Src Tyrosine Kinase Regulates Adhesion and Chemotaxis in Waldenstrom Macroglobulinemia

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Abstract

Purpose: Waldenstrom macroglobulinemia is a lymphoplasmacytic lymphoma characterized by widespread involvement of the bone marrow. Despite different options of therapy, Waldenstrom macroglobulinemia is still incurable. Src tyrosine kinase has been shown to play a central role in the regulation of a variety of biological processes, such as cell proliferation, migration, adhesion, and survival in solid tumors. We sought to determine whether the protein tyrosine kinase Src regulates adhesion, migration, and survival in Waldenstrom macroglobulinemia.

Experimental Design: We tested the expression of Src tyrosine kinase in Waldenstrom macroglobulinemia and normal cells, and the effect of the specific Src inhibitor AZD0530 on the adhesion, migration, cell cycle, and survival of a Waldenstrom macroglobulinemia cell line and patient samples. Moreover, we tested the effect of AZD0530 on cytoskeletal and cell cycle signaling in Waldenstrom macroglobulinemia.

Results: We show that Src is overexpressed in Waldenstrom macroglobulinemia cells compared with control B cells, and that the use of the Src inhibitor AZD0530 led to significant inhibition of adhesion, migration, and cytoskeletal signaling induced by SDF1. Moreover, inhibition of Src activity induced G1 cell cycle arrest; however, it had minimal effect on survival of Waldenstrom macroglobulinemia cells, and no significant effect on survival of normal cells.

Conclusions: Taken together, these results delineate the role of Src kinase activity in Waldenstrom macroglobulinemia and provide the framework for future clinical trials using Src inhibitors in combination with other drugs to improve the outcome of patients with Waldenstrom macroglobulinemia. (Clin Cancer Res 2009;15(19):6035–41)

Src was the first oncogene to be discovered, as well as the first protein tyrosine kinase (1–5). It is a nonreceptