Oncogenes and Tumor Suppressors

FGFR3 Translocations in Bladder Cancer: Differential Sensitivity to HSP90 Inhibition Based on Drug Metabolism

Jaime Acquaviva1, Suqin He1, Chaohua Zhang1, John-Paul Jimenez2, Masazumi Nagai1, Jim Sang3, Manuel Sequeira1, Donald L. Smith1, Luísa Shin Ogawa1, Takayo Inoue1, Noriaki Tatsuta1, Margaret A. Knowles2, Richard C. Bates1, and David A. Proia1

Abstract
Activating mutations and/or overexpression of FGFR3 are common in bladder cancer, making FGFR3 an attractive therapeutic target in this disease. In addition, FGFR3 gene rearrangements have recently been described that define a unique subset of bladder tumors. Here, a selective HSP90 inhibitor, ganetespib, induced loss of FGFR3-TACC3 fusion protein expression and depletion of multiple oncogenic signaling proteins in RT112 bladder cells, resulting in potent cytotoxicity comparable with the pan-FGFR tyrosine kinase inhibitor BGJ398. However, in contrast to BGJ398, ganetespib exerted pleiotropic effects on additional mitogenic and survival pathways and could overcome the FGFR inhibitor–resistant phenotype of FGFR3 mutant–expressing 97-7 and MHG-U3 cells. Combinatorial benefit was observed when ganetespib was used with BGJ398 both in vitro and in vivo. Interestingly, two additional FGFR3 fusion-positive lines (RT4 and SW480) retained sensitivity to HSP90 inhibitor treatment by the ansamycins 17-AAG and 17-DMAG yet displayed intrinsic resistance to ganetespib or AUY922, both second-generation resorcinol-based compounds. Both cell lines, compared with RT112, expressed considerably higher levels of endogenous UGT1A enzyme; this phenotype resulted in a rapid glucuronidation-dependent metabolism and subsequent efflux of ganetespib from SW780 cells, thus providing a mechanism to account for the lack of bioactivity.

Implications: Pharmacologic blockade of the molecular chaperone HSP90 represents a promising approach for treating bladder tumors driven by oncogenic gene rearrangements of FGFR3. Furthermore, UDP-glucuronosyltransferase enzyme expression may serve as a predictive factor for clinical response to resorcinol-based HSP90 inhibitors. Mol Cancer Res; 12(7); 1042–54. ©2014 AACR.

Introduction
Bladder cancer represents the fifth most common malignancy worldwide and a major cause of cancer-related morbidity and death. Incidence and mortality rates have remained relatively constant over the past four decades, with an estimated 72,570 new cases and 15,210 deaths predicted for 2013 in the United States alone (1). A vast majority of patients, more than 70%, present with non–muscle-invasive bladder cancer (NMIBC), previously referred to as "superficial" bladder cancer (2). A proportion (around 10%–20%) of these tumors may ultimately progress to invasive disease, but even the bulk percentage of these neoplasms have a characteristic high risk of recurrence which presents an immense challenge for the clinical management and ongoing surveillance of patients, often over many years. The remaining cases are diagnosed as muscle-invasive bladder cancer (MIBC), a more aggressive stage of the disease that is associated with a higher risk of metastasis and for which the 5-year survival rates are only around 50% (2). Indeed, progression to metastatic bladder cancer, whether from recurrent NMIBC or MIBC, results in significantly poorer survival outcomes and is generally considered incurable (3). In contrast to other malignancies, advances in the management and treatment of bladder cancer have been limited (4); consequently, there exists a considerable unmet need for novel therapeutic approaches to improve patient outcomes in this disease indication.

FGFR3 is a member of a structurally related family of tyrosine kinase receptors (FGFR1–4) that orchestrate a diverse variety of cellular activities, including proliferation, differentiation, and survival (5). Ligand binding promotes receptor dimerization, transphosphorylation of key tyrosine residues, and recruitment of adaptor proteins, ultimately leading to the activation of multiple downstream signaling...
cascades, including PI3K/AKT, RAS/MAPK, STATs, and phospholipase Cγ (PLCγ; ref. 5). Somatic mutation of the FGFR3 gene is one of the most frequent genetic alterations seen in bladder cancer, occurring in around 75% of all cases of NMIBC (6, 7). Interestingly, most of the missense mutations identified in bladder tumors are identical to germline gain-of-function mutations responsible for autosomal-dominant human skeletal disorders and dwarfism syndromes (5, 8). FGFR3 mutations are less prevalent in muscle-invasive tumors, which more commonly exhibit dysregulated FGFR3 function via overexpression of the FGFR3

