Dasatinib, a small molecule inhibitor of the Src kinase, reduces the growth and activates apoptosis in pre-neoplastic Barrett’s esophagus cell lines: Evidence for a noninvasive treatment of high-grade dysplasia

Landon J. Inge, PhD, a Aaron J. Fowler, BA, a Kimberly M. Paquette, MS, b Amanda L. Richer, BS, a Nhan Tran, PhD, b and Ross M. Bremner, MD, PhD a

Background: Only local ablation (radiofrequency ablation, cryotherapy) or esophagectomy currently is available to treat high-grade dysplasia in Barrett’s esophagus. Alternative treatments, specifically chemopreventive strategies, are lacking. Our understanding of the molecular changes of high-grade dysplasia in Barrett’s esophagus offers an opportunity to inhibit neoplastic progression of high-grade dysplasia in Barrett’s esophagus. Increased activity of the Src kinase and deregulation of the tumor suppressor p27 are features of malignant cells and high-grade dysplasia in Barrett’s esophagus. Src phosphorylates p27, inhibiting its regulatory function and increasing cell growth and proliferation. We hypothesized that a small molecule inhibitor of Src might reduce the growth and reverse Src-mediated deregulation of p27 in Barrett’s esophagus cells.

Methods: Immortalized Barrett’s esophagus cell lines established from patient biopsies were treated with the Src kinase inhibitor dasatinib and evaluated for p27 localization and protein levels, as well as for effects on the cell cycle and apoptosis using flow cytometry, viability assays, and protein and RNA markers.

Results: Dasatinib reduced both Src activation and p27 phosphorylation and increased p27 protein levels and nuclear localization. These effects correlated with decreased proliferation, cell-cycle arrest, and activation of apoptosis. Analysis of biopsies of patients with Barrett’s esophagus revealed the presence of phosphorylated p27 in high-grade dysplasia, consistent with in vitro findings.

Conclusions: Dasatinib has considerable antineoplastic effects on Barrett’s esophagus cell lines carrying genetic markers associated with dysplasia, which correlates with the reversal of p27 deregulation. These findings suggest that dasatinib has potential as a treatment for patients with high-grade dysplasia and Barrett’s esophagus and that p27 holds promise as a biomarker in the clinical use of dasatinib in patients with high-grade dysplasia and Barrett’s esophagus. (J Thorac Cardiovasc Surg 2013;145:531-8)

Barrett’s esophagus (BE) is the primary risk factor for esophageal adenocarcinoma, one of the fastest growing malignancies in the Western world. 1,2 Progression to early adenocarcinoma in nondysplastic BE is low (0.5%-1% per year). 1 However, this risk increases to 7.4% per year in BE with high-grade dysplasia (HGD), with invasive cancer developing in 5 years in 50% of patients with HGD-BE. 3,4 This increased risk has led to the current treatment paradigm for BE, specifically, annual or biannual endoscopic surveillance for HGD and removal of HGD lesions by endoscopic resection, radiofrequency/cryoaulation, or esophagectomy. 5 The invasive nature of these procedures has precipitated investigations into chemopreventive targets and agents as alternatives to noninvasively treat or control progression of HGD-BE. 6,7

The Src family of kinases (cSrc, Lyn, Fyn, Yes, Lck, Blk, Hck) is a group of protein tyrosine kinases that function in the regulation of growth in eukaryotic cells. 8 Activity of Src kinases is regulated by a variety of stimuli, and increased cSrc (or Src) kinase activity is a feature of several solid tumor types, 8 including esophageal adenocarcinoma. Increased activity and expression of Src are also features of HGD-BE. 9-12 Src targets a diverse number of proteins within the cell, one of which is the tumor suppressor p27. Responsible for regulation of the cell cycle, p27 has been found to be tyrosine phosphorylated by Src at 2 discrete sites (tyrosine 74 and 88), 13 leading to increased p27 proteolysis, reduced p27 inhibitory binding of cyclin E-Cdk2, impaired p27 inhibition of cyclin D-cdk4 kinase activity, and correlation with p27 cytoplasmic localization...
in malignant tissues.\textsuperscript{13,14} The deregulation of p27 by Src results in increased cell proliferation. Small molecule inhibition of Src reverses these effects, restoring p27’s ability to regulate the cell cycle and reduce growth.\textsuperscript{13} A retrospective analysis of samples from patients with HGD-BE has revealed increased p27 cytoplasmic localization (48\% of samples),\textsuperscript{12} suggesting involvement of p27 deregulation in the progression of BE. On the basis of these previous observations in HGD-BE, we hypothesized that Src inhibition may have value in controlling HGD-BE and that Src alters the function of p27 in HGD-BE cells. To test this hypothesis, we investigated the effects of a small molecule inhibitor of the Src kinase (dasatinib) on the growth and regulation of p27 in immortalized BE cell lines.

