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Spotlight on Clinical Response

Activity of dasatinib against *L576P KIT* mutant melanoma: Molecular, cellular, and clinical correlates

Scott E. Woodman,[1,6] Jonathan C. Trent,[2] Katherine Stemke-Hale,[3,6] Alexander J. Lazar,[4] Sabrina Pricl,[7] Giovanni M. Pavan,[7] Maurizio Fermeglia,[7] Y.N. Vashisht Gopal,[6] Dan Yang,[2] Donald A. Podoloff,[5] Doina Ivan,[4] Kevin B. Kim,[1] Nicholas Papadopoulos,[1] Patrick Hwu,[1] Gordon B. Mills,[3,6] and Michael A. Davies[1,3,6]

Departments of [1]Melanoma Medical Oncology, [2]Sarcoma Medical Oncology, [3]Systems Biology, [4]Pathology, and [5]Radiology, [6]The Kleberg Center for Molecular Markers, M. D. Anderson Cancer Center, Houston, Texas; and [7]Molecular Simulation Engineering Laboratory, DICAMP, University of Trieste, Trieste, Italy

Abstract

Point mutations in the *KIT* receptor tyrosine kinase gene have recently been identified in mucosal, acral lentiginous, and chronically sun-damaged melanomas. We have identified the first human melanoma cell line with an endogenous L576P mutation, the most common *KIT* mutation in melanoma (30-40%). *In vitro* testing showed that the cell viability of the L576P mutant cell line was not reduced by imatinib, nilotinib, or sorafenib small molecule KIT inhibitors effective in nonmelanoma cells with other *KIT* mutations. However, the viability of the mutant cells was reduced by dasatinib at concentrations as low as 10 nM ($P = 0.004$). Molecular modeling studies found that the L576P mutation induces structural changes in KIT that reduce the affinity for imatinib ($\Delta\Delta G_{bind} = -2.52$ kcal/mol) but not for dasatinib ($\Delta\Delta G_{bind} = +0.32$ kcal/mol). Two metastatic melanoma patients with the L576P *KIT* mutation were treated with dasatinib, including one patient previously treated with imatinib. Both patients had marked reduction (>50%) and elimination of tumor F18-fluorodeoxyglucose (FDG)-avidity by positron emission tomography (PET) imaging after dasatinib treatment. These data support the selective inhibitory effect of dasatinib against cells harboring the most common *KIT* mutation in melanoma, and thus has therapeutic implications for acrallentiginous, chronic sun-damaged, and mucosal melanomas. [Mol Cancer Ther 2009;8(8):2079–85]

Introduction

Patients with metastatic melanoma have a median survival of 6 to 8 months (1). Unfortunately multiple clinical trials with chemotherapy, immunotherapy, and biochemotherapy have failed to significantly improve survival. Protein kinase inhibitors are beneficial in diseases with highly prevalent oncogenic events (e.g., CML, GIST), and within selected subpopulations (e.g., HER2-amplified breast cancer; ref. 2). More than 50% of melanomas arising from areas without chronic sun damage harbor activating mutations of BRAF (3). However, BRAF mutations are extremely rare in other melanoma subtypes: acral lentiginous (AL), chronic sun-damaged (CSD), and mucosal (4). Interestingly, amplification of chromosomal region 4q12 was seen frequently and selectively in AL, CSD, and mucosal melanomas. Interrogation of candidate genes in this region led to the discovery of frequent mutations and or amplifications of the *KIT* tyrosine kinase receptor gene in these subtypes (5).

The identification of *KIT* mutations in melanoma has direct therapeutic implications. Activating *KIT* mutations are present in about 85% to 90% of gastrointestinal stromal tumors (GIST; ref. 6). Treatment with the KIT inhibitor imatinib significantly improved survival in GIST patients, and it is now the standard of care for this disease (7). Three previous clinical trials using imatinib in unselected melanoma patients failed to show clinical benefit (8–10). However, it is possible that KIT inhibitors will be beneficial to the subset of melanoma patients with *KIT* mutations. Recent case reports have described clinical responses following treatment

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Requests for reprints: Michael A. Davies, Department of Melanoma Medical Oncology and Department of Systems Biology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 904-953-7283; Fax: 904-953-1412. E-mail: mdavies@mdanderson.org

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with imatinib in two patients with metastatic mucosal melanoma with *KIT* K642E and *KIT* 577 PYDHKWE duplication mutations, respectively (11, 12). We reported a complete response in a metastatic mucosal melanoma patient with a *KIT* V560D mutation who was treated with a sorafenib-based regimen, which also inhibits KIT (13).