Materials and Methods

Cell lines, antibodies, and reagents

The RT4 and SW780 cell lines were obtained from the American Type Culture Collection (ATCC). Each was maintained according to manufacturer’s instructions, authenticated by routine company DNA typing, and used within six months of receipt. RT112 cells were obtained from Sigma-Aldrich. The 97-7 and MGH-U3 lines are established in the Knowles laboratory and cell line identity was verified by short tandem repeat DNA typing using a Powerplex 16 Kit (Promega). The remaining fifteen bladder lines listed in Table 1 are part of a collection assembled by the Center for Molecular Therapeutics (Massachusetts General Hospital Cancer Center, Boston, MA) who performed the drug sensitivity analysis. As part of the establishment of this cell line collection, a panel of 92 SNPs was profiled for each cell line and short tandem repeat analysis was also performed. All primary antibodies were purchased from Cell Signaling Technology with the exception of the FGFR3 (B9) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, Inc.), UGT1A9

Table 1. In vitro cytotoxicity values of ganetespib in bladder cancer cell lines

<table>
<thead>
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<th>Cell line</th>
<th>Ganetespib IC50 (nmol/L)</th>
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Viability assays

Cellular viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer protocols. Bladder cancer cell lines were seeded into 96-well plates based on optimal growth rates determined empirically for each line. All viability assays were performed in triplicate. Twenty-four hours after plating, cells were dosed with graded concentrations of ganetespib for 72 hours. CellTiter-Glo was added (50% v/v) to the cells, and the plates incubated for 10 minutes before luminescent detection in a SpectraMax Plus 384 Microplate Reader (Molecular Devices). Data were normalized to percent of detection in a SpectraMax Plus 384 Microplate Reader (Molecular Devices). Data were normalized to percent of control and IC50 values used to determine the sensitivity of each line. For the comparative analyses with ganetespib and BGJ398, RT112 cells were treated with graded concentrations of each compound for 72 hours and cell viability measured as above. For the evaluation of apoptotic induction, caspase activity was assessed using the Caspase-Glo 3/7 assay (Promega) according to manufacturer protocols. Twenty-four hours after plating, RT112 cells were treated with graded concentrations of ganetespib for 24 hours. Caspase-Glo was added to cells (50% v/v) and the plates incubated for 1 hour before luminescent detection.

Western blotting

Following in vitro assays, tumor cells were disrupted in lysis buffer on ice for 10 minutes. Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before transfer to nitrocellulose membranes (Bio-Rad). Membranes were blocked with Star-tingBlock T20 blocking buffer (Thermo Scientific) and immunoblotted with the indicated antibodies. Antibody–antigen complexes were visualized using an Odyssey system (LI-COR).

Reverse-phase protein array

RT112 cells were treated with dimethyl sulfoxide (DMSO, control) or 100 nmol/L ganetespib for 24 hours. Lysates were prepared as recommended by the M.D. Anderson Cancer Center (Houston, TX) with cellular proteins denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by an Aushon 2470 Arrayer (Aushon BioSystems). A total of 5,808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody (19). Only antibodies with a Pearson correlation coefficient between RPPA and Western blotting of more than 0.7 were used in reverse-phase protein array analysis. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using Microvigen software (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model developed by the Department of Bioinformatics and Computational Biology (M.D. Anderson Cancer Center). The fitted curve is plotted with the signal intensities, both observed and fitted, on the y axis and the log2–concentration of proteins on the x axis for diagnostic purposes. The protein concentrations of each set of slides were normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

In vivo xenograft tumor models

CD-1 nude mice (Charles River Laboratories) at 7 to 12 weeks of age were maintained in a pathogen-free environment and all in vivo procedures were approved by the Synta Pharmaceuticals Corp. Institutional Animal Care and Use Committee. RT112 cells (5 × 106) were subcutaneously implanted in female mice and animals bearing established tumors (~150 mm3) were randomized into treatment groups of 8. Mice were intravenously dosed with ganetespib (150 mg/kg) once weekly or per os dosed with BGJ398 (10 mg/kg) daily, either alone or in combination, for 3 weeks. Tumor growth inhibition was monitored by tumor volume measurements twice weekly. As a measurement of in vivo efficacy, the %T/C value was determined from the change in average tumor volumes of each treated group relative to the vehicle-treated animals. Statistical significance was determined using two-way ANOVA followed by Bonferroni post tests.

Pharmacodynamics

Nude mice bearing established RT112 xenografts were randomized into groups of 3 and administered a single bolus injection of ganetespib (150 mg/kg) or vehicle. At 24 and 72 hours posttreatment, tumors were resected and homogenized in lysis buffer. Expression changes in cell signaling pathway components were interrogated using Pathscan RTK Signaling and Pathscan Intracellular Signaling Array Kits (Cell Signaling Technology) according to the manufacturer’s protocols. Fluorescence readouts were measured using an Odyssey system (LI-COR) and average changes in signaling protein expression were calculated for each cohort.

