The Novel HSP90 Inhibitor, IPI-493, Is Highly Effective in Human Gastrointestinal Stromal Tumor Xenografts Carrying Heterogeneous KIT Mutations

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Abstract

Purpose: KIT activity is crucial for gastrointestinal stromal tumors (GIST). Imatinib (IMA) and sunitinib (SUN) are very effective KIT-inhibitors in patients with advanced GIST but have no curative potential. We evaluated the efficacy of the novel HSP90 inhibitor IPI-493 alone, or in combination with IMA or SUN in GIST xenografts with KIT mutations.

Experimental Design: Nude mice (n = 98) were grafted bilaterally with human GIST carrying KIT exon 11 (GIST-PSW), KIT exon 9 (GIST-BOE), or double, KIT imatinib-sensitive exon 11 and imatinib-resistant exon 17 mutations (GIST-48). Mice were divided into six treatment groups and dosed orally for 15 days as follows: (i) control group, sterile water; (ii) IMA alone; (iii) SUN alone; (iv) IPI-493 alone; (v) IPI-493 + IMA; and (vi) IPI-493 + SUN.

Results: Treatment with IPI-493 resulted in tumor growth stabilization, variable proliferation arrest, induction of apoptosis and necrosis, and downregulation of KIT and its signaling cascade, especially in the GIST-BOE model. Significant reduction of vessel density was observed with IPI-493 treatment, and was equal to SUN treatment in GIST-PSW and GIST-BOE xenografts. IPI-493 treatment effects were enhanced in combination with TKIs, especially with IPI-493 + SUN. In our hands, IPI-493 showed dose-dependent liver damages.

Conclusions: When administered as a single agent in a xenograft model, the HSP90 inhibitor IPI-493 has consistent antitumor activity and induces KIT downregulation in GISTs with heterogeneous KIT mutations. IPI-493 synergizes with TKIs that are commonly used for the treatment of advanced or IMA-resistant GIST. The antitumor response of IPI-493 is particularly enhanced in combination with SUN.

Clin Cancer Res; 17(17); 5604–14. ©2011 AACR.

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal malignancies of the digestive tract (1). By immunohistochemistry, about 95% of GISTs express the receptor tyrosine kinase KIT (2). Activating mutations in KIT or in the platelet derived growth factor receptor alpha (PDGFRα) are the principal oncogenic events in GIST (1). GIST cells are extremely sensitive to tyrosine kinase inhibitors (TKI), such as imatinib (IMA) or sunitinib (SUN) (3, 4). The type of KIT/PDGFRα mutation determines tumor response to TKIs treatment, which is rarely curative (3). Gradually, the majority of GIST patients become refractory to TKIs (3). Resistance to TKIs in GIST is mainly caused by “secondary” mutations that show site-dependent and heterogeneous sensitivity to alternative KIT inhibitors, alternatively amplification of the KIT gene is also implicated (5, 6). Resistant mutations may be present synchronously in the same patient at different metastatic sites, or even within the same metastasis (7). The development of novel therapeutic strategies is thus of utmost importance in GIST.

HSP90 facilitates the correct folding of other proteins, aids in protein activation and function, and protects the cell from undesired aggregation of proteins. The activity of HSP90 is crucial for the function of more than 200...
Translational Relevance

The data presented in this study show that IPI-493 (an orally bioavailable HSP90 inhibitor) has strong antitumor potential against gastrointestinal stromal tumors (GIST). In combination with imatinib or sunitinib the therapeutic effect of IPI-493 is enhanced. The pharmacologic inhibition of the chaperone activity of HSP90 offers the opportunity to impair the activity of a number of signaling protein kinases and other oncoproteins that are crucial for the survival of cancer cells. GIST strongly depends on the activity of mutated KIT receptor, which is dependent on HSP90 for its maturation. Hence, HSP90 inhibitors represent promising compounds for GIST therapy. The heterogeneous molecular mechanisms conferring resistance to tyrosine kinase inhibitors in GISTs make the search for novel therapeutic strategies a major challenge. The combined therapy with tyrosine kinase inhibitors and HSP90 inhibitors constitutes an interesting strategy that could potentially overcome resistance to imatinib or sunitinib in GIST patients.

proteins, known as client proteins. Notably in cancer cells, HSP90 is hyperactivated and overexpressed, and many of its client proteins are deregulated causing uncontrolled proliferation and oncogenic signaling. Thus, targeting HSP90 activity represents a promising therapeutic strategy against certain malignancies (8–11). The KIT receptor is one of the HSP90 client proteins (9). HSP90 inhibition in human malignant mast cell lines results in KIT inhibition and downregulation (12). Similarly, in diverse GIST cell lines with IMA-sensitive or -resistant KIT mutations, HSP90 inhibition proved to be an efficient cytotoxic agent (13). In addition, cell lines expressing GIST-associated PDGFRA mutations showed high sensitivity to HSP90 inhibitors (HSP90-i; ref. 14). Thus, the rationale exists to target HSP90 in GIST.