Although these early responses are encouraging, there are characteristics of *KIT* mutations in melanoma that suggest imatinib resistance may be an issue in the treatment of these patients. In GIST, the majority (~80%) of *KIT* mutations occur in the juxtamembrane regulatory domain encoded by exon 11. Most of these mutations have been characterized both *in vitro* and clinically as being imatinib-sensitive. In contrast, imatinib resistant mutations occur in exons 13 and 17, the kinase domains of the protein. These mutations are rare in GIST (exon 13, <1%; exon 17, 1%). The *KIT* mutations identified in melanomas occur in the same exons as those affected in GIST. However, there is a greater prevalence of mutations in exon 13 (20%) and exon 17 (10%; refs. 5, 11–19). Additionally, in GIST >90% of the observed mutations are deletions or insertions, whereas >90% of the *KIT* mutations in melanoma are point mutations. The L576P *KIT* mutation, which is the most common *KIT* mutation reported to date in melanoma (~30-40% of mutations), is located at the C-terminus of *KIT* exon 11, although KIT exon 11 deletions in GIST occur mostly at the N-terminus of *KIT* exon 11 (5). These differences in the type and localization of *KIT* mutations may impact drug efficacy.

Here we report the identification and characterization of the first human melanoma cell line (WM3211) with a L576P *KIT* mutation. The L576P mutation is the most common KIT mutation identified to date in melanoma, and represents approximately 30% to 40% of the reported point mutations. Despite the location of the L576P mutation in exon 11, which is generally associated with imatinib sensitivity in GIST, we observed that the WM3211 cells were markedly resistant to the growth inhibitory effects of imatinib. In contrast, the cell line was sensitive to dasatinib, a structurally distinct inhibitor of KIT. We also report here the results of molecular modeling studies to investigate the structural effects of the mutation on the interaction with KIT inhibitors, and describe the treatment of two metastatic mucosal melanoma patients with this mutation.

Materials and Methods

Cell Culture and Reagents

The WM3211 human melanoma cell line was provided by Dr. Meenhard Herlyn. A375 and MEWO human melanoma cell lines were obtained from American Type Culture Collection. All cells were maintained in RPMI media with 5% FCS. The imatinib, dasatinib, nilotinib, and sorafenib were purchased from Eurasias Chemicals.

Mutation Detection

A mass spectroscopy-based approach evaluating single nucleotide polymorphisms (SNPs; ref. 20) was used to detect *KIT* mutations (A829P, D816H, D816V, K642E, L576P, N556D, R634W, V559A, V559D, V560D, V825A, Y553N). PCR and extension primers for *KIT* were designed using Sequenom, Inc. Assay Design (Sequenom, San Diego, CA). PCR-amplified DNA was cleaned using EXO-SAP (Sequenom), and primer was extended by IPLEX chemistry, desalted using Clean Resin (Sequenom), and spotted onto Spectrochip matrix chips using a nanodispenser (Samsung). Chips were run in duplicate on a Sequenom MassArray MALDI-TOF MassArray system. Sequenom Typer Software and visual inspection were used to interpret mass spectra. Reactions in which >15% of the resultant mass ran in the mutant site in both reactions were scored as positive. Traditional Sanger sequencing of exons 11, 13, and 17 of *KIT* was done as previously described (6). Genomic DNA samples were isolated from paraffin-embedded or frozen tissue, polymerase chain reaction was done, and mutations were identified by a 3730 9 1 DNA Analyzer (Applied Biosystems, Foster City, CA) at the MDACC Nucleic Acid Core Facility.

In vitro Inhibitor Testing

Cells were plated in 96-well plates and treated the following day with increasing concentrations of imatinib, nilotinib, sorafenib, dasatinib, (0-1 μM doses), or equamolar dimethyl sulfoxide (DMSO) in triplicate. After 48 hours, cell viability was determined by the Cell Titer-Blue Cell Viability Assay (Promega, Madison, WI) per the manufacturer's instructions. The relative growth of each cell line for each treatment was determined relative to mock treated control. DMSO vehicle at equamolar concentrations had no significant effect on cell viability in all lines. Calculations and graphs were made using Microsoft Excel. Statistical comparison was made using Student's *t* test.