Bioanalysis

In the first set of experiments, RT112 and SW780 cells were treated with graded concentrations of ganetespib or 17-DMAG (10–1,000 nmol/L) for 1 and 24 hours. Cell lysates were prepared for measurement of intracellular concentrations of the respective HSP90 inhibitors. RT112 and SW780 cells were also treated with 1 μmol/L ganetespib for 15 minutes, 1, 4, 8, and 24 hours. At each time point, cell
lysates were prepared and media was collected for analysis of intracellular and secreted concentrations of ganetespib and its glucuronidated metabolites. Bioanalysis was performed on equal protein concentrations from cell lysates or equal volumes of media. All samples were ultimately extracted by protein precipitation and analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS) using an Agilent 1100 HPLC interfaced to an API 4000 tandem mass spectrometer (Applied Biosystems). A Phenomenex Kinetex 2.6 μm C18 (30 × 2.1 mm) column was used with a run time of 3.5 minutes per sample.

**UGT1A gene expression analysis**

Total RNA was extracted from untreated SW780 and RT112 cells using the RNeasy Kit (Qiagen Inc.). cDNA (1 μg) was synthesized using an iScript cDNA synthesis kit (Bio-Rad). Real time-PCR was performed using custom primers for UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (Bio-Rad). The cycling conditions were 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Data were normalized to two housekeeping genes [hypoxanthine phosphoribosyltransferase 1 (HPRT1) and GAPDH] and analyzed by comparing 2−ΔΔC of the normalized sample. PCR was performed on a Bio-Rad iCycler iQ5 (Bio-Rad).

**siRNA knockdown assay**

The Nucleofector transfection system (Lonza) was used for cell transfections using standard manufacturer protocols. Briefly, for each siRNA construct 3–5 × 10^5 SW780 cells resuspended in 100 μL supplemented nucleofector solution were transiently transfected with siRNA-negative control or 300 nmol/L siRNA-UGT1A7, 8, 9, 10 mix (30 pmol/sample, Invitrogen). Transfected cells were grown for 72 hours. Cell cultures were then exposed to graded concentrations of ganetespib (0, 100, 1,000 nmol/L) for 24 hours before lysis and analysis by Western blot.

**Competitive UGT1A9 inhibitor assay**

SW780 cells were cultured with 0.5 μmol/L ganetespib, 100 and 300 μmol/L propofol, both alone and in combination, or vehicle (DMSO) for 24 hours. At the end of the incubation, cells were harvested, lysed, and protein expression changes evaluated by Western blot analysis.

**Results**

**Ganetespib displays potent cytotoxic activity in FGFR3-driven bladder cancer lines**

Sensitivity to targeted HSP90 inhibition by ganetespib was evaluated using a panel of 20 bladder cancer cell lines of diverse genetic FGFR3 backgrounds (Table 1). In the majority of lines examined, ganetespib reduced cellular viability with low nanomolar potency, including all that expressed activating point mutations. Furthermore, ganetespib treatment could overcome an intrinsic FGFR inhibitor-resistant phenotype displayed by mutant FGFR3-expressing 97–7 and MGH-U3 cells (Supplementary Fig. S1). These lines harbor FGFR3^{S249C} and FGFR3^{V375C} mutations, respectively, and were insensitive to the pan-FGFR inhibitor BGJ398 yet both remained susceptible to the cytotoxic effects of ganetespib exposure (Supplementary Fig. S1).

**Suppression of multiple oncogenic signaling cascades in FGFR3 fusion-driven RT112 bladder cancer cells by ganetespib**

From the initial analysis, RT112 cells were found to be acutely sensitive to ganetespib treatment (IC50, 9 nmol/L; Table 1). Long considered overexpressers of wild-type FGFR3, the recent identification of the FGFR3-TACC3 fusion gene product in these cells accounts for their critical dependence on FGFR3 activity for growth and survival (9). Moreover, bladder cell lines now known to be FGFR3 fusion-positive, including RT112, have been reported to be highly sensitive to BGJ398 (20). In this regard, ganetespib was equipotent to selective FGFR inhibition in reducing cell viability in this line (Fig. 1A). This loss of viability was concurrent with activation of apoptosis as shown in Fig. 1B. RT112 cells were exposed to increasing concentrations of ganetespib and viability measured at 72 hours. This profile was compared with apoptotic induction determined by activated caspase-3/7 levels assessed 24 hours posttreatment which showed that ganetespib-induced cytotoxicity was mediated by an irreversible commitment to apoptosis.