The activity of HSP90 is orchestrated by diverse cofactors (known as cochaperones protein), and by conformational changes that require ATP hydrolysis by the intrinsic ATP-ase activity of HSP90 (11). Twelve HSP90-i are currently undergoing early clinical evaluation (9). So far, all HSP90-i share a common mechanism of action, which is the competitive blockage of ATP hydrolysis, resulting in client protein degradation (9, 11). The benzoquinone ansamycins (BA) family of HSP90-i is the best characterized. The prototype molecule geldanamycin and its 2 analogues 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) showed unacceptable toxicities or unsatisfactory pharmacological properties (9–11). The hydroquinone hydrochloride salt of 17-AAG (IPI-504, retaspimycin hydrochloride), has a more favorable pharmacologic profile (15–16). More recently, a related compound, the orally bioavailable IPI-493 [17-amino-17-demethoxygeldanamycin (17-AG)] has shown promising results in preclinical models (17).

This study is the first in vivo evaluation of the antitumor activity of IPI-493, alone or in combination with IMA or SUN, in 3 human GIST xenograft models that carry either primary IMA-sensitive, or secondary IMA-resistant KIT mutations.

Materials and Methods

Cell lines, biopsy, and generation of mouse GIST xenografts

The GIST-PSW (carrying KIT exon 11 mutation) and the GIST-BOE (carrying KIT exon 9 mutation) cell lines were used to generate heterotopic human GIST xenografts, as previously described (18). The GIST-48 cell line with primary KIT exon 11 homozygous and secondary KIT exon 17 heterozygous mutations was a kind gift of Dr. Jonathan Fletcher (13). GIST-48 cells (5 × 10^6) mixed with Cultrex matrix-gel (Trevigen) were injected bilaterally under the skin of the mice (NMRI nude mice). The GIST-48 tumors reached a volume of about 500 mm^3 in about 8 months; tumors were then sequentially retransplanted from mouse to mouse 3 times. For this study, we used 32 GIST-PSW (8th passage), 36 GIST-BOE (5th passage), and 30 GIST-48 (4th passage) mice.

Experimental design, evaluation of response to treatment and adverse events

All animal experiments were approved by the ethical committee of the Catholic University Leuven (Leuven, Belgium). A total of 98 mice bearing bilateral GIST tumors of approximately 390 mm^3 on average were grouped as follows: group 1 control mice (sterile water; GIST-PSW n = 5; GIST-BOE n = 6; GIST-48 n = 5); group 2 IMA treatment (GIST-PSW n = 5; GIST-BOE n = 6; GIST-48 n = 5); group 3 SUN treatment (GIST-PSW n = 5; GIST-BOE n = 6; GIST-48 n = 5); group 4 IPI-493 treatment (GIST-PSW n = 6; GIST-BOE n = 6; GIST-48 n = 5); group 5 IPI-493 + IMA combination (GIST-PSW n = 6; GIST-BOE n = 6; GIST-48 n = 5); group 6 IPI-493 + SUN combination (GIST-PSW n = 5; GIST-BOE n = 6; GIST-48 n = 5). IMA, SUN, and IPI-493 were administered by oral gavage at 50 mg/kg twice a day, 40 mg/kg daily and 100 mg/kg 3 times weekly, respectively in GIST-BOE. The IPI-493 dose was reduced to 80 mg/kg with unchanged schedule in GIST-PSW and GIST-48, to lower drug intolerance. In IPI-493 + IMA and IPI-493 + SUN groups, IPI-493 was dosed at 50 mg/kg with the same schedule, and the TKI schedules/doses were unchanged. Importantly, a 2-hour interval was maintained between TKI and IPI-493 administration. The treatment lasted 15 days, tumor volume and body weights 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 treatment administration on day 15. Specimens of tumor were partly snap frozen and partly fixed in formalin. Necropsy was done on each animal, and organs were collected to evaluate related adverse events (at least 1 mouse...
per group). Multiple complementary techniques were adopted to assess response to treatment on tumor tissue, as previously described (18). In addition, the KIT genotype and copy number changes in specific passages of xenografts used in this study was confirmed by sequencing and FISH analysis, and the histologic profile of the GIST xenografts used was evaluated using routine hematoxilin/eosin (H&E) staining and immunohistochemistry. On histology, we assessed histologic response (HR), proliferation, and apoptosis as previously reported (18). Given the known high efficacy of SUN toward VEGF receptors, and the antiangiogenic activity of IPI-493, SUN, and IPI-493+SUN were assessed by measuring the tumor vessel density of the GIST xenografts. The vessel density was defined as the number of vessels counted in 5 microscopic fields (200× magnification). Specific monoclonal rat antibodies against mouse epitopes of CD34 (in all models) and CD31 (only in GIST-BOE) were used to mark the mice vessels. Western blot analysis was used to measure KIT protein concentrations. Detailed information about reagents, drugs, evaluation of treatment response, and xenograft characterization is provided in Supplementary Methods.