**MATERIALS AND METHODS**

**Cell Lines and Patient Tissue**

The human Barrett’s esophageal metaplastic (CP-A) and high-grade dysplastic (CP-D) human cell lines were obtained from American Type Culture Collection (Manassas, Va), maintained in recommended media and grown and cultured as previously described.\textsuperscript{15} Both cell lines harbor characteristic BE chromosomal abnormalities, increased ploidy, and p16/CDKN2 inactivation, but differ in p53 status (CP-A-p53 wt, CP-D p53 mutant). Although the CP-A cell line was established from a patient with metaplasia, a recent report has suggested caution in interpreting these cells as comparable to metaplasia because they have characteristics inconsistent with metaplastic BE.\textsuperscript{16} Patient tissues were obtained under a protocol approved by the institutional review board and managed by the Human Specimen Procurement Service (St Joseph’s Hospital and Medical Center, Phoenix, Ariz).

**Drug Treatment**

Dasatinib (Selleck Chemicals, Houston, Tex) was dissolved in dimethyl sulfoxide (DMSO) to appropriate working concentrations with final DMSO less than 1\%. All controls were treated with an equivalent volume of DMSO diluted in culture media.

**Biochemical Analysis**

Total protein lysates from treated and untreated cells were isolated as described previously.\textsuperscript{17} Sample protein, 25 to 40 \(\mu\)g, was separated on 12\% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and subsequently transferred to nitrocellulose at 4\(^{\circ}\)C overnight. Immunoblotting was performed and developed as described previously.\textsuperscript{17} Antibodies specific to p-p27-S10, p-p27-T198, and p-p27-T157 were obtained from Abcam (Abcam, Cambridge, Mass). The p-p27-T187 antibody was acquired from LifeSpan Biosciences (LifeSpan Biosciences, Inc, Seattle, Wash). Antibodies to p27/Kip1, Src, phosphorylated Src (Y416), cyclin D, cyclin E, and cleaved Parp were obtained from Cell Signaling (Cell Signaling Technology, Boston, Mass).

**Quantitative Reverse Transcriptase Polymerase Chain Reaction**

Total RNA isolated with the RNeasy Mini Kit (QIAGEN Inc, Valencia, Calif) was used for first-strand cDNA synthesis using SuperScript III (Invitrogen, Carlsbad, Calif) and oligo(dT) (Invitrogen). Custom designed gene-specific primers (Invitrogen) were suspended to 10 \(\mu\)mol/L, combined with LightCycler 480 SYBR Green I Master (Roche Applied Science, Indianapolis, Ind) and 2 \(\mu\)L of the cDNA reaction. The samples were analyzed in triplicate on the LightCycler 480 (Roche Applied Science) and normalized to GAPDH. Fold change analysis was performed as previously described.\textsuperscript{18}

**Cell Viability**

A total of 250 cells per well were plated on a 96-well plate with 8 replicates per treatment dose and allowed to attach overnight. Cells were treated with the appropriate concentration of dasatinib diluted in cell culture media for 24 hours. Viability was assessed with the CellTitier Glo Luminescent Cell Viability Assay (Promega Corp, Madison, Wis) according to the manufacturer’s instructions. Luminescence was read on the DTX880 Multimode Detector (Beckman Coulter Inc, Indianapolis, Ind). Viability of treated cells was determined relative to vehicle-treated cells.