Next, we examined molecular changes in client and signaling protein pathways associated with FGFR3-TACC3 in ganetespib-treated RT112 cells (Fig. 1C). Ganetespib induced a robust and dose-dependent destabilization of FGFR3-TACC3 expression, in terms of both total and phosphorylated protein levels, suggesting that the FGFR3 fusion protein was highly responsive to HSP90 inhibition. Importantly, targeted degradation of this oncogenic driver was accompanied by loss of downstream signaling effector activity (as evidenced by loss of phosphorylated ERK and Akt levels) and induction of BIM, an additional marker of apoptosis (Fig. 1C). Consistent with its alternative mode of action as a specific tyrosine kinase inhibitor, an effective dose of BGJ398 (100 nmol/L) did not affect total FGFR3-TACC3 expression but resulted in a similar abrogation of autophosphorylated FGFR3-TACC3 activity, disruption of oncogenic pathways, and induction of apoptosis. When the kinetics of client protein loss in response to HSP90 inhibition were examined (Fig. 1D), it was found that destabilization of the FGFR3-TACC3 fusion and the congruent alterations in downstream signaling were relatively rapid, occurring within 4 hours of ganetespib exposure. Indeed, the destabilization profile for the FGFR3-TACC3 protein was similar to that of HER2, a highly sensitive HSP90 client.

A feature of targeted HSP90 blockade is the simultaneous disruption of multiple cellular signaling cascades and processes that are exquisitely dependent on the chaperoning function of the molecule. Therefore, we performed a more extensive reverse-phase protein array analysis of ganetespib effects in RT112 cells. In addition to the expected down-regulation of client receptor tyrosine kinases such as HER2, MET and EGFR, ganetespib treatment also selectively
altered the expression of a number of proteins involved in MAPK, AKT, and mTOR signaling and cell-cycle regulation, along with predicted increases in the apoptotic markers caspase-7 and BIM (Table 2). Thus, the potent and selective disruption of FGFR3-mediated signaling coupled with coordinate effects on additional mitogenic and survival pathways in RT112 cells accounts for the potent cytotoxic activity of ganetespib in this line.

Ganetespib in combination with FGFR3 tyrosine kinase inhibition confers superior antitumor activity in vitro and in vivo

On the basis of their distinct mechanisms of action on FGFR3-TACC inhibition, we examined whether combining ganetespib and BGJ398 would lead to increased activity in FGFR3 fusion-dependent bladder cancer cells. To do this, we first evaluated the effects on cellular viability following combination exposure in the RT112 cell line (Fig. 2A). The bladder cancer cells were treated with increasing concentrations of ganetespib and/or BGJ398 and viability assessed after 72 hours. The percentages represent the degree of cell death at each individual dose level, which revealed that combinatorial benefit was achieved at all concentrations tested. For example, at the approximate EC_{50} concentrations for each inhibitor (9.6 nmol/L for ganetespib and 5 nmol/L for BGJ398), combined exposure resulted in 71% cell killing. Overall, all combinations of ganetespib and BGJ398 showed improved cell killing activity over single-agent treatment alone.

To determine whether these effects on cell viability in vitro would translate to improved efficacy in vivo, RT112 xenograft bearing mice were treated with ganetespib and BGJ398, both as single agents and in combination. We have previously determined that the maximally tolerated dose of ganetespib on a weekly dosing regimen is 150 mg/kg (17). Weekly administration of ganetespib at this dosing level was comparable with daily dosing of BGJ398 at 10 mg/kg, with each compound inducing a similar degree of tumor regression (T/C values of −23% and −20%, respectively; Fig. 2B). Concurrent treatment with both drugs resulted in a significant enhancement of antitumor activity causing 66% tumor regression. In addition, combination treatment was well tolerated, with no significant changes in body weights seen after 3 weeks of treatment (data not shown). Thus, ganetespib and BGJ398, when combined, displayed superior antitumor efficacy compared with monotherapy in RT112 bladder tumor xenografts.

Next, pharmacodynamic analysis was performed in additional mice bearing RT112 xenografts to confirm that the ganetespib-induced tumor response correlated with target modulation in vivo. Animals were treated with a bolus injection of ganetespib at 150 mg/kg and tumors harvested 24 and 72 hours later. Control group mice were administered a single injection of vehicle and tumors excised at the same time points. Expression changes in components of multiple signaling pathways, including receptor tyrosine kinases and their effector proteins, were investigated using multiplexed antibody arrays; average changes for each

Figure 1. Cellular viability, apoptosis induction, and pathway modulation in FGFR3-TACC3-expressing RT112 cells following ganetespib treatment. A, RT112 cells were treated with increasing concentrations of ganetespib or BGJ398 and viability assessed after 72 hours. B, RT112 cells were treated with graded concentrations of ganetespib, as indicated. Caspase-3/7 activity was measured at 24 hours and viability assessed at 72 hours posttreatment. RLU, relative luminescence units. C, RT112 cells were exposed to increasing concentrations of ganetespib or BGJ398 at 100 nmol/L, for 24 hours. The expression levels of FGFR3-TACC3, phosphorylated FGFR3, ERK and AKT (p-FGFR, p-ERK, p-AKT), and BIM were determined by immunoblotting. D, RT112 cells were treated with 200 nmol/L ganetespib and harvested at 2, 4, 6, and 24 hours. Lysates were immunoblotted with the indicated antibodies. V, vehicle control at 24-hour time point.
Overall, these data show that single-dose ganetespib exerts a potent and rapid destabilizing effect on the FGFR3-TACC3 fusion kinase and its effectors in RT112 xenografts.