Statistics
Comparisons between tumor volumes on day 0 versus day 14 were done with the Wilcoxon matched pair test. Comparison between groups was done using the Mann–Whitney U test, which was adopted also for assessing histologic measurements and KIT concentration values from cell lysates. Statistically significant differences were defined as P < 0.05. Bonferroni correction was used for multiple testing. The software STATISTICA (StatSoft) was used for analysis.

Results
Characterization of mouse xenografts
Histopathologic features of untreated GIST-PSW and GIST-BOE tumors were identical to those previously described (18). Microscopically, the GIST-48 tumors were mainly composed of epithelioid tumor cells.

The specific biomarkers for interstitial cells of Cajal (KIT, ANO1, CD44, ETV1, and CD34) were studied by immunohistochemistry (Table 1 and Supplementary Fig. S1A; refs. 19, 20). Mutational and FISH analysis done on GIST-PSW and GIST-BOE confirmed our previous results (18). In GIST-48, we confirmed the presence of heterozygous KIT double mutation and diploid KIT copy number (13).

As shown by immunoblotting, KIT expression was heterogeneous in the 3 xenografts, and HSP90 the target of IPI-493 was equally expressed in all xenografts. The enzyme NQO1 was expressed only by GIST-PSW and GIST-48 xenografts, whereas the cochaperone protein HSP27 was expressed by GIST-PSW and GIST-BOE, but not by GIST-48 xenografts (Supplementary Fig. S1B).

Tumor volume assessment
The GIST-PSW xenografts present a common IMA-sensitive KIT mutation (KIT exon 11), whereas the GIST-BOE (KIT exon 9 mutation) and the GIST-48 (KIT exon 11 and exon 17 double mutations) xenografts carry mutations that are associated with dose dependent and secondary resistance to IMA, respectively.

Control tumors grew steadily in all models more than 15 days. The GIST-PSW and GIST-48 grew more rapidly than the GIST-BOE xenografts (Fig. 1A–C).

As expected, IMA caused the most remarkable tumor regression in the GIST-PSW (74% reduction from the starting volume; 95% CI: 59%–89%; P < 0.01; Wilcoxon test; Fig. 1A). In the GIST-BOE model, despite IMA treatment, the tumor burden increased almost as much as in control tumors (under IMA 126% of baseline, 95% CI: 103%–150%; P = 0.27 vs. control; U test; Fig. 1B). Surprisingly, IMA treatment stabilized the GIST-48 tumors. Thus, tumor burden was 93% of baseline (95% CI: 44%–143%; Fig. 1C), being significantly different from control tumors (P < 0.01; U test).

Regardless of the type of xenograft, 15 days of SUN treatment led to consistent tumor regression in all models (P < 0.01 in all xenografts; Wilcoxon test). Tumor volume

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<th>Table 1. GIST xenograft characterization</th>
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<td><strong>Immunophenotype</strong></td>
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NOTE: The histology, immunophenotype, FISH, genotype and description of chromosomal changes is described for each xenograft model.

Abbreviations: Sp, spindled cell type; Ep, epithelioid type. The immunostaining is graded according to the intensity and distribution as follows: +++, strongly expressed; ++, moderately expressed; +++, weakly expressed; −−−, diffuse expression; f, focal expression. For FISH analysis, the status of the KIT gene on chromosome 4 is briefly described: No ampl, no amplification of the KIT. In the mutational analysis: hom, homozygous, htz, heterozygous mutation.
was 21% (95% CI: 2%–40%), 36% (95% CI: 30%–41%) and 44% (95% CI: 26%–62%) of baseline in GIST-PSW, GIST-BOE, and GIST-48 xenografts, respectively (Fig. 1A–C). Of note, responses to SUN were significantly better than responses to IMA in all xenografts (at least $P < 0.05$; U test).