^{18}FDG PET Imaging

Patients underwent ^{18}FDG PET imaging as described previously (21). Images were interpreted using volumetric and multiple orthogonal projection analysis then quantified using vendor specific software. SUVmax was measured from a region(s) of interest (ROI) representative of tumor on pretreatment images and corresponding ROIs on posttreatment images. Images were reviewed and measured by an experienced radiologist (D.A.P.). PET response was defined as >25% decrease in SUVmax (22).

Molecular Dynamics Simulations

Molecular dynamics (MD) simulations were carried out using the AMBER 9 (*sander* and *pmemd* module) suite of programs and the parm99 all-atom force field working in parallel on 64 processors of the IBM/BCX calculation cluster of the CINECA calculation center of Bologna, as described previously (23, 24). Briefly, starting geometries for both wild-type and mutant simulations used the crystallographic coordinates of the active KIT structure in complex with imatinib. The crystallographic structure of the complex was then modified by substituting imatinib with dasatinib. The binding free energy (ΔG_{bind}) for each inhibitor is calculated using the molecular mechanics-Poisson-Boltzmann surface area method as the sum of the electrostatic, van der Waals, polar solvation, nonpolar solvation, and entopic contributions (See Supplementary Methods).

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Results

Identification and Characterization of L576P KIT Mutant Cell Line

Mass spectroscopy-based genotyping was used to screen a panel of 65 human melanoma cell lines for point mutations in *KIT* previously reported in melanoma and other diseases (Supplementary Table 1). We identified one cell line, WM3211, that harbors a point mutation at nucleic acid residue 1,727 in exon 11 of *KIT*, which results in the L576P amino acid substitution (Fig. 1A). Sanger DNA sequencing of *KIT* exons 11, 13, and 17 confirmed this finding, and failed to identify any other mutations (Fig. 1B). Additional analysis of the WM3211 cell line by mass spectroscopy-based genotyping failed to identify activating mutations in either *BRAF* or *NRAS*, which is consistent with the pattern of these mutations in melanoma (Supplemental Table 1).

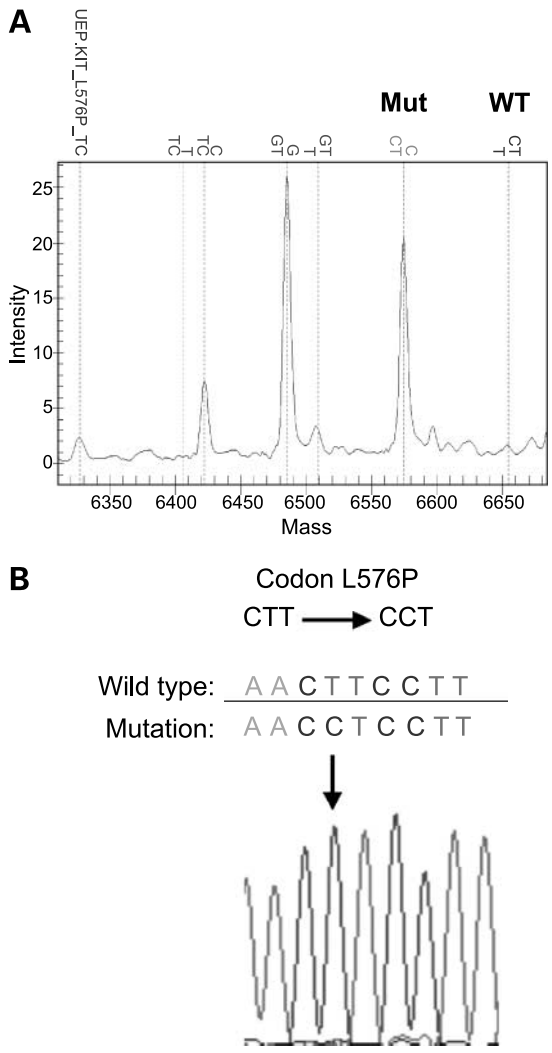

Figure 1. L576P KIT mutation in a melanoma cell line. **A,** mass spectroscopy-based detection of the L576P KIT mutant allele(s) in the WM3211 cell line. A peak is correlated with the L576P mutant KIT (Mut), whereas there is no peak for the wild-type KIT (WT). **B,** sanger sequencing of exon 11 of *KIT* in the WM3211 cells. Arrow, T-to-C base pair change.