Differential sensitivity of FGFR3 fusion-positive bladder cancer lines to HSP90 inhibitors

As part of a previous study, it was shown that RT112 and RT4 bladder cancer cells, which also express the FGFR3-TACC3 fusion protein, were sensitive to targeted HSP90 inhibition by the first-generation ansamycin compound 17-AAG (17-allylamino-17-deethoxygeldanamycin) (21). However, RT4 cells were largely insensitive to ganetespib exposure (Table 1). To explore this lack of activity with ganetespib we evaluated the cytotoxicity of four HSP90 inhibitors (HSP90i) in RT4 and SW780 cells: 17-AAG, the closely related ansamycin analog 17-DMAG (17-dimethylaminoethylamino-17-deethoxygeldanamycin), ganetespib, and another resorcinol-based second-generation inhibitor AUY922 (Fig. 3A). In RT4 cells, exposure to an ansamycin-based HSP90i resulted in dose-dependent cytotoxicity and low nanomolar IC50 values. In contrast, ganetespib and AUY922 were minimally effective at reducing cellular viability with IC50 values greater than 1 μmol/L (Fig. 3A).

At the molecular level, 17-DMAG and 17-AAG both effectively destabilized FGFR3 and higher weight FGFR3-TACC3 fusion protein expression in RT4 cells (Fig. 3B). This was accompanied by a concomitant loss of downstream ERK activity (Fig. 3C). In addition, two other sensitive HSP90 client proteins, HER2 and CDC2, were similarly degraded in a concentration-dependent manner. Consistent with the viability results, a corresponding dose-dependent increase in BIM expression was observed supporting the premise that both of these first-generation HSP90i induced apoptosis in RT4 cells (Fig. 3C). As predicted by the sensitivity profile of this line, ganetespib had negligible effects on the expression of any of the same molecular markers (Fig. 3D).

This pattern was repeated in the SW780 line which harbors an FGFR3-BAI1AP2L1 gene rearrangement. The two resorcinol-based inhibitors were again only weakly cytotoxic yet the cells remained acutely sensitive to 17-AAG and 17-DMAG treatment (Fig. 4A). In stark contrast to 17-AAG, neither ganetespib nor AUY922 were found to significantly affect FGFR3-BAI1AP2L1 fusion protein levels, wild-type FGFR3, downstream effector pathways (p-ERK, p-AKT), or other established HSP90 client proteins (MET, HER2; Fig. 4B). These data were additionally supported by the dose-dependent analysis of ganetespib activity shown in Fig. 4C. No changes in FGFR3-BAI1AP2L1, phosphorylated FGFR3, ERK, AKT or MEK levels were observed, suggesting that the HSP90 inhibitory activity of the compound was compromised in this cell line. Taken together, these data show that the FGFR3-TACC3 and FGFR3-BAI1AP2L1 gene fusion products were sensitive HSP90 client proteins, as evidenced by the potent effects of 17-AAG and 17-DMAG on protein destabilization and cell viability in RT4 and SW780 cells, respectively. However, the lack of

### Table 2. Fold-changes in protein expression following ganetespib treatment in RT112 bladder cancer cells using reverse-phase protein array analysis

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<th>Cellular target</th>
<th>Protein</th>
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treatment cohort are presented in Fig. 2C. Ganetespib exposure resulted in the deactivation of endogenous FGFR3-TACC3 activity, as evidenced by the significant repression of p-FGFR3 levels and congruent repression of phosphorylated ERK and AKT by 24 hours. These effects were sustained over time with recovery occurring at 72 hours posttreatment. Similar kinetics were observed for signaling intermediates of the mTOR pathway (phosphorylated S6 ribosomal protein and PRAS40), consistent with what was observed following targeted HSP90 inhibition in vitro (Table 2). Overall, these data show that single-dose ganetespib exerts a potent and rapid destabilizing effect on the FGFR3-TACC3 fusion kinase and its effectors in RT112 xenografts.
client protein modulation and cytotoxic activity seen with ganetespib and AUY922 suggested that these two cell lines exhibited an intrinsic level of resistance to the resorcinol-based, second-generation HSP90i.

High expression of UGT1A enzyme expression in FGFR3 fusion-positive bladder cells results in glucuronidation and efflux of ganetespib

To dissect the mechanisms underlying the differential sensitivities to specific HSP90i exhibited by the FGFR3 fusion-positive cell lines, we first compared the intracellular concentrations of ganetespib and 17-DMAG in the “resorcinol-sensitive” RT112 and “resorcinol-resistant” SW780 cell lines as a function of time (Fig. 4A). In RT112 cells, both compounds accumulated in a dose-dependent manner 1 hour posttreatment, albeit with comparatively higher total ganetespib levels than 17-DMAG at each concentration tested. Moreover, the intracellular levels for each HSP90i seen after 1 hour appeared to approach saturation as no significant increases were observed at the 24-hour time point (Fig. 5A, left). In the SW780 cell line, the initial accumulations of ganetespib and 17-DMAG were indistinguishable, with each HSP90i readily entering and accumulating to near identical concentrations at 1 hour. By 24 hours, levels of 17-DMAG were maintained and modestly increased; in stark contrast virtually no ganetespib was detectable within the intracellular compartment of SW780 (Fig. 5A, right).