**Immunofluorescence and Brightfield Imaging**

2 \(\times\) 10\(^5\) cells were seeded onto glass coverslips and allowed to attach overnight, followed by treatment with 100 nM dasatinib. Drug was diluted from prepared stock and changed every 24 hours. Cells were fixed with 4\% paraformaldehyde, permeabilized with 0.1\% saponin/phosphate-buffered saline, and blocked with CAS Block (Invitrogen). Primary (p27) and AlexaFluor488 conjugated secondary antibodies were diluted in 0.5\% bovine serum albumin/phosphate-buffered saline and incubated overnight at 4\(^{\circ}\)C or 2 hours at room temperature, respectively. Nuclei were stained and coverslips mounted onto glass slides with Prolong Gold with DAPI (Invitrogen) according to the manufacturer’s instructions. Stained cells were visualized with a Zeiss 710 laser scanning confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) equipped with a 63\(\times\)/1.4NA plan-apochromat objective. AlexaFluor488 (p27) was identified with 488 nm excitation and 505 to 525 nm emission. DAPI was identified with 405 nm excitation and 410 to 460 nm emission. Emission spectra were collected separately to minimize fluorophore overlap. Images were acquired with the confocal aperture set to 1 airy unit and managed in Zen 2009 v 5.5.0.375 software (Carl Zeiss Microscopy, LLC). Brightfield images were viewed on a Zeiss Axiosvert 200M (Carl Zeiss Microscopy, LLC, Thornwood, NY) and captured with an AxioCam ERS5s camera (Carl Zeiss Microscopy, LLC) at 10\(^{\times}\) magnification.

**Flow Cytometry**

To analyze the effects of dasatinib treatment on the cell cycle and apoptosis of BE cells, flow cytometry was performed on the Accuri C6 Flow Cytometer (BD Biosciences, San Jose, Calif) with a threshold at 80,000 and 20,000 events collected per sample. Cells (1 \(\times\) 10\(^6\) cells/100-mm dish) were allowed to attach overnight and treated with 100 nM dasatinib for the indicated times. Assays were performed in triplicate and repeated. Cell-cycle analysis was conducted using propidium iodide to stain DNA content. RNA was eliminated with RNaseA. To determine apoptosis, phosphatidylserine externalization was used as an apoptotic marker and visualized using the Dead Cell Apoptosis Kit with Annexin V FITC (Invitrogen) per the manufacturer’s recommended protocol. Phosphatidylserine was visualized by Annexin V FITC conjugation indicative of apoptosis. For comparison, cells were treated with camptothecin to induce apoptotic death to establish proper gating. Compensations were set to FL1-FL3 6\%, FL3-FL1 5\%.
RESULTS
Dasatinib Reduces Src Activation in Immortalized Barrett’s Esophagus Cells and Reverses p27 Deregulation