Dasatinib, but not Imatinib, Nilotinib, or Sorafenib Inhibits Cell Viability in WM3211 Cells

The WM3211 cell line was tested for sensitivity to a panel of U.S. Food and Drug Administration (FDA)-approved, clinically utilized small molecule KIT inhibitors. The effect on cell viability in WM3211 cells was compared with effects on two human melanoma cell lines that have no mutations in *KIT*, A375 (V600E mutant BRAF, wild-type NRAS) and Mewo (wild-type BRAF and NRAS). Surprisingly, imatinib failed to inhibit the viability of WM3211 cells at concentrations up to 1 μM (<10% decrease compared with untreated cells) with similar results in the A375 and Mewo cell lines (Fig. 2A). WM3211 also showed no statistically significant ($P > 0.05$) difference in sensitivity to sorafenib and nilotinib versus the KIT wild-type melanoma cell lines (Fig. 2B and C). However, dasatinib, a structurally distinct small molecule inhibitor of KIT, reduced WM3211 cell viability ~25% ($P = 0.004$ versus vehicle) at 10 nM and ~50% ($P = 0.00001$) at 1 μM concentration (Fig. 2D). In contrast, minimal inhibition of viability of the A375 and Mewo cell lines (<15%, $P = 0.9$) was observed up to 1 μM concentration of dasatinib. Treatment of the T1 GIST cell line containing a heterozygous V560_Y579del in exon 11 confirmed that each of the inhibitors used for these experiments was active against a known sensitive *KIT* mutation (data are not shown).

Modeling the Effects of the L576P KIT Mutation

In order to investigate whether the differential sensitivity of imatinib and dasatinib observed in the WM3211 cell line may be due to altered KIT-binding affinity, molecular dynamic studies were done to determine the effects of the L576P mutation on the structure of KIT and its interactions with the inhibitors. The free energy of binding of both inhibitors to wild-type KIT was relatively similar ($\Delta G_{bind,}$ −10.28 kcal/mol for imatinib; $\Delta G_{bind,}$ −9.61 kcal/mol for dasatinib; Fig. 3A and C). Modeling of the L576P mutant KIT protein showed a structure that was similar to the active conformation of the molecule, which in previous studies has been associated with a diminished binding affinity for imatinib (25). Consistent with these data, imatinib had a less favorable free energy of binding for the L576P mutant form of KIT ($\Delta G_{bind,}$ −7.75 kcal/mol) as compared with the wild-type protein, whereas the free energy of binding of dasatinib was essentially unchanged ($\Delta G_{bind,}$ −9.93 kcal/mol) (Fig. 3C and D).

Treatment of Two Patients with L576P KIT Mutant Melanoma

Patient A is a 55-year-old woman who had resection of a labial mucosal melanoma. She received adjuvant interferon therapy for 1 year, but developed a recurrence in the inguinal lymph node bed, and underwent lymphadenectomy. Molecular analysis of a lymph node metastasis identified the presence of a L576P *KIT* mutation. The patient was then treated with adjuvant imatinib 400 mg daily, with a dose reduction to 300 mg daily after 3 months for periorbital edema. After 8 additional months of adjuvant imatinib therapy a positron emission tomography (PET)-computerized