These data suggested that ganetespib resistance was not due to differences in HSP90 biology but likely attributable to drug efflux and/or metabolism mechanisms. Similar to AUY922 (22), ganetespib is susceptible to metabolism by the UGT1A family of UDP-glucuronosyltransferase enzymes that transform small lipophilic molecules into water-soluble, excretable metabolites (23). Therefore, the intracellular and secreted levels of ganetespib and its glucuronidated metabolites were measured in RT112 and SW780 cells as a function of time (Fig. 5B). No glucuronidated form of ganetespib was detectable within the intracellular compartment of SW780 (Fig. 5A, right).

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that ganetespib was being rapidly metabolized in these cells. In agreement with the data shown in Fig. 5A, intracellular SW780 ganetespib concentrations decreased to undetectable levels by 24 hours; this was concomitant with a marked accumulation of metabolite secretion into the culture media with time (Fig. 5B, right).

When overall UGT1A protein expression was evaluated in the three FGFR3 fusion-positive bladder lines it was found that both SW780 and RT4 cells expressed considerably higher endogenous enzyme levels compared with RT112 (Fig. 5C). Furthermore, UGT1A protein expression was virtually undetectable in 97-7 bladder cancer cells, which were also sensitive to ganetespib exposure (Supplementary Fig. S1). We subsequently performed a comparative PCR analysis assessing relative mRNA levels of individual UGT1A family enzymes to examine differences in metabolism-related gene expression between the RT112 and SW780 cell lines (Fig. 5D). From this screen, it was found...
that SW780 cells showed significantly higher basal UGT1A family expression compared with the RT112 line, most notably the UGT1A7, UGT1A8, UGT1A9, and UGT1A10 isoforms, which represent a cluster of genes encoding highly homologous enzymes within the UGT1A subfamily (24). Of note, we have previously identified UGT1A9 as the primary UGT enzyme responsible for ganetespib glucuronidation in liver microsome assays (data not shown). To confirm the PCR findings, UGT1A9 and 1A10 protein expression were individually evaluated in the panel of lines, with UGT1A9- and UGT1A10-transfected 293T cells serving as a positive control (Fig. 5E). As predicted, the SW780 and RT4 cell lines expressed considerably higher endogenous levels of both enzymes compared with RT112 cells. These data indicated that of the three FGFR3 fusion-positive lines, SW780 and RT4 cells had a higher intrinsic propensity to metabolize ganetespib than RT112, and suggested that this phenotypic variation contributed to the differential sensitivities seen following drug exposure. Taken together, our findings support the premise that high endogenous UGT1A enzyme expression in SW780 cells promoted the rapid glucuronidation and subsequent efflux of ganetespib, sufficient to account for its lack of bioactivity in this line.
Targeted reductions in UGT1A activity by RNA interference or competitive inhibition sensitize SW780 bladder cells to ganetespib

To confirm this putative mechanism of action, we performed a series of reversion experiments in SW780 bladder cells. In the first, UGT expression was modulated by transfecting SW780 cells with a non-target (control) or UGT1A7, 8, 9, 10 siRNA probe for 72 hours. HSP90 inhibitory activity was then compared with control and UGT1A-deficient SW780 cells exposed to increasing concentrations of ganetespib for a further 24-hour period (Fig. 6A). Immunoblotting revealed that targeted RNA interference effectively resulted in the complete abrogation of UGT1A9 and UGT1A10 protein expression. Upregulation of HSP70 was used as a surrogate marker of HSP90 inhibition (25) and effects on two well-established client proteins, EGFR and cyclin-dependent kinase 1 (CDK1), were assayed. Control cells were largely insensitive to the effects of ganetespib exposure, reflecting the phenotype of the parental SW780 line. In stark contrast, UGT1A9/1A10-deficient SW780 cells showed marked increases in HSP70 expression, alongside dose-dependent destabilization of both EGFR and CDK1. Moreover, ganetespib treatment at the highest dose resulted in the induction of apoptosis, as evidenced by cleaved PARP expression. Taken together, these data showed that siRNA knockdown of UGT1A9 and 1A10 expression could overcome intrinsic ganetespib resistance in SW780 bladder cells.