To investigate the effects of Src inhibition and p27 function in BE, we treated 2 immortalized BE cell lines established from patient tissue, one diagnosed as without dysplasia (CP-A) and one diagnosed with HGD (CP-D), with dasatinib, an inhibitor of Src kinase and Bcr-ABL oncogene, which is currently approved by the Food and Drug Administration as a second-line treatment for chronic myeloid leukemia. CP-D and CP-A cells were treated with 100 nM dasatinib and analyzed by immunoblot for active (phosphorylated) Src (p-src Y416). Dasatinib treatment results in reduced active Src. CP-D and CP-A cells were probed with antibodies specific to phosphorylated-T187 p27 and p27. CP-D cells display increased basal phosphorylated-T187 p27, compared with CP-A cells. β-Actin was used as a loading control. Blot is representative of 3 independent experiments. Treatment with dasatinib for 24 hours reduced p27 phosphorylation and increased total p27 protein levels, consistent with observations that phosphorylation at T187 results in p27 proteosomal degradation. p27 is phosphorylated at multiple sites in addition to T187 (serine 10-S10, threonine 157-T157, threonine 198-T198), which can contribute to p27 deregulation. We immunoblotted dasatinib-treated BE protein lysates with antibodies specific to phosphorylation at these sites (S10, T157, T198) to ascertain whether Src inhibition had any relative to the metaplastic CP-A cell line. On the basis of the observed increased basal Src activity in CP-D and CP-A cells, we performed an immunoblot analysis of p27 for phosphorylation at threonine 187 (T187), a site shown to require Src tyrosine phosphorylation. Immunoblotting with an antibody specific to p27 T187-phosphorylation showed increased basal phosphorylation of p27 at the T187 site in CP-D cells, compared with CP-A cells (Figure 1, bottom). Treatment with dasatinib for 24 hours reduced p27 T187 phosphorylation and increased total p27 protein levels (Figure 1, B), consistent with observations that phosphorylation at T187 results in p27 proteosomal degradation. We immunoblotted dasatinib-treated BE protein lysates with antibodies specific to phosphorylation at these sites (S10, T157, T198) to ascertain whether Src inhibition had any...
Dasatinib Induces Cell-Cycle Arrest in Barrett’s Esophagus Cell Lines

Because treatment with dasatinib resulted in nuclear re-localization and increased protein levels of p27 in BE cell lines, we next investigated whether these changes correlated with alterations in the cell cycle. p27 directly associates and regulates cyclins D and E, 2 proteins necessary for progression through the cell cycle. Expression analysis of CP-A and CP-D revealed that treatment with dasatinib resulted in considerable decreases in the mRNA of both cyclins D and E in CP-D and CP-A cell lines and particularly for cyclin D (Figure 2, A). To confirm these findings, protein levels of cyclins D and E were assessed in CP-D and CP-A BE cells treated with dasatinib for 24 and 96 hours. As shown in Figure 2, B, both cell lines displayed increases in protein levels of cyclins D and E after 24 hours of treatment. However, after 96 hours of dasatinib treatment, both BE cell lines showed dramatic decreases in cyclins D and E protein levels, consistent with the quantitative polymerase chain reaction data at 96 hours (Figure 2, C). We next analyzed the DNA content and cell-cycle profile of CP-D cells treated with dasatinib by flow cytometry. As shown in Table 1 and Figure 2, C, CP-D cells displayed distinct changes in the percentages of cells in each cell-cycle phase on treatment with dasatinib. Specifically, dasatinib treatment resulted in a decrease in S (37.60%) at

**FIGURE 2.** Dasatinib treatment results in cell-cycle arrest. A, Total RNA from vehicle-treated and dasatinib- (96 hours) treated CP-D and CP-A underwent expression analysis of cyclins D and E. Fold change is relative to expression of cyclin D or E in vehicle-treated cells. Data represent the mean of 2 independent experiments performed in duplicate. Bars represent standard error. B, Immunoblot analysis of cyclins D and E in CP-D and CP-A cells treated with 100 nM dasatinib for 24 or 96 hours. Asterisk indicates nonspecific band migrating below cyclin E. β-Actin was used as a loading control. Blot is representative of 3 independent experiments. C, Cell-cycle analysis of CP-D cells after 96 hours of dasatinib (100 nM) treatment. CP-D cells were stained and analyzed as described in “Materials and Methods.” Control represents cell-cycle profile of CP-D cells at the beginning of the experiment (0 hours). Data are the mean of 2 independent experiments.

| TABLE 1. Dasatinib induces cell-cycle arrest at G1 in Barrett’s esophagus cells |
|-----------------|----------|----------|----------|----------|----------|
|                | 0        | 24       | 48       | 72       | 96       |
| G1             | 37.60%   | 45.41%   | 64.48%   | 69.04%   | 73.27%   |
| S              | 34.99%   | 26.3%    | 25.13%   | 24.94%   | 22.77%   |
| G2             | 37.13%   | 33.67%   | 20.95%   | 14.52%   | 14.36%   |

CP-D cells were treated with dasatinib for 24, 48, 72, and 96 hours. Cell-cycle distribution was determined using propidium iodide as described in “Materials and Methods.” Results are the mean of 2 independent experiments.
Prolonged Dasatinib Treatment Results in Apoptosis in Barrett’s Esophagus Cell Lines

We next tested the response of CP-D and CP-A cells to a range of dasatinib concentrations. After treatment for 24 hours, both CP-D and CP-A showed a significant decrease in the number of viable cells to all concentrations of dasatinib, compared with vehicle-treated cells (Figure 3, A). CP-D cells, derived from HGD, responded to lower concentrations of dasatinib than CP-A cells. We observed significant numbers of detached cells within the media of treated cells (Figure 3, B), suggesting that cells also could be activating apoptosis in response to dasatinib treatment. To investigate this possibility, dasatinib-treated CP-D and CP-A cells were analyzed by immunoblot using an antibody specific to cleaved Parp, a marker of apoptosis. Dasatinib treatment resulted in an increase in cleaved Parp at 48 hours, with maximal levels occurring after 96 hours of treatment (Figure 3, C). To confirm these findings, we assessed Annexin V staining, an early marker of apoptosis, after dasatinib treatment. Dasatinib treatment resulted in an increase in Annexin V staining after 24 hours in both cell lines, which remained throughout treatment (Figure 3, D).

Phosphorylation of p27 in Barrett’s Esophagus Patient Tissue

On the basis of the observations of increased p27 phosphorylation by Src in CP-A and CP-D cells, we subsequently investigated p27 phosphorylation in patient tissue samples. Total protein lysates from patient biopsy samples with pathologic diagnoses of metaplastic BE (n = 4), HGD-BE (n = 4), or esophagitis (n = 3) were immunoblotted for p-T187 p27, p-T157 p27, p-S10 p27, total p27, and Src kinase. As shown in Figure 4, levels of Src were increased in patients with BE and HGD compared with patients with esophagitis, which is consistent with previous observations.10,11 Phosphorylation of p27 at T187 was present at increased levels in all BE tissues and 2 of 4 HGD tissues, compared with esophagitis (Figure 4, A).
whereas 3 of 4 HGD-BE samples showed increased S10 phosphorylation compared with BE and esophagitis (Figure 4). Tissues of patients with HGD demonstrated increased total levels of p27 compared with BE (Figure 4), shown to be a consequence of p27 present throughout the entire BE intestinal gland in HGD and restriction of p27 to superficial regions of the BE gland. Analysis of p-T157 was inconclusive, with little to no staining in any of the tissues (data not shown).

DISCUSSION

The present study explored the effects of a Food and Drug Administration–approved, small molecule inhibitor of the Src kinases, dasatinib, on BE cells. Treatment of immortalized BE cell lines (metaplasia and HGD) in vitro with dasatinib results in reduced proliferation, cell-cycle arrest, and apoptosis. These observations correlate with inhibition of Src, p27 nuclear relocalization, increased protein levels of p27, and reduction of p27 phosphorylation. These findings collectively suggest that dasatinib and other Src kinase inhibitors may have potential as chemopreventive agents for HGD-BE and whose efficacy can be assessed by evaluation of p27.

Preclinical studies indicate that dasatinib and other Src inhibitors have substantial chemotherapeutic activity in malignant cells, inhibiting cell-cycle progression, proliferation, and metastasis, and activating apoptosis. The current study found that dasatinib treatment results in cell-cycle arrest after 96 hours of treatment (Figure 2, C, and Table 1), increases cyclins D and E after 24 hours of treatment, and decreases cyclin D and E expression at 96 hours (Figure 2, A and B) and apoptosis (Figure 3) in pre-malignant BE cells. Further, these characteristics also are found with p27-induced growth arrest and forced p27 expression in cells. These findings suggest that the antineoplastic effects of dasatinib observed within BE cell lines might be due to reversal of Src-mediated deregulation of p27 and support a potential model for the effects of dasatinib in our BE cell lines (Figure 4, B and C). Evidence indicates that tyrosine phosphorylation of p27 by Src permits T187-phosphorylation of p27 by cyclin E-cdk2, promoting disassociation of p27 from the cyclin E-cdk2 complex and proteosomal degradation, as well as being necessary for activation of the cyclin D-cdk4/6 complex (Figure 4, B). Combined, these events result in cell-cycle