IPI-493 treatment resulted in a slight but insignificant regression of GIST-PSW and GIST-BOE tumors, with a 22%
IPI-493 treatment of GIST-48 xenografts resulted in a significant stabilization rather than tumor regression with the use of Clinical Cancer Research (95% CI: 91%–147%). However, this value was significantly different from control tumors, and it was interpreted as partial tumor growth arrest (P < 0.05; U test; Fig. 1C). Interestingly, IPI-493 was better than IMA in GIST-BOE (P < 0.01; U test).

Generally speaking, in GIST-PSW and GIST-BOE xenografts both combination arms showed similar effects to those observed in the respective TKI single-agent arms (Fig. 1A and B). In GIST-48 tumors both combination treatments were very effective, yielding more than 50% tumor regression from baseline (P < 0.01 in both combination arms; Wilcoxon test; Fig. 1C). Importantly, both combination treatments were also better than IMA or IPI-493 (at least P < 0.01 in both comparisons; U test) but equal to SUN in reducing GIST-48 tumor burden.

Histopathology
We first assessed the magnitude of HR in the tumors on day 14 (n = 171 tumors). In treated tumors, HR was heterogeneous and mostly did not correlate with tumor shrinkage. Majority of IMA-treated tumors showed grade 1HR (21/26 tumors; Fig. 2). The HR was more pronounced with SUN (3-4 HR in 5/29 tumors; Fig. 2). Almost half of the tumors treated with IPI-493 showed wide areas of necrosis (12/29 tumors with grade 2–3 HR; Fig. 2), suggesting a cytotoxic effect. With IPI-493+IMA treatment, we observed grade 3 to 4 HR in 3/9 of GIST-PSW tumors (Fig. 2), but in GIST-BOE and GIST-48 tumors only grade 1 to 2 HR were recorded. The best HR scores across all tumors were observed with IPI-493+SUN treatment (grade 3–4 HR was observed in 9/26 tumors; Fig. 2). This result was especially evident in GIST-48, because 4/9 GIST-48 tumors showed grade 4 HR (Fig. 2).

Subsequently, we assessed proliferative and apoptotic activity in GIST xenografts, by means of mitotic and apoptotic count (H&E), Ki-67, and cleaved caspase 3 (CC3) immunostainings. We identified 32 mitoses per 10 HPF (400 times magnification; 95% CI: 22–42 mitosis/10 HPF) in GIST-PSW, 17 mitoses/10 HPF (95% CI: 12–20 mitoses/10 HPF) in GIST-BOE, and 16 mitoses/10 HPF (95% CI: 12–19 mitoses/10 HPF) (Fig. 3A) in GIST-48, indicating highly aggressive tumors.

IMA treatment significantly reduced the mitotic count of all the xenografts at day 14 (at least P < 0.01 compared with control). Proliferation was reduced by 21.6-fold in GIST-PSW tumors, while in GIST-48 and GIST-BOE tumors it was reduced by 2.6- and 1.6-fold, respectively (Fig. 3A). IMA treatment also caused a significant induction of apoptosis in GIST-PSW tumors (more than 2-fold increase compared with control, P < 0.01), but not in GIST-BOE or GIST-48 tumors (1.4-fold increase compared with control, P = 0.3; Fig. 3B).

In all xenografts exposed to SUN, the proliferative activity was profoundly affected (at least P < 0.001 vs. control); mitoses were 32-, 7.4-, and 4-fold lower than control in GIST-PSW, GIST-BOE, and GIST-48 tumors, respectively (Fig. 3A). The proapoptotic activity of SUN was prominent in GIST-PSW and GIST-48 (7.5- and 1.6-fold higher than controls, respectively; at least P < 0.05) but not in GIST-BOE tumors (P = 0.16; Fig. 3B).

Treatment with IPI-493 reduced mitotic activity by 11.6-fold in GIST-PSW tumors (P < 0.0001). The antiproliferative activity of IPI-493 was insignificant in the other 2 models (reduction by 1.4-fold vs. control; Fig. 3A). IPI-493 significantly induced apoptosis in GIST-PSW (2-fold increase), whereas in GIST-BOE IPI-493 was the sole treatment to stimulate significantly apoptosis (2.4-fold increase vs. control; at least P < 0.01 vs. all other groups; Fig. 3B). IPI-493 had no significant apoptotic effect in GIST-48 tumors.

Treatment with IPI-493+IMA reduced the mitotic activity in all xenografts; it was virtually absent in GIST-PSW (more than 50-fold decrease vs. control; P < 0.001), in GIST-48 it was reduced by 5.1-fold (P < 0.0001) and in GIST-BOE it was reduced by 1.6-fold (P < 0.01; Fig. 3A). IPI-493+IMA remarkably increased the apoptotic activity in GIST-PSW (more than 5-fold vs. control; P < 0.0001), but not in GIST-BOE and GIST-48 (Fig. 3B).