Reductions in glucuronidation activity were also assessed by treating SW780 cells with ganetespib in the presence or absence of propofol, a competitive inhibitor of UGT1A9 activity (26). As expected, ganetespib treatment alone had only minor effects on HSP70 induction and client protein stability; propofol exposure (100 and 300 μmol/L concentrations) exhibited no discernable effects on HSP90 activity (Fig. 6B). However, in the presence of propofol, SW780 cells were sensitized to ganetespib treatment as shown by robust HSP70 upregulation, more complete degradation of both client proteins, and marked elevations in cleaved PARP levels. Together with the siRNA data, these observations provide further support for glucuronidation-based metabolism as representing the primary mechanism of ganetespib resistance in this bladder model.

Finally, we reasoned that the differences in metabolic profiles exhibited by the FGFR3 fusion–positive bladder cancer lines may also confer differential sensitivity to a wider variety of therapeutic agents, in addition to HSP90i. To examine this possibility, we interrogated the Genomics of Drug Sensitivity in Cancer Project database (http://www.cancerrxgene.org). Clinically validated, targeted compounds that showed 3-fold or higher changes in IC50 cytotoxicity values for SW780 cells compared with the RT112 line are listed in Supplementary Table S1. In agreement with the findings shown in Fig. 4A, AUY922 was identified in the screen, as was another resorcinol-based HSP90i CCT018159. In general terms, targeted agents that exhibited the highest potential for phase II glucuronidation metabolism were associated with higher IC50 ratios, indicating that SW780 cells were far less sensitive to drug treatment than RT112. Overall, these data suggest that metabolism-based resistance linked to UGT1A expression may represent a more general consideration for the evaluation of variety of anticancer compounds in bladder tumors.

Discussion

FGFR3 has long been considered an attractive therapeutic target in bladder cancer based on its high frequency of dysregulated activity, particularly through mutation and/or overexpression (11). Importantly, FGFR3 has been implicated to play a key oncogenic role in both noninvasive and invasive disease (7). The recent discovery of genomic FGFR3 rearrangements in a subset of bladder tumors provides another pathogenic mechanism for aberrant FGFR3 activation in this disease and one with particular relevance for the identification of patients who may ultimately benefit from...
targeted FGFR inhibition (9). The fusion gene products generated by rearrangements of FGFR3 with either TACC3 or BAI1AP2L1 are tumorigenic and create dependency in cell lines harboring such alterations (9, 10). Moreover, transforming fusions of FGFR and TACC genes have also been found in glioblastoma (27), suggesting that translocation may represent a more universal mechanism of FGFR dysregulation that is conserved across tumor types. Of note, constitutively active fusion gene products are well characterized as critical driver proteins in other cancer types, such as EML4-ALK in non–small cell lung cancer and BCR-ABL in chronic myeloid leukemia (28–30). Indeed, the clinical successes of agents directed against such proteins (e.g., crizotinib for EML4-ALK and imatinib for BCR-ABL) have served as paradigms for the design and application of molecularly targeted therapy in cancer. A number of small molecules with activity against FGFRs are currently under development, the majority of which are multikinase inhibitors originally designed as antiangiogenic agents against other growth factor receptors (31). Recently, more selective and potent pan-FGFR inhibitors, including BGJ398 (20), have entered early-stage clinical evaluation in bladder and other tumor types (31).

In this report, we show that bladder cancer cell lines harboring FGFR3-TACC3 and FGFR3-BAI1AP2L1 gene rearrangements are sensitive to HSP90 inhibition. Previously, 17-AAG has been reported to exert in vitro cytotoxic activity in RT112 and RT4 cells (21); however, the presence and significance of the FGFR3-TACC3 product expressed in these lines were not recognized in that study. Here we provide the first demonstration that FGFR3 fusion proteins expressed by bladder cancer cells are reliant on the chaperoning activity of the HSP90 molecule for their stability and function, as evidenced by their pronounced destabilization following HSP90i treatment (in an inhibitor-dependent context), ultimately leading to loss of tumor cell viability. While this finding alone does not unequivocally identify FGFR3 fusions as direct HSP90 substrates, it is important to note that the FGFR3 kinase itself is a bona fide HSP90 client protein (32, 33). Indeed, within the FGFR family of receptor tyrosine kinases there is a hierarchical degree of destabilization induced by selective HSP90 inhibitors, with FGFR3 representing the member most sensitive to chaperone inhibition (33). In RT112 cells, ganetespib exposure resulted in the rapid and potent degradation of FGFR3-TACC3 protein expression, loss of downstream effector signaling, and subsequent induction of apoptosis. HSP90 blockade was as effective as direct kinase inhibition afforded by BGJ398 treatment and, importantly, the in vitro antitumor activity of both compounds was recapitulated in vivo. BGJ398 administration induced tumor regressions in FGFR3-TACC3–driven RT112 xenografts, a response that was both consistent with, and superior to, what has been observed with the small-molecule FGFR kinase inhibitor PD173074 in RT4 and SW780 models (10). A weekly dosing schedule of ganetespib was equally efficacious as the BGJ398 regimen. We extended these findings by combining the two modalities of HSP90 inhibition and selective FGFR3 targeting in RT112 tumors, which resulted in improved efficacy over that achieved with either agent alone. Together with the broader spectrum of biologic activity conferred by ganetespib exposure, these data strongly suggest that HSP90 inhibition offers an alternative and potentially complementary strategy to selective kinase inhibition in FGFR3–driven bladder cancer.

