The heat shock protein 90 inhibitor IPI-504 induces KIT degradation, tumor shrinkage and cell proliferation arrest in xenografts models of gastrointestinal stromal tumors

Giuseppe Floris1*, Maria Debiec-Rychter2, Agnieszka Wozniak1, Cristiana Stefan1, Emmanuel Normant4, Gavino Faa5, Kathleen Machiels2, Ulla Vanleeuw1, Raf Sciot3, Patrick Schöffski1

1Laboratory of Experimental Oncology and Department of General Medical Oncology, 2Department of Human Genetics, and 3Department of Pathology, University Hospital Gasthuisberg, Catholic University of Leuven, Leuven, Belgium; 4Infinity Pharmaceuticals, Inc., Cambridge, MA, USA; 5Department of Pathology, University of Cagliari, Cagliari, Italy.

*Corresponding author: Giuseppe Floris, Laboratory of Experimental Oncology and Department of General Medical Oncology, University Hospital Gasthuisberg, Catholic University of Leuven, Herestraat 49, bus 815; 3000 Leuven; Belgium. Phone: +32(0)16346900; Fax: +32(0)16346901; e-mail: giuseppe.floris@uzleuven.be

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Abstract

The activity of the receptor tyrosine kinase KIT is crucial for gastrointestinal stromal tumor (GIST) growth and survival. Imatinib (IMA) and sunitinib (SUN) 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 IMA or SUN 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 IMA (50 mg/kg twice daily), SUN (40 mg/kg once daily), IPI-504 (100 mg/kg 3 times per week), IPI-504+IMA or IPI-504+SUN. We evaluated tumor volume, proliferation and apoptosis, KIT expression and activation, as well as treatment adverse events. 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 IMA or SUN. On histology, liver damages were frequently observed in animals exposed to combination treatments. In conclusion, IPI-504 demonstrates consistent anti-tumor activity and induces KIT downregulation in GIST, as a single agent, and is more potent in combination with IMA or SUN. The sequence of drug administration in the combination arms warrants further studies.
Introduction

Gastrointestinal stromal tumors (GIST) are the most common sarcomas of the digestive system (1). About 95% of GISTs express the receptor tyrosine kinase KIT (2). About 80-85% of GISTs carry somatic activating mutations in the KIT gene or in the platelet derived growth factor receptor alpha (PDGFRA) gene, which are causative events in GIST development (1-2). Selective tyrosine kinase inhibitors (TKIs), such as imatinib mesylate (IMA), have become standard palliative care for patients with advanced, inoperable disease (3-4). However, IMA shows only limited curative potential and is maintained indefinitely until emergence of intolerance or resistance (1; 5). Interestingly, response to IMA is significantly dependent on the tumor genotype and not all patients respond equally to the standard IMA dose (i.e. 400 mg/daily) (6). Thus, while the 400 mg schedule is sufficient to achieve a significant clinical benefit in GISTs harbouring KIT exon 11 mutations, the tumors with mutation in KIT exon 9 might require higher IMA dose. Patients with GIST carrying mutations causing primary resistance to IMA (such as PDGFRAp.D842V) do not benefit from IMA therapy. Accordingly, IMA treatment should be tailored to the type of KIT/PDGFRA mutation (6). GIST patients refractory or intolerant to IMA are treated with another TKI, sunitinib malate (SUN) (4). Eventually, SUN also ceases to be effective, leaving GISTs patients without approved alternative treatment options. Resistance to TKIs is either pre-existing 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 co-factors (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 of 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 proved to efficiently kill tumor cells (15). Additionally, 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-i are natural or semi-synthetic compounds. The natural antibiotics benzoquinone ansamycins (BA) are the best characterized HSP90-i. Geldanamycin is the prototype molecule, but has unacceptable toxicities (17). Two semisynthetic analogs known as 17-AAG (17-allylamino-17-demethoxygeldanamycin) and 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin) 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 4000-fold more soluble. It can be given intravenously and has a favorable pharmacological 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 IMA or SUN, in two 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), p42/44 MAPK (pc) were from Cell
Signaling (Leiden, The Netherlands). Antibodies to KIT (CD117) (pc), HRP-polyclonal rabbit anti-mouse immunoglobulins, HRP-polyclonal goat anti-rabbit immunoglobulins, the anti-rabbit Envision+ System-HRP labeled polymer and 3’-diaminobenzidine-tetrahydrochloride (DAB) were purchased from DAKO (Glostrup, Denmark). Antibodies to HSP90 (pc), CDK4 (mc), HSP70 (pc) and NQO1 (pc) were from Stressgen (now Enzo Life, Farmingdale, NY), Invitrogen (Carlsbad, CA), and Santa Cruz (Santa Cruz, CA), respectively. b-actin (pc) and tubulin (mc) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Western Lightning™ chemiluminescence reagent came from PerkinElmer (Boston, MA, USA). The Bradford protein assay and the PVDF membranes were from Biorad (Hemel Hempstead, UK). Electrophoresis was carried out on NuPAGE gels from Invitrogen (Carlsbad, CA, USA). IMA was purchased from Sequoia Research Products (Pangbourne, UK), and dissolved in water. SUN was purchased from Selleck Chemicals (Houston, TX, USA), and was dissolved in citrate buffer at pH 3.5. Retaspimycin (IPI-504) was provided by Infinity Pharmaceuticals (Cambridge, MA, USA).