FIGURE 4. Abnormal phosphorylation of p27/Kip1 and increased expression of Src are present in biopsies of patients with Barrett’s esophagus (BE) and high-grade dysplasia (HGD). A, Standard biopsies from patients diagnosed with metaplastic BE (n = 4), HGD (n = 4), or esophagitis (Es) (n = 3) were used to isolate total protein as described in “Materials and Methods.” Lysates were separated using standard procedures and immunoblotted with antibodies specific to p27 phosphorylated at T187 or S10. Lysates also were probed for total protein levels of Src and p27. β-Actin was used as a loading control. B and C, Potential model for dasatinib in BE cells. B, p27 phosphorylation by Src reduces p27 inhibitory functions and results in cell-cycle progression and p27 degradation. C, Inhibition of Src with small molecule inhibitors (eg, dasatinib) stabilizes p27 and allows p27 to inhibit function of cyclins E and D and induce cell-cycle arrest.
progression. Inhibition of Src by dasatinib stabilizes p27 protein levels and nuclear relocalization, allowing p27 to inhibit progression of the cell cycle and induce cell-cycle arrest (Figure 4, C). However, because of the broad effects of Src within eukaryotic cells, as well as dasatinib’s ability to functionally inhibit other tyrosine kinases, it is possible that dasatinib could be producing the observed responses via inhibition of an Src-dependent or Src-independent pathway. Our observations of reduction in other p27 phosphorylation sites may reflect this possibility because phosphorylation at these sites (S10, T157, T198) has been linked to activity of other kinases. Thus, it will be important in future investigations to definitively implicate p27 as a mediator of dasatinib’s antineoplastic effects in BE cells, a topic that is currently being pursued in our laboratory.

Although dasatinib has displayed minimal efficacy in late-stage solid tumors as a monotherapy, our evidence suggests that dasatinib (even at low doses) could have benefits for control of HGD-BE, a premalignant lesion. The current state of treatment for HGD-BE focuses on local ablation or surgical removal of the lesion. Although efficacious, these procedures are expensive and often require multiple treatments. In addition, the invasive nature of these procedures can be unattractive to patients. Development of chemopreventive strategies that limit and perhaps even eradicate the HGD-BE lesion would have considerable patient benefit, providing a safe, noninvasive method to control their BE. Such an approach is not unforesworn, as evidenced by the Aspirin and Esomeprazole Chemoprevention (AspECT) in BE trial currently ongoing in Europe and smaller studies investigating the chemopreventive potential of aspirin and other nonsteroidal anti-inflammatory drugs. However, despite the moderate efficacy of nonsteroidal anti-inflammatory drugs in limiting BE progression, the cardiovascular, gastrointestinal, and renal side effects of these agents have limited their clinical application and led to the pursuit of alternative modalities.

CONCLUSIONS

Our in vitro studies with dasatinib are an early preliminary step toward an alternative chemopreventive agent for HGD-BE. Our observations of reduced growth and apoptosis at low doses of dasatinib could indicate that patients with HGD-BE may benefit from dasatinib at low doses or short durations of treatment. Further, because our studies show the occurrence of Src-dependent p27 phosphorylation and increased Src expression in HGD-BE, evaluation of p27 localization or p27 phosphorylation would be a useful marker for Src inhibition in both preclinical and clinical studies. However, despite the beneficial effects of dasatinib in BE cell lines, substantial studies are necessary before dasatinib can be applied clinically. Specifically, testing the efficacy of low-dose dasatinib in controlling BE progression, as well as developing dosing and treatment regimens in surgical rodent models of BE, will be important before attempting patient studies. Such studies are critical, because a chemopreventive strategy for HGD-BE would beneficially affect early adenocarcinoma by providing multiple avenues for early treatment.

References