An interesting feature of FGFR fusion–positive tumor lines is their enhanced susceptibility to pharmacologic inhibition by selective FGFR kinase inhibitors over activating point mutations (10). In this regard, ganetespib retained full potency against two BGJ398-resistant bladder cell lines, 97-7 and MGH-U3, which bear mutant FGFR3S249C and FGFR3Y357C receptors, respectively. Furthermore, ganetespib was also highly potent in reducing cell viability in a number of bladder cell lines with wild-type FGFR3 status, highlighting the therapeutic potential of pharmacologic HSP90 blockade for affecting this validated and actionable disease target. Clinically, the field of FGFR-targeted therapy is still in its infancy and the translational benefit of any specific agent is yet to be realized. However, a recurring theme in the clinical experience with many targeted kinase inhibitors, including imatinib and crizotinib, is that their long-term efficacy is often hampered by the invariable development of acquired resistance, commonly arising due to the acquisition of secondary mutations in their respective kinase targets (34, 35). We and others have previously shown that HSP90 inhibition is an effective approach for overcoming oncogenic EML4-ALK activity in non–small cell lung cancer (18, 36, 37). Significantly, ganetespib also displayed potent activity against a variety of acquired ALK mutations that confer crizotinib resistance in multiple experimental models and in the clinical setting (18). In light of the broader activity profile of ganetespib in affecting both activating mutations and translocations of FGFR3, it is reasonable to suggest that HSP90 blockade may warrant further investigation as a means to overcome acquired resistance should similar mechanisms arise that allow escape from targeted FGFR agents.

A significant finding of this study was the differential sensitivities exhibited by the fusion–positive bladder lines to alternate classes of HSP90 inhibitors. In particular, cell fate following HSP90i exposure in RT4 and SW780 cells varied according to the chemical class of inhibitor used. Both of the first-generation ansamycin inhibitors 17-AAG and 17-DMAG potently induced cell death, and loss of viability was associated with destabilization of the individual FGFR3 fusions expressed by each line. However, and in contrast to RT112 cells, both lines were largely resistant to ganetespib treatment or exposure to AUY922, a second resorcinol-based HSP90i. Mechanistically, the rapid metabolism (via glucuronidation) and subsequent efflux of ganetespib from SW780 cells was sufficient to account for the lack of HSP90 inhibitory activity. This was validated by inhibiting the high endogenous levels of UGT1A9 and UGT1A10 expression in SW780 by RNA interference, as well as competitive inhibition of the UGT1A9 isoform by propofol. Both experimental strategies overcame the intrinsically resistant
phenotype of this bladder line, and conferred sensitivity to ganetesib exposure. This is the first demonstration of ganetesib metabolism as a contributing factor to drug resistance and thus has clear implications for the clinical application of the compound. Overall, response to the second-generation resorcinol inhibitors appeared intimately linked to the endogenous level of UGT1A family enzyme expression, the primary catalysts of glucuronidation reactions in multiple human tissues (23). Interestingly, the UGT1A gene cluster has been identified as a novel susceptibility locus for bladder cancer using genome-wide association studies (38). Furthermore, experimental evidence has shown that UGT1A expression becomes downregulated during bladder cancer progression in animal models (39), consistent with the hypothesis that UGT variants mapping to this region may provide a protective, tumor-suppressive function in bladder cells (40). FGFR3 fusion protein expression is likely to be an important biomarker to aid in patient selection when evaluating novel bladder cancer treatments, due to an increased susceptibility of fusion-bearing tumor cells to targeted FGFR therapies (9). On the basis of the findings presented here, UGT1A expression in bladder tumors may represent an additional marker with direct translational relevance for the clinical evaluation of HSP90 inhibitor-based strategies. Moreover, we have observed high levels of UGT1A in subsets of patients with lung cancer (D. Proia, unpublished observations). Combining these results with patient responses in ongoing clinical trials of ganetesib may be informative as to the potential use of UGT1A as a predictive biomarker for future studies.

In summary, this study provides preclinical proof-of-concept that targeting FGFR3 gene fusions via selective HSP90 inhibition represents a rational therapeutic strategy for bladder tumors oncogenically driven by this mechanism of aberrant receptor activation. On the basis of select advantages of this approach over FGFR-specific kinase inhibitors, these findings are likely to provide a framework for the development of new targeted agents and biomarkers designed to improve therapeutic outcomes for patients with bladder cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the other authors.

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References


FGFR3 Translocations in Bladder Cancer: Differential Sensitivity to HSP90 Inhibition Based on Drug Metabolism

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