI-504 were illustrated by the
Table 2. Hepatic histologic changes observed in the experimental groups

<table>
<thead>
<tr>
<th>Histologic changes</th>
<th>Treatment</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td>Grade 1 (minimal)</td>
<td>3</td>
</tr>
<tr>
<td>Grade 2 (mild)</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3 (moderate)</td>
<td>0</td>
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<td>Grade 4 (severe)</td>
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<td>Treatment regimen</td>
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</tr>
<tr>
<td></td>
<td>b.i.d oral</td>
</tr>
<tr>
<td>GIST-PSW or -882</td>
<td>Both</td>
</tr>
<tr>
<td>Pilot or current</td>
<td>Current</td>
</tr>
<tr>
<td>Total number of animals</td>
<td>10</td>
</tr>
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</table>

NOTE: For each group, the number of animals with none, minimal, mild, moderate, or severe histologic changes in the liver are presented. In the IPI-504 group, 2 cohorts are compared: the column on the left represents animals that received doses of 150 mg/kg i.p. during pilot studies; the column on the right represents animals that were included in the current study (100 mg/kg orally). In the IPI-504 + imatinib group, the cohort of mice that received the 2 drugs simultaneously is listed on the left, whereas on the right are listed the animals that received the 2 drugs with a 2- to 4-hour delay (delay). Information about treatment regimen, xenograft model (GIST-PSW or GIST-882), type of study, and total number of animals is also provided in the last 3 rows of the table.

Abbreviations: b.i.d., twice a day dosing; qd, once a day dosing.
significant reduction in mitotic activity and induction of wide areas of necrosis in the tumor sections, suggesting a more cytotoxic effect of IPI-504. Surprisingly, in both models the apoptotic activity was not significantly influenced by IPI-504. We speculate that the HSP70 upregulation may be responsible for the lack of significant apoptotic activity in our study. The upregulation of the co-chaperone HSP70 is a standard biomarker for HSP90-i activity and confirms the availability and activity of HSP90-i in tumor samples (11, 12). However, it has been suggested that HSP70 upregulation might decrease the efficacy of HSP90-i by preventing cancer cells from entering apoptosis, thereby lowering antitumor effects (12).

By assessing the KIT expression levels, we show that treatment with IPI-504 results in partial downregulation and inhibition of the KIT protein. This finding is in line with previous results obtained in vitro in mastocytosis and GIST cell lines, and is consistent with the hypothesis that KIT is a HSP90 client protein (14, 15). As observed in vitro, the interaction between HSP90 and KIT oncogenes may vary from tumor to tumor (15). Similarly in this study, we show that, in GIST-882, the level of KIT degradation was more visible than in the GIST-PSW tumors. This diversity might be related to 3 different scenarios. First, intrinsic molecular differences (e.g., type of KIT mutation or other distinctive molecular features) of the 2 GIST models may determine the sensitivity to HSP90-i. Second, higher levels of KIT expression in certain tumors could mask the actual degradation of the protein. Third, the ratio between wild-type KIT/oncogenic KIT could influence sensitivity to HSP90-i because the KIT mutation is homozygous in GIST-882, and heterozygous in GIST-PSW (unpublished results).

As expected, imatinib treatment resulted in a significant reduction of tumor burden in both xenograft models. However, the efficacy of the standard treatment was clearly better in the GIST-PSW tumors than in the GIST-882 tumors. The KIT exons 13 mutation present in GIST-882 tumors is a rare type of mutation identified in ~2% of KIT-mutant GIST patients. It is not clear yet whether higher doses of imatinib may provide better clinical outcomes (6, 28). However, a comparison between the results reported from the present study and those from our previous study may suggest that patients with this type of mutation could benefit from higher doses of imatinib (23). Thus, in the study where we tested the efficacy of the histone deacetylase
inhibitor panobinostat, the dose of imatinib was 3 times higher than now and yielded, in GIST-882, a 3-times higher reduction of tumor burden along with a more significant induction of apoptosis and proliferation arrest (23). In contrast, in GIST-PSW tumors, imatinib efficacy was not influenced by the different dose (23). As observed in the clinic, GIST patients with KIT exon 11 mutations do not require higher doses of imatinib to achieve clinical benefit (6).

Sunitinib is established as a second-line therapy for GIST patients who are intolerant or have tumors that progressed under imatinib. However, little is known about sunitinib activity as a first-line agent in imatinib-sensitive GIST. In this study, we showed that sunitinib treatment reduces the KIT oncogenic signaling more efficiently than imatinib, and produces higher degrees of HR in a highly aggressive patient-derived GIST xenograft. These results may suggest that sunitinib is more potent than imatinib and warrant further evaluation as a first-line treatment in patients with advanced GISTs.

We tested the hypothesis that IPI-504 in combination with TKIs could induce enhanced antitumor effects. In GIST-882 we clearly showed that IPI-504 and imatinib may provide additive effects by inducing higher levels of KIT degradation, tumor regression, and proliferation arrest. This was less obvious in GIST-PSW; however, the morphologic parameters evaluated in this study (e.g., higher degrees of HR and higher apoptotic activity) support the rationale for combining TKI with HSP90-i in GISTs.

The activity of intravenous IPI-504 has recently been studied in a phase III clinical trial in patients with GIST resistant to at least imatinib or sunitinib (RING trial; ref. 29). The trial was prematurely halted due to a higher than anticipated mortality in the treatment arm (29). Liver failure with severe elevation of the liver enzymes was the most common abnormality observed in these patients (29). The intriguing RING trial outcome prompted us to review the histologic changes observed in livers collected from IPI-504–treated mice. Our examination of livers from selected IPI-504- and TKI-treated mice found that changes in histology consisting of foci of spotty necrosis and apoptotic hepatocytes were observed at a greater incidence and severity compared with control animals when IPI-504 was administered i.p. at 150 mg/kg, 3 times per week (range: none to moderate). In addition, the liver histology was more severe in mice that received simultaneous treatment with IPI-504 + imatinib (range: none to severe). The histologic changes that occurred in these animals were not observed when IPI-504 was administered orally at 100 mg/kg, when TKIs were administered alone, or when combination treatments with IPI-504 were staggered over 2 to 4 hours. The presence of foci of spotty necrosis and apoptotic hepatocytes may account for the episodes of transient transaminitis described by other authors (17–20). These observations suggest a careful evaluation of doses and sequence of administration of these drugs whenever combined treatment is considered.

The exact mechanism of IPI-504–related liver toxicity is elusive as yet, but it might in part depend on the presence of a quinone moiety in its molecular scaffold. The metabolism of such molecules through P450 reductases may generate a number of unstable molecules capable of redox cycles that eventually result in hepatocellular toxicity (11, 12). For this reason, the recent efforts of the pharmaceutical industry were concentrated on the modeling of synthetic HSP90-i, which retains similar or increased potency to that observed with natural compounds although lacking the quinone moiety (11, 12). Similar drugs, such as AT-13387 or STA-9090, are expected to have a safer pharmacologic profile and are currently being tested in phase I/II trials in GIST patients (30). However, given the wide array of HSP90 clients’ protein, it is still possible that some of the side effects observed in the RING trial are inevitable intrinsic consequences of HSP90-i, especially in heavily pretreated patients with a high burden of liver metastases. Future studies with HSP90-i will likely help us to understand whether dose-limiting toxicities are class-specific (generalized to all HSP90-i) or drug-specific (synthetic HSP90-i vs. natural compound) in GIST, and to select the safest, clinically more efficient dosing schedule and combination treatment.

In conclusion, we show that IPI-504 has remarkable antitumor activity in GIST xenografts, by inducing consistent KIT degradation, inhibition of KIT signaling, tumor necrosis, and arrest of tumor cell proliferation. In combination with imatinib or sunitinib, most of the described effects were variably enhanced. Intrinsically molecular features of GISTs may influence response to IPI-504, warranting further studies to evaluate interactions between HSP90 and diverse KIT oncoproteins. In addition, we showed that there was a potential for liver injury when mice were treated with high doses of IPI-504 (150 mg/kg i.p.) or when IPI-504 (150 mg/kg i.p. or 100 mg/kg orally) was administered simultaneously with imatinib. Current and future clinical trials with IPI-504 administered intravenously will explore different doses at a less intense dose schedule (i.e., weekly) with particular attention paid to liver enzyme monitoring. Careful evaluation of the dose and schedule of IPI-504 in combination with a TKI may require further studies.

Disclosure of Potential Conflicts of Interest

P. Schöffski received research grants from Infinity Pharmaceuticals. E. Normant is employed by Infinity Pharmaceuticals whose drug was herein tested.

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The Heat Shock Protein 90 Inhibitor IPI-504 Induces KIT Degradation, Tumor Shrinkage, and Cell Proliferation Arrest in Xenograft Models of Gastrointestinal Stromal Tumors

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