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tomography (CT) showed a new F^{18}-fluorodeoxyglucose (FDG)-avid right internal iliac lymph node representing a recurrence of melanoma while on imatinib. The patient was subsequently treated with several standard and experimental regimens for metastatic melanoma, including CRO11-vcMMAE (AntiGPNMB antibody conjugated to auristatin E), high-dose bolus IL-2, sorafenib and temsirolimus, and combination chemotherapy with CVD (cisplatin, vinblastine, and dacarbazine). A repeat biopsy after progression on the sorafenib and temsirolimus regimen confirmed continued presence of the L576P *KIT* mutation, and failed to identify any secondary mutations in KIT. Based on the clinical activity of dasatinib in imatinib-resistant or -intolerant patients in other cancers, the decision was made to treat the patient off clinical protocol with dasatinib. After undergoing a pretreatment PET scan, the patient was started on dasatinib 70 mg twice daily. After one month, the patient reported a marked improvement in her symptoms, which primarily consisted of inguinal and or pelvic pain. Repeat PET/CT imaging showed an overall 53.5% reduction in average maximal standardized uptake value (SUVmax; Fig. 4A and B). Unfortunately, despite this early response and clinical benefit, the patient developed progressive disease at the same sites in the pelvis after 4 months of therapy. No secondary *KIT* mutations were identified in material obtained from a biopsy of the deep pelvic tumor.

Patient B is a 61-year-old woman with anal melanoma who received neoadjuvant radiation therapy (5 Gy) in five fractions followed by tumor resection. A PET/CT done just prior to radiation therapy did not show any evidence of metastatic disease. A follow-up PET/CT 4 months after surgery revealed two FDG-avid lesions in the right lobe of the liver. DNA sequencing of the original biopsy showed the presence of a L576P *KIT* mutation. After one month of dasatinib therapy the lesion in the inferior aspect of the right liver lobe (SUVmax = 8.5) could not be identified (Fig. 4C and D) and the lesion in the superior aspect of the right liver lobe was no longer well-circumscribed and showed a 54% reduction in SUVmax (SUVmax 11.1 → 5.1; Fig. 4E and F). The patient tolerated dasatinib therapy well, with the only complication being the development of asymptomatic pleural effusions.

The patient was maintained on dasatinib for 3 months, then treatment was stopped for the development of progressive disease at the same sites within the liver.

Discussion

More effective treatments are desperately needed for patients with metastatic melanoma. The recent discovery of *KIT* mutations in AL and mucosal melanoma tumors and those arising in CSD skin suggests that small molecule KIT inhibitors may have efficacy in these tumor types. The assumption of general imatinib-sensitivity in these tumors is largely based on the clinical experience in GIST, which is also characterized by *KIT* mutations. The data presented here suggest that the management of *KIT*-mutant melanoma may need to be further refined.