**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 (Boston, MA, USA) in 2003. GIST-882 cells were tested and authenticated every 6 months in our laboratory by standard karyotyping and by 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, France. Human tumor xenografts were sequentially passed from mouse to mouse. For this study, we used 59 GIST-882 mice (4th passage) and 44 GIST-PSW mice (7th passage).

**Experimental design**

All animal experiments were approved by the ethical committee for laboratory animals of the Catholic University Leuven (Belgium). A total of 103 mice bearing bilateral tumors...
of ~600 mm³ on average were assigned to 6 experimental groups as follows: 1) control
mice receiving only sterile water (GIST-882, n=14; GIST-PSW, n=9); 2) IMA treatment
(GIST-882 n= 12; GIST-PSW n=6); 3) SUN treatment (GIST-PSW n= 6); 4) IPI-504
treatment (GIST-882, n=18; GIST-PSW, n=12); 5) IPI-504+IMA combination (GIST-
882, n=14; GIST-PSW, n=6); 6) IPI-504+S UN combination (GIST-PSW, n=6). The
group assignment of the animals was partly dependent on the grafts growth potential and
associated re-graftment 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 not synchronous timing for experiments in both models. As an exploratory study,
SUN was arbitrarily given to animals carrying GIST-PSW , because this xenograft
originated from a clinically progressive on imatinib tumor, while GIST-882 cells derived
from an imatinib-naïve GIST. The IMA, SUN and IPI-504 were administered by oral
gavage at 50 mg/kg twice a day, 40 mg/kg once daily and 100mg/kg three times a week,
respectively. The same doses of drugs were used for the combination treatment groups;
in GIST-882 the two drugs were given simultaneously, whereas in GIST-PSW, there was
a 2-4 hour interval between each drug administration. Notably, for both TKI, 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
every other day. The ellipsoid formula was used for tumor measurement (tumor
volume*π/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 IMA in 17 GIST-PSW mice. In these studies, IPI-504 was administered
intraperitoneally (ip) at 150 mg/kg, 3 times per week (n=8), and in the combination arm
the drugs were administered simultaneously (n=9). The IMA dose was 50 mg/kg administered orally twice daily.

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

**Histology**

Paraffin sections (5 μm) were used for H&E staining and immunohistochemistry. Mitoses, apoptosis and histologic response were assessed as previously described (23, 25). Microscopy was performed with an Olympus LH-30M microscope; pictures were taken with an Olympus digital camera Color View, and analyses were performed with the Olympus Cell D imaging software (Münster, Germany).

**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 semi-quantitatively measured by densitometry using AIDA software (Raytest; Straubenhardt, Germany). The optical density of each band was assessed on digital pictures, and values were normalized against β-actin or tubulin. Finally, the relative values compared to non-treated tumors were calculated.

**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 p values less than 0.05, and Bonferroni correction was used for multiple testing. The STATISTICA software (StatSoft, Hamburg, Germany) was used for calculations.
Results

Tumor volume assessment

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

By the end of the experiment, control tumors had doubled (GIST-882; Figure 1b) or almost quadrupled their starting volume (GIST-PSW; Figure 1c). Both GIST-882 and GIST-PSW xenografts were IMA-sensitive, as previously observed (23). After 15 days of IMA treatment, we observed a statistically significant reduction in GIST-882 tumor volume of about 15% from baseline (95% confidence interval [95% CI]: 2-29%; p<0.05) (Figure 1b). In GIST-PSW tumors, IMA reduced the tumor volume by 83% (95% CI: 75-98%; p<0.01), whereas SUN treatment resulted in a 66% reduction from baseline values (95% CI: 49-84%; p<0.01) (Figure 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 (Figure 1b,c). Interestingly, no statistically significant difference was observed between IMA and IPI-504 treatment in
the GIST-882 model, while in the GIST-PSW model, IMA and SUN treatment resulted in significantly greater tumor regression than IPI-504 (p<0.001 in both pair wise comparisons).

The combination of IPI-504+IMA in GIST-882 had a greater effect than either treatment alone, yielding a 66% tumor regression (95% CI=54-78%; p<0.001; Figure 1b), and it showed additive antitumor activity in comparison with IMA or IPI-504 (p<0.001 in all pair-wise comparisons). Both combination treatments (IPI-504+IMA and IPI-504+SUN) in GIST-PSW efficiently reduced the tumor burden (p<0.01 in both groups), but the antitumor activity did not differ significantly from IMA or SUN alone (at least p=0.14) (Figure 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 cytoplasmatic staining with scattered tumor cells displaying a “Golgi-like” pattern. The intensity of the staining was higher in the GIST-PSW model (Supplementary Figure 1).

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 high power fields at 400x magnification [HPF]; 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 (Figure 2a,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; U-test). On average, IMA treatment reduced the mitotic index in GIST-882 by 4.8 fold. In GIST-PSW, IMA and SUN treatment were equally efficient in reducing the mitotic index (p=0.77), yielding a 17.9- and 24.2-fold reduction, respectively (Figure 2a-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 IMA treatment (p=0.43; Figure 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; Figure 2b), but its activity was less remarkable than IMA or SUN alone.
In GIST-882 tumors, the IPI-504+IMA combination had better anti-mitotic 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+IMA treatment; however, the anti-proliferative activity did not differ significantly from IMA or SUN alone (p=0.08). Interestingly, the IPI-504+SUN combination was better than any other GIST-PSW treatment (reduction by over 300-fold; at least p<0.001 in the comparisons; Figure 2a,b).

None of the treatments induced significant apoptosis in the GIST-882 model (Figure 2c). In the GIST-PSW model, a significant increase in apoptosis was observed only in the IMA and SUN 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 control (Figure 2d). Both combination treatments further increased the apoptotic activity in GIST-PSW (up to 5.35-fold), however, only the combination of IPI-504+IMA was better than IPI-504 or IMA single agents alone (p<0.001; Figure 2d).

Next, we assessed on H&E the grade of histological response (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, IMA treatment induced minimal HR in most tumors. Although IMA was more efficient at decreasing the average tumor volume in GIST-PSW, higher degrees of HR were observed in GIST-882 xenografts (about 10% of tumors with grade 3HR; Figure 2e). SUN treatment led to grade 4HR in one GIST-PSW tumor, showing on histology more evident effects than IMA. Treatment with IPI-504 resulted mainly in tumor necrosis yielding grade 3HR in one GIST-PSW tumor, and grade 2HR in about half of the GIST-882 tumors, suggesting a prevalent cytotoxic effect (Figure 2e). In tumors treated with the combination IPI-504+IMA and IPI-504+SUN regimens, we observed higher degrees of HR than in the single treatment arms, suggesting a more efficient tumor cell death in the combination groups (Figure 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 IMA) and in GIST-PSW (IMA and SUN). 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+IMA treatment the loss of KIT was even more remarkable in GIST-882 (Supplementary Figure 2), 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 semi-quantitatively by densitometry as described in material and methods (Figure 3a-d). With IMA treatment, KIT was partially down-regulated in GIST-PSW, and remained almost unchanged in GIST-882 (Figure 3a, c). SUN treatment reduced KIT expression more consistently than IMA in GIST-PSW tumors (reduced by 75% in comparison to control; Figure 3c-d).

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

With IPI-504+IMA treatment, KIT expression was nearly absent in GIST-882 tumors (90% reduction versus control; Figure 3a-b); however, the combination yielded only partial down-regulation of KIT in GIST-PSW tumors (Figure 3c-d). A more pronounced loss of KIT expression was observed upon IPI-504+SUN treatment in GIST-PSW tumors (55% reduction versus control; Figure 3c-d).
The level of AKT was visibly down-regulated only in the GIST-882 tumors (57% decrease; Figure 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 up-regulated in both xenografts in all IPI-504 treated tumors, both in the single treatment and in the combination arms (Figure 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-iBA derivatives (26).

All 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 up-regulation. Interestingly, the GIST-882 model showed higher sensitivity than the GIST-PSW model in terms of KIT/AKT down-regulation.

**Phosphorylation levels of KIT and its downstream molecules.**

The activation of KIT and its main downstream molecules by phosphorylation was assessed by Western immunoblotting. As expected, both TKIs led to consistent inhibition of KIT and its signaling pathway in both models (Figure 3a-d). In GIST-PSW tumors, although IMA treatment reduced the phosphorylation level of KIT by 80%, AKT still showed some residual activity (~40% reduction; Figure 3c-d). With SUN 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 (Figure 3a-d). Accordingly, we observed substantial inhibition of AKT, whereas the inhibition of MAPK was more apparent in the GIST-PSW model (Figure 3a-d).

The combination of IPI-504 +IMA 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+IMA treatment than with single agent IMA or IPI-504 treatment. In GIST-PSW tumors, only AKT was efficiently inhibited by the combination of the two drugs. The combination IPI-504+SUN led to a significant inhibition of both KIT and downstream molecules in GIST-PSW (Figure 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 the type of treatment, the heart, lungs, kidneys, spleen and bowel showed no specific alterations. Livers collected from IMA 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 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 three times per week at 100 mg/kg resulted in liver histopathology nearly identical to the control group. Changing the dose and route of administration to 150 mg/kg IPI-504 administered three times weekly via ip injection resulted in an increase in the number of animals with moderate liver changes (n=2).

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

Examination of the livers collected from animals exposed to IPI-504 at 150 mg/kg ip or as part of the IPI-504+IMA simultaneous combination showed varying degrees of tissue damage. In particular, the histological changes were localized around the terminal veins (centrolobular portion of the liver), while the portal tracts were unaffected. Frequent foci of spotty necrosis and apoptotic hepatocytes were observed (Figure 4a-c). Changes around terminal veins of the sub-capsular region of the liver were often more
prominent (Figure 4d). On histology, 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 (Figure 4e). Also wide areas of sub-capsular necrosis of the left liver lobe were observed (Figure 4f). Interestingly, these morphological 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 two GIST xenograft models with distinctive KIT mutations, originating either from the IMA-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, IMA or the combination of the two drugs. Animals with the GIST-PSW tumors were treated also with SUN, alone or in combination with IPI-504. Additionally we illustrate a compendium of histologic changes observed in the liver of animals treated with IPI-504.

Up to date only few laboratories succeeded in the establishment of GIST xenografts, the majority of which are IMA-sensitive models. In our hands GIST tumors carrying KIT single mutation, when grafted in mice, grow two or three times faster than those with KIT double mutations. Unfortunately, at the time of our experiments we did not yet succeed in the establishment of xenografts with imatinib-resistant mutation. Nevertheless, testing novel therapeutic strategies in IMA-sensitive models may have two significant clinical implications. Firstly, it proofs the efficacy of compounds that as single agents could potentially substitute 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 histology, additional signs of the efficacy of IPI-504 were illustrated by the 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 up-
regulation may be responsible for the lack of significant apoptotic activity in our study. The up-regulation 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 up-regulation might decrease the efficacy of HSP90-i by preventing cancer cells from entering apoptosis, thereby lowering anti-tumor 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 oncoproteins 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 three different scenarios. First, intrinsic molecular differences (e.g., type of KIT mutation or other distinctive molecular features) of the two 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, since the KIT mutation is homozygous in GIST-882, and heterozygous in GIST-PSW (unpublished results).

As expected, IMA 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 exon 13 mutation present in GIST-882 tumors is a rare type of mutation present in about 2% of KIT mutant GIST patients. It is not clear yet whether higher doses of IMA may provide better clinical outcomes (6, 28). However, a comparison between the results reported here and those from our previous study may suggest that patients with this type of mutation could benefit from higher doses of IMA (23). Thus in the study where we tested the efficacy of the histone deacetylase inhibitor panobinostat, the dose of IMA was three times higher than now and yielded in GIST-882 a three times higher reduction of tumor burden along with a more significant induction of apoptosis and proliferation arrest (23). In contrast, in GIST-PSW tumors, IMA 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 IMA to achieve clinical benefit (6).

SUN is established as a second line therapy for GIST patients who are intolerant or have progressed under IMA. However, little is known about SUN activity as a first-line agent in IMA-sensitive GIST. Here we show that SUN treatment reduces the KIT oncogenic signaling more efficiently than IMA, and produces higher degrees of HR in a highly aggressive patient-derived GIST xenograft. These results may suggest that SUN is more potent than IMA 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 anti-tumor effects. In GIST-882 we clearly showed that IPI-504 and IMA 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 morphological parameters evaluated here (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 IMA or SUN (RING trial; 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 histological 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 to control animals when IPI-504 was administered ip 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+IMA (range: none-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 2-4 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.

As of yet, the exact mechanism of IPI-504 related liver toxicity is elusive, 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 instable molecules capable of redox cycles that eventually result into 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 while 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 also 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 pre-treated 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 versus 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 anti-tumor activity in GIST xenografts, by inducing consistent KIT degradation, inhibition of KIT signaling, tumor necrosis, and arrest of tumor cell proliferation. In combination with IMA or SUN, most of the described effects were variably enhanced. Intrinsic 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 is a potential for liver injury when mice are treated with high doses of IPI-504 (150 mg/kg ip) or when IPI-504 (150 mg/kg ip or 100 mg/kg orally) was administered simultaneously with IMA. 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.
Acknowledgements

We are grateful to Dr. Erna Dewil for the logistic support offered for the animal facility. We thank Lieve Ophalvens and Jasmien Wellens for their excellent technical assistance. Results of the study were partially presented at the annual meetings of the American Association for Cancer Research (Denver, CO USA April 18th-23rd 2009; (31)) and of the American Society of Clinical Oncology (Orlando, FL May 29th- June 2nd 2009(32)).

Bibliography


24. Rappaport AM. The structural and functional unit in the human liver (liver acinus) Anat Rec 1958; 130:673-89


Figure legend

**Figure 1:** Tumor volume assessment. Chemical structures of IMA, SUN and IPI-504 (a) Tumor volume was assessed in GIST-882 (b) and in GIST-PSW (c), at day 0, 3, 6, 9, 12 and 14. For each time point the standard error of the mean is plotted on the graph.

**Figure 2:** Histopathologic assessment. Mitoses and apoptotic bodies were counted on H&E slides in 10 fields at high power magnification (400 fold magnification). Results are presented in panels’ a to d as box plots showing maximum, minimum, interquartile range and median. GIST-882 control tumors showed a brisk mitotic activity which was promptly decreased by all type of treatment (a). Similar results were observed in GIST-PSW (b). The apoptotic activity in GIST-882 was not significant regardless the type of treatment (c). The contrary was observed in GIST-PSW, in which the combination treatment yielded the best results in term of apoptotic induction (d). Histologic response (HR) was graded based on the magnitude of necrosis, myxoid degeneration, or fibrosis as: grade 1 (0-10%), 2 (10%-50%), 3 (50%-90%), and 4 (>90%) respectively, in the GIST-882 and GIST-PSW on H&E slides (e)

**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: 

\[
\text{[(densitometry treated tumor/densitometry control tumor)-1].}
\]