The IPI-493+SUN regimen showed the best antiproliferative activity, causing reduction of mitoses by 64-, 13-, and 11-fold from control, respectively in GIST-PSW, GIST-BOE, and GIST-48 tumors (P < 0.01; Fig. 3A). After IPI-493+SUN treatment apoptosis increased more than 3-fold compared with control in GIST-PSW tumors (P < 0.001). In GIST-BOE it was unchanged, and in GIST-48 tumors we recorded an more than 7-fold increase versus control (P < 0.001), which was better than any other treatment (at least P < 0.01 in the 4 pairwise comparisons; Fig. 3B).
The Ki-67 and CC3 immunostainings confirmed the previously described results in all groups and xenograft models (Fig. 3C and D).

SUN treatment reduced tumor vessel density in both GIST-PSW and GIST-BOE tumors \( (P < 0.05 \text{ vs. controls}) \). Similar results were observed with IPI-493 treatment \( (P < 0.05 \text{ vs. controls}) \). Similarly, IPI-493+SUN treatment reduced vessel density only in GIST-PSW and GIST-BOE tumors \( (P < 0.01 \text{ vs. control in both models}; \text{Fig. 4}) \). Regardless of the type of treatment, in GIST-48 tumors we observed a slight but not significant increase in vessel density when compared with control (Fig. 4). The CD31 immunostaining confirmed our findings in the GIST-BOE model (data not shown).

**Evaluation of HSP90 inhibition**

As shown by immunohistochemistry, the level of KIT expression was only minimally affected in animals treated with IPI-493 (both single agent and in combination arms; data not shown). In these animals we identified a slight change in the intensity of the staining, that is, KIT immunostaining was unevenly distributed, showing focal areas with low KIT expression (data not shown).

Next, total KIT levels were assessed by Western immunoblotting and densitometry analysis. With IMA treatment, KIT was partially downregulated in GIST-PSW and GIST-48 xenografts, whereas in GIST-BOE KIT was slightly upregulated (up to 20%; Fig. 5A, E). SUN treatment induced a more consistent downregulation of total KIT regardless of the type of xenograft model (Fig. 5). KIT expression was minimally downregulated by IPI-493 in all models (up to 25% reduction compared with control; Fig. 5); the IPI-493+IMA combination yielded equivalent results (Fig. 5). IPI-493+SUN treatment led to a more visible reduction of total KIT, especially in the GIST-PSW and GIST-48 tumors (Fig. 5). Thus, 2 of 6 GIST-PSW tumors and 1 of 5 GIST-48 tumors analyzed showed very low KIT expression (Fig. 5A, E).
Subsequently, by using the ELISA technique we were able to measure the concentrations of total KIT in tumor lysates (3 to 5 tumors for each group). In untreated GIST-PSW tumors, we measured the highest KIT concentration (6.52 ng/mL 95% CI: 4.72–8.32 ng/mL), followed by GIST-BOE (4.98 ng/mL 95% CI: 3.62–6.34 ng/mL) and the lowest in GIST-48 (3.02 ng/mL 95% CI: 0.88–5.17 ng/mL; Supplementary Fig. S2). The ELISA assay confirmed the pattern of changes assessed by densitometry. However, in comparison with control, the only statistically significant changes were observed in GIST-BOE and GIST-PSW treated with SUN and IPI-493 + SUN, respectively (in both xenografts \( P < 0.05 \), U test; Supplementary Fig. S2).

Finally, we observed variable degrees of HSP70 upregulation in all xenografts treated with IPI-493, a standard feature observed upon HSP90-i treatment (in both single and combination regimens; ref. 21). Notably, SUN treatment led to even higher upregulation of HSP70 in all xenograft models (Fig. 5).

Activation of KIT and its main downstream molecules

Further immunoblotting studies were done to assess the activation of KIT and its main downstream molecules. Regardless the type of treatment, we observed uniform inhibition of KIT and its main downstream signaling intermediates in GIST-PSW. Although IMA treatment visibly inhibited KIT (p-KITY705 and p-KITY719) inhibition by 72% and 80%, respectively) and its downstream signaling intermediates, some AKT expression was still detected (p-AKTY473 was decreased by 37%). Treatment with SUN and IPI-493 provided higher degrees of inhibition of the oncogenic signaling pathway (reduction by at least 80% of p-KIT and p-AKT for both drugs). The combination treatment regimens were very effective in inhibiting KIT and AKT, but did not exert the same level of ERK1/2 inhibition (Fig. 5A and B).

In GIST-BOE, none of the single treatment arms resulted in complete inhibition of KIT. KIT phosphorylation was reduced by 58%, 66%, and 67% by SUN, IMA, and IPI-493, respectively. Similar levels of inhibition were observed for AKT and ERK1/2. Treatment with the combination of IPI-493 + IMA did not provide any advantage in terms of KIT or AKT inhibition, but it was better than any other treatment at inhibiting ERK1/2. In contrast, considerable inhibition of the whole oncogenic signaling pathway was observed with IPI-493 + SUN treatment (Fig. 5C and D).

In the GIST-48 model, all treatment but IPI-493 + IMA yielded less than 40% of inhibition of KIT and its signaling cascade. The IPI-493 + IMA combination treatment inhibited KIT and AKT by 77% and 65%, respectively. Activation of ERK1/2 remained unchanged in all groups in comparison to control (Fig. 5E and F).

Treatment-emergent adverse events

The TKIs were well tolerated; no severe body weight loss or major changes in behavior, skin appearance, hydration, or signs of diarrhea were recorded. The 80 mg/kg 3 times per week dose of IPI-493 was also well tolerated (up to 3% body weight loss on average, data not shown). In contrast, 3 of 6 GIST-BOE mice treated with 100 mg/kg 3 times per week IPI-493 showed severe body weight loss of up to 25% loss, skin dehydration and rashes which led us to stop treatment in these animals on day 10. Following treatment discontinuation, 1 mouse recovered but the other 2 died.
Similar side effects were observed in 1 GIST-BOE mouse receiving IPI-493þIM, however, treatment discontinuation between days 6 and 10 resulted in prompt recovery and therapy rechallenge. Except for the mouse mentioned above, both combination treatments (IPI-493þIM and IPI-493þSUN) were well tolerated. Necropsy revealed no gross changes in the organs of the animals, including the 2 GIST-BOE mice that had died during the experiment.

On histology, the heart, lungs, kidneys, spleen, and bowels collected from mice did not show specific alterations regardless of the type of treatment. The histology of livers collected from single agent TKI treated mice did not differ from control mice (data not shown). The livers of mice treated with IPI-493 at 80 mg/kg 3 times weekly, IPI-493þIM or IPI-493þSUN showed only limited signs of damage. Namely, foci of spotty necrosis and apoptotic hepatocytes around terminal veins were observed whereas portal tracts were unaffected. These signs were more evident in mice receiving higher IPI-493 doses (100 mg/kg 3 times weekly in GIST-BOE; data not shown).

Severe liver damage was observed in the 2 GIST-BOE mice treated with 100 mg/kg 3 times per week IPI-493 that had died despite drug discontinuation. In these animals, massive necrosis/apoptosis of the parenchyma between adjacent terminal veins (known as bridging necrosis with terminal/terminal pattern) was recorded, which left few unaffected peri-portal hepatocytes (data not shown).

In the livers collected from the GIST-BOE mice that had recovered after a period of therapy discontinuation, we found simultaneous presence of hepatocyte proliferation (mitosis) and of apoptosis/necrosis (data not shown). This sign was interpreted as postnecrotic hepatocellular regeneration, and it was observed in about 20% of the remaining mice treated with IPI-493 alone or in combination.

Figure 5. Western blot and densitometry analysis. Whole tumor lysates were used to assess total expression and phosphorylation of KIT protein and its downstream signaling molecules AKT and ERK1/2. HSP70 was used as a biomarker to check activity and viability of IPI-493 in the tumors. Densitometry was measured as described in Materials and Methods; results are presented according to the formula: (densitometry treated tumor/densitometry control tumor)-1 and expressed as a percentage. In panel A, C, and E, representative pictures of the KIT signaling cascade in GIST-PSW, GIST-BOE, and GIST-48 models, respectively are illustrated; in the remaining panels the densitometry analysis for each xenograft model is reported. Note: Densitometric assessment does not necessarily correspond to blot shown, because control tumor were run multiple times to better compare differences versus each different type of treatment.
Discussion

The rationale for targeting HSP90 in GIST stems from diverse in vitro evidence. KIT degradation and inhibition has been reported in human cell lines of mastocytosis carrying diverse KIT mutations treated with 17-AAG (12). Interaction between HSP90 and KIT is important for KIT activation in the GIST-T1 cell line (22). 17-AAG was active in diverse KIT expressing GIST cell lines, inducing KIT inhibition and degradation, downregulation of the oncopgenic pathways, cell proliferation arrest and apoptosis (13). Ba/F3 cells transduced to express mutated PDGFRα are sensitive to the HSP90 inhibitor IPI-504 (14). In this study, we provide further in vivo evidence in support of this therapeutic approach in GIST. Specifically, we have tested for the first time the antitumor activity of the oral HSP90 inhibitor IPI-493 against 3 human GIST xenografts carrying heterogeneous KIT mutations that show differential sensitivity to IMA. Moreover, we explored the antitumor activity of combination treatments of IMA or SUN with IPI-493 in these models.

The activity of IPI-493 as a single agent was more evident in the GIST-PSW and GIST-BOE models than the GIST-48 model. We observed substantial tumor burden stabilization during treatment in these highly proliferating tumors. Clinically, GIST patients with progressive disease benefit from the induction of stable disease under TKI therapy (23, 24). On histology, the efficacy of IPI-493 was further supported by remarkable induction of HR, the proapoptotic capacity of the agent in both models and the more variable arrest of tumor cell proliferation. In the GIST-48 model, treatment with IPI-493 did not lead to tumor growth delay rather than tumor regression, and neither proliferation activity nor apoptosis were influenced. However, as proven by HR, mild degrees of necrosis were induced in 50% of the GIST-48 tumors treated with IPI-493, suggesting a cytotoxic activity in this model. Notably, the presence of necrosis may lead to underestimation of the actual response of a tumor to therapy, since replacement of tumor tissue with necrosis may sometimes result in volumetric increase rather than objective tumor regression (25). Nevertheless, tumor growth delay may benefit GIST patients failing standard treatment. In this scenario, targeting HSP90 may represent a salvage strategy in case of failure after treatment with different TKIs.

High expression levels of HSP27 and lack of NQO1 enzyme were reported to negatively influence the response to HSP90-i (9, 26, 27), but our results did not show any significant correlation between response to IPI-493 and expression of these proteins. In addition, upregulation of the cochaperone protein HSP70 and degradation of HSP90 client proteins (including KIT) are standard requirements of HSP90 inhibition. In our study, however, expression of total KIT was minimally affected by IPI-493 despite HSP70 upregulation, as assessed by densitometric analysis and ELISA assay. Notwithstanding, we documented a remarkable inhibition of KIT activity and its signaling intermediates upon IPI-493 treatment. To date, it is still unclear whether the activity and efficacy of HSP90-i in GIST is dependent on KIT degradation. Review of the literature indicates that inhibition of KIT and its main downstream intermediates, and consequently also antitumor HSP90-i activity, are often independent of KIT degradation in GIST. Moreover, KIT degradation is heterogeneous in GIST upon treatment with HSP90-i (13, 22, 28). Because the interactions between HSP90 and the diverse isoforms of KIT oncoproteins may vary, it is possible that this heterogeneity is dependent on GIST tumor genotype, which may eventually influence the response to HSP90-i. Our previous experiences with the HSP90-i IPI-504 may support this hypothesis (28). GIST xenografts with KIT exon 13 mutation (i.e., the GIST-882 model) were more sensitive to IPI-504 than GIST-PSW xenografts, by showing better KIT degradation and greater tumor regression (28 and unpublished results). Similarly, in vitro, the GIST-882 cell line shows the most remarkable levels of KIT degradation in comparison to the GIST-48 and GIST-430 cell lines upon 17-AAG treatment (13). Notably, mutations occurring in other HSP90 client proteins than KIT may affect the affinity of a given client protein to HSP90, and therefore influence sensitivity to HSP90-i (29). Formal crystallographic studies, which are beyond the scope of this study, should address our hypothesis. Finally, other biological features of individual tumors could influence the response to HSP90-i in GIST. Our observations could guide a personalized approach to treating GIST with HSP90-i, based on the pretherapeutic characterization of KIT oncoprotein.

An important step in drug testing in preclinical models of cancer is represented by the validation of the predictive value of the xenograft model with standard treatments approved in the clinic (i.e., IMA and SUN in GIST; ref. 30). The KIT genotype is the major predictor of response to treatment with IMA and SUN in GIST (31). We show that GIST-PSW (KIT exon 11 mutant) was the most IMA-sensitive xenograft of the 3 tested in this study, while GIST-BOE (KIT exon 9 mutant) showed tumor growth rather than tumor regression under IMA, most likely due to the IMA dose used. Indeed, doses of IMA 3 times higher than the one used here remarkably increased the efficacy of IMA in GIST-BOE but not in GIST-PSW, as indicated by previous studies (18). This observation is consistent with observations in the clinic. Thus, while in KIT exon 11 mutant GIST the clinical outcome is not influenced by higher IMA dose, the clinical benefit increases under higher doses of IMA in KIT exon 9 mutants GIST (31, 32).