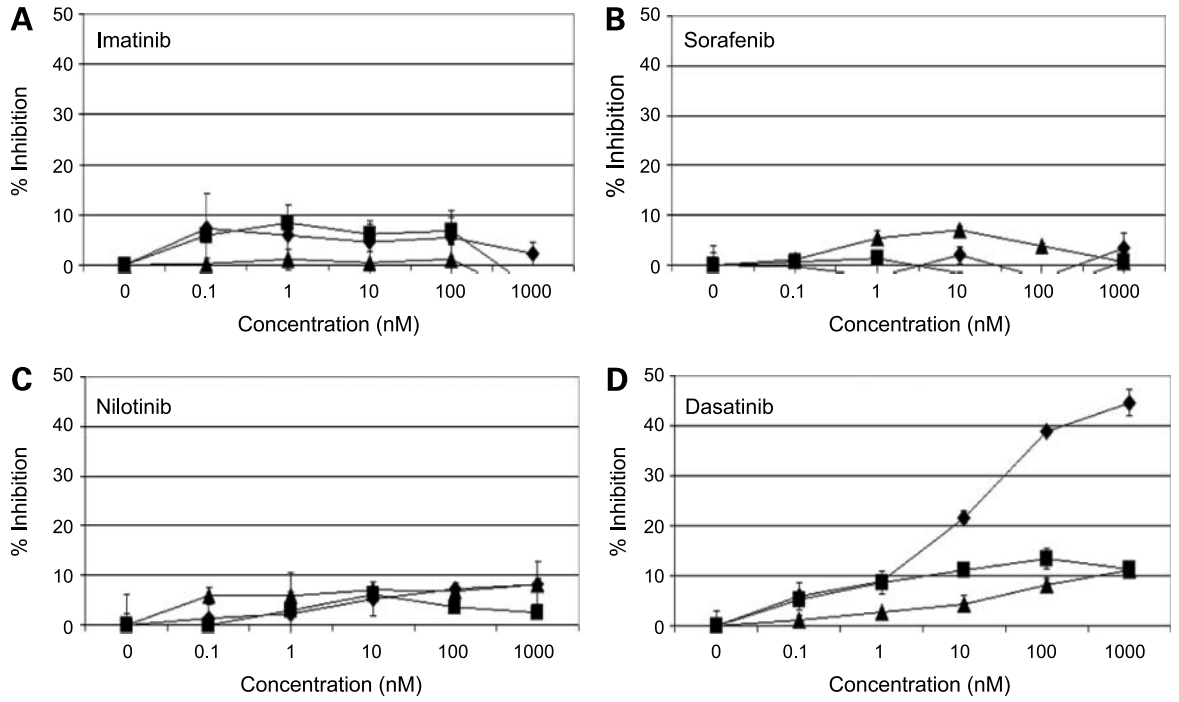

Figure 2. Effect of KIT inhibitors on WM3211 cell line viability. WM3211 (♦), MEWO (■), and A375 (▲) human melanoma cells were treated with increasing doses of the KIT inhibitors, **A,** imatinib, **B,** sorafenib, **C,** nilotinib, or **D,** dasatinib, *x*-axis, drug concentration (nM); *y*-axis, percent reduction in cell viability. Each data point represents the average of three replicates, error bars represent the standard deviation.

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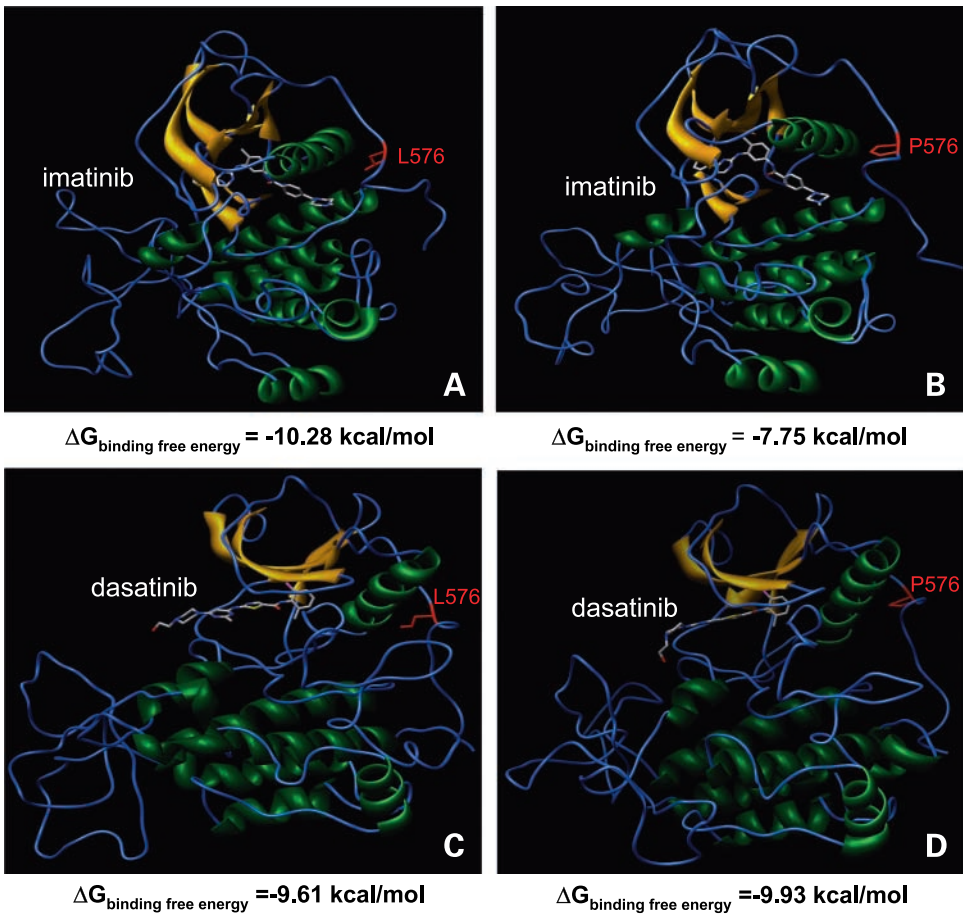

Figure 3. Effects of L576P KIT mutation on protein structure and drug binding. The interaction of imatinib with **A,** wild-type versus **B,** L576P KIT reveals a ΔΔGbind = −2.52 kcal/mol, reflecting a decrease in affinity induced by the mutation. The interaction of dasatinib with **C,** wild-type versus **D,** L576P KIT reveals a ΔΔGbind = +0.32 kcal/mol. The free binding energy for L576P KIT is more favorable for dasatinib (−9.93 kcal/mole) than for imatinib (−7.75 kcal/mole), indicating a greater affinity for dasatinib.

We have identified a human melanoma cell line (WM3211) with the L576P *KIT* mutation, the most common *KIT* mutation in melanoma. This mutation occurs in exon 11, a region in which the vast majority of mutations in GIST occur, and to date have been associated with imatinib sensitivity. Surprisingly, we found that the WM3211 cell line was resistant to the growth inhibitory effects of imatinib and to structurally similar inhibitors. The resistance to imatinib is at least in part due to conformational changes induced by the L576P substitution that reduces the affinity of imatinib for its binding pocket within the receptor. In contrast, the viability of WM3211 cells was reduced by nanomolar concentrations of dasatinib, a small-molecule inhibitor used in other diseases in the setting of imatinib-resistance and or -intolerance. Despite the similar binding energy for both the wild-type and L576P KIT, dasatinib had no significant effect on the viability of Mewo or A375 melanoma cell lines that express only the wild-type KIT protein. These findings are congruent with those of Buettner and colleagues, who showed that dasatinib had no effect on the cell viability of multiple melanoma cell lines (26). These data support the functional dependence upon dasatinib-sensitive signaling in this *KIT* mutant melanoma.

The differential effects of dasatinib and imatinib on melanoma cells with the L576P *KIT* mutation are consistent with the report of Antonescu and colleagues who showed that stable transfection of the L576P KIT protein into murine pro-B-cell Ba/F3 cells yielded clones that were more sensitive to dasatinib than imatinib (14). Ba/F3 cells exogenously expressing the V559D *KIT* mutation in this model showed no difference in sensitivity between dasatinib and imatinib, indicating the specificity of the effect of dasatinib on the L576P mutant. These results correspond with the recent observation that human melanoma cells harboring a *KIT* V559A mutation showed growth inhibition to imatinib (27).

Dasatinib is a multikinase inhibitor with targets in addition to KIT. It is possible that inhibition of these additional targets along with KIT may synergize to produce the growth suppression observed in the WM3211. This possibility is an ongoing area of research. However, we did not observe significant growth inhibition in the WM3211, or a differential effect as compared the wild-type KIT melanoma cell lines, following treatment with a small molecule inhibitor of Src family kinases (data are not shown), which are known targets of dasatinib. The critical nature of KIT as a target is also supported by the fact that in the work by Antonescu and colleagues dasatinib only inhibited the Ba/F3 cells that were transfected with the mutant KIT constructs.[8]

[8] C.R. Antonescu, personal communications.