In GIST-882 KIT expression and inactivation were affected by IPI-504 exposure, but the combination IPI-504+IMA further enhanced these results (a). Densitometry evaluation in GIST-882 shows HSP70 up-regulation together with down-regulation of KIT and AKT in IPI-504 treated tumors (b). IPI-504 and TKI exposure significantly decrease the activity of KIT and downstream intermediates in GIST-PSW (c). Despite a significant HSP70 up-regulation, in GIST-PSW only KIT showed some degree of downregulation upon IPI-504 treatment (d).
Figure 4: Liver histological changes in IPI-504 treated mice. Liver histology of IPI-504 treated mice was reviewed on H&E staining. (a) (H&E 200x) Apoptotic hepatocytes (arrows) are frequently found around terminal veins (T), portal tract are unaffected (P). (b-c) (H&E 200x) Apoptotic hepatocytes (arrow) together with foci of spotty necrosis are observed close. (d) (H&E 200x) Groups, or isolated apoptotic hepatocytes are frequently found in the subcapsular region of the liver. (e) (H&E 100x) Moderate to severe liver necrosis (N) is observed between terminal veins (T) in an IPI+IMA treated mouse; the black line highlight the border between necrotic parenchyma and the few plates of vital hepatocytes around portal tracts (P). (f) (H&E 40x) Wide areas of necrosis (N) are mainly concentrated in the subcapsular region of the liver in a mouse treated with IPI-504 during pilot studies.
**TABLE 1: Semiquantitative scoring system for liver damage.** Histological changes were first evaluated in the zone 1 (periportal) and zone 3 (periterminal) of the Rappaport acinus (24); next hepatocellular abnormalities were recorded. Each feature is scored according to the severity. The grade of liver damage was defined by the sum of each histological change.

<table>
<thead>
<tr>
<th>Lobular changes</th>
<th>Hepatocellular changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score for zone 1 (changes present in &gt;3 portal tracts)</td>
<td>Score for zone 3 (changes present in &gt;3 terminal veins)</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>

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

<table>
<thead>
<tr>
<th>Score 1-3, minimal</th>
<th>Score 4-6, mild</th>
<th>Score 7-9, moderate</th>
<th>Score 10-12, severe</th>
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</thead>
<tbody>
<tr>
<td>GRADE 1</td>
<td>GRADE 2</td>
<td>GRADE 3</td>
<td>GRADE 4</td>
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**TABLE 2:** Hepatic histological changes observed in the experimental groups. For each group the number of animals with either none, minimal, mild, moderate or severe histological changes in the liver are presented. In the IPI-504 group two cohorts are compared: the column on the left represents animals that received doses of 150 mg/kg intraperitoneally 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+IMA group the cohort of mice that received the two drugs simultaneously (simult.) is listed on the left, while on the right the animals received the two drugs with a 2-4 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 three rows of the table.

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>Histol. changes</th>
<th>control</th>
<th>IMA</th>
<th>SUN</th>
<th>IPI-504</th>
<th>PI504+IMA</th>
<th>PI504+SUN</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>3</td>
<td>7</td>
<td>4</td>
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<tr>
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<td>2</td>
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<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2 (mild)</td>
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<td>2</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Grade 3 (moderate)</td>
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<td>0</td>
<td>2</td>
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<td>1</td>
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<tr>
<td>Grade 4 (severe)</td>
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<td>2</td>
</tr>
<tr>
<td>Treatment Regimen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IPI-504</td>
<td>PI504+IMA</td>
<td>PI504+SUN</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150 mg/kg, 3x/wk, ip</td>
<td>100 mg/kg, 3x/wk, oral</td>
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<tr>
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<td>both</td>
<td>-PSW</td>
<td>-PSW</td>
<td>both</td>
<td>-both</td>
<td>-PSW</td>
</tr>
<tr>
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<td>6</td>
<td>3</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>

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 three rows of the table.
Figure 2
Molecular Cancer Therapeutics

The heat shock protein 90 inhibitor IPI-504 induces KIT degradation, tumor shrinkage and cell proliferation arrest in xenografts models of gastrointestinal stromal tumors

Giuseppe Floris, Maria Debiec-Rychter, Agnieszka Wozniak, et al.

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