Unexpectedly, the GIST-48 xenograft model, which carries secondary, IMA- and SUN-resistant KIT exon 17 mutation, shows response upon treatment with TKI, particularly with SUN, as evidenced by tumor stabilization or shrinkage. However, the whole protein lysates of GIST-48, in contrast with the other 2 xenograft models, showed high levels of KIT activation and its oncogenic signaling despite TKI treatment. This finding is similar to in vitro data, in which a minor decrease of KIT signaling, but more than 40% GIST-48 cell loss is observed after 72 hours treatment with IMA (33).
We recorded the best responses with SUN treatment regardless of type of xenograft. However, in GIST-BOE, the apoptotic activity was not stimulated by SUN, suggesting a more cytostatic rather than cytoidal effect in this model. An interesting and new observation was the remarkable HSP70 upregulation in GIST treated with SUN. This feature is documented also in tumors treated with HSP90-i, and it may be related to inefficient induction of apoptosis by HSP90-i (11). Specific HSP70 inhibitors that are currently under development may circumvent this unwanted feature, if combined with HSP90-i. Thus, the combination of SUN with HSP70 inhibitors could represent an innovative therapeutic strategy that warrants further studies in GIST.

We found that IPI-493 caused significant reduction of host vessel density in our xenografts suggesting a broader spectrum of anticancer activities for IPI-493. Our findings are consistent with previous reports, and further support the hypothesis that the anticancer activity of HSP90-i might be in part mediated by antiangiogenic effects (34).

The combination of IPI-493 with either IMA or SUN enhanced most of the therapeutic features analyzed. This result was particularly evident with the IPI-493+SUN regimen, which led to the highest degree of response. Importantly, this outstanding outcome was more evident in the GIST-48 model, which carries KIT mutations that are associated with TKI resistance in the clinic. Our results suggest that the combination IPI-493+SUN is more efficient at affecting GIST cells, and support the rationale for this therapeutic strategy in IMA-resistant tumors.

In GIST-BOE mice, the high dose of IPI-493 was not tolerated in about half of the animals. Despite treatment discontinuation some animals died, most likely due to drug-related toxicities in the liver. The reduction by 20% of the IPI-493 dose significantly improved the tolerability of the drug, without affecting its efficacy. Impaired liver function is commonly observed under treatment with BA-derived HSP90-i (10). However, the molecular scaffold of IPI-493, which is related to 17-AAG, shows improved pharmacologic properties (35). According to in vitro data, IPI-493 presents higher potency in comparison with other HSP90-i (17). Thus, the higher in vitro potency could be mirrored by a greater potential for drug toxicity. However, it is important to mention that NCR nu/nu mice tolerate well high doses of IPI-493 (17). Thereby, we do not exclude the possibility that the different genetic background carried by the strain of mice here tested (NMRInu/nu) might have had a negative influence on the metabolism of the drug, resulting in increased liver damage.

In conclusion, we provide the first in vivo evidence of the antitumor activity of IPI-493 in GIST. As a single agent [in xenograft], IPI-493 induces consistent inhibition of KIT signaling, tumor necrosis, apoptosis, and arrest of tumor cell proliferation. Importantly, the efficacy of IPI-493 is better than IMA in GIST carrying a KIT exon 9 isofrom. The combination of IPI-493 with SUN remarkably enhances antitumor effects in GIST, especially in tumors carrying double KIT mutations. The combination of SUN with IPI-493 should be explored in the clinic, for example, in patients refractory/intolerant to IMA.

Disclosure of Potential Conflicts of Interest

P. Schöflski has advisory functions for the Infinity Pharmaceuticals and E. Normant is employed by Infinity Pharmaceuticals, whose drug was herein tested.

Acknowledgments

We thank Dr. Erna Dewil for the logistic support offered for the animal facility. Our gratitude goes also to Stefania Crippa for the kind advice on the ELISA assay. We also thank Lieve Ophalvens, Ulla Vanleeuw, and Kathleen Machiels for their excellent technical assistance.

Grant Support

This work is supported by research grants from Life Raft Group (M. Debie-Rychter) and from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO grant # G. 0510.06 P. Schöflski). P. Schöflski received research grants from Infinity Pharmaceuticals.

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Received February 28, 2011; revised June 22, 2011; accepted June 27, 2011; published OnlineFirst July 7, 2011.

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The Novel HSP90 Inhibitor, IPI-493, Is Highly Effective in Human Gastrointestinal Stromal Tumor Xenografts Carrying Heterogeneous \textit{KIT} Mutations

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cite{Clinc Cancer Res 2011;17:5604-5614. Published OnlineFirst July 7, 2011.}

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