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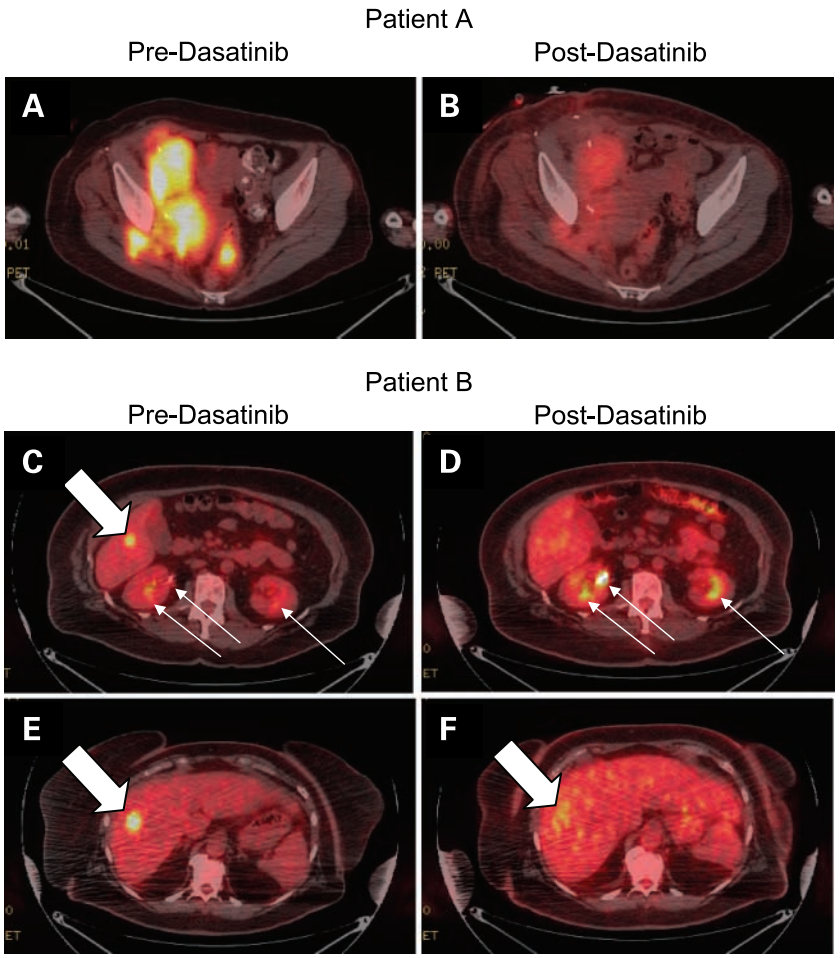

Figure 4. Treatment of L576P KIT mucosal melanoma patients with dasatinib. FDG-PET/CT images of Patient A, **A** and **B,** and Patient B, **C–F,** metastatic mucosal melanoma patients, with L576P KIT mutant tumors. Images were obtained before, **A, C, E,** and after, **B, D, F,** treatment with 70 mg twice daily of dasatinib for 1 mo. Solid block white arrows indicate FDG-avid tumors; narrow white arrows indicate physiologic FDG excretion in renal collecting system.

We did not see a complete elimination of cell viability of the WM3211 with dasatinib treatment. It is possible that dasatinib-induced cell death is cell-cycle specific and thus only kills a fraction of the melanoma cells while having a cytostatic effect on cells in other phases of the cell cycle. Alternatively, the WM3211 cell line may be composed of a heterogeneous collection of cells with differential sensitivity to dasatinib. Combinatorial approaches may be required to achieve optimal results in patients with KIT mutations. Consistent with this, both metastatic melanoma patients with L576P KIT mutations treated with dasatinib showed initial marked reduction in PET positivity, but had reemergence of metabolic activity and tumor growth after ~4 months of treatment. The identification of a melanoma cell line with the most common *KIT* mutation in this disease presents a powerful tool for future investigations.

Patient A had a melanoma recurrence in the setting of adjuvant imatinib therapy without evidence of a secondary mutation in *KIT*. Given the infrequency of *KIT* L576P mutations in GIST (<1% of cases), there are few reported cases of treatment with imatinib, and none with dasatinib, for GIST patients with this mutation. Of the cases reported, stable disease or partial response was achievable, but often at higher doses of imatinib (28–30). As the experience with treating *KIT*-mutant melanoma is in its infancy, it will be critical to treat patients in the setting of clinical trials, with documented evaluation of the responsiveness of different mutations allowing for the optimization of patient management in the future.

In summary, we have identified the first melanoma cell line that has a naturally occurring L576P *KIT* mutation, the most common *KIT* mutation observed in melanoma. Our *in vitro* and clinical data show that melanoma cells with this mutation are sensitive to dasatinib, but they are relatively resistant to imatinib. Our results also suggest that resistance to therapy with KIT inhibitors is likely to be an important issue in melanoma. These data have implications for the development of therapeutic strategies for AL, CSD, and mucosal melanoma patients. The results also support previous data that dasatinib may have selective activity in specific subsets of melanoma patients, which should be investigated in the setting of ongoing clinical trials with this agent. Importantly, this work represents an example of the application of bench-to-bedside research to develop new therapeutic approaches. We believe that similar interrogations of the functional alterations that are identified in this disease will be the key to improving outcomes in patients.

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Disclosure of Potential Conflicts of Interest

A.J. Lazar: honoraria from Novartis Speakers Bureau. No other potential conflicts of interest were disclosed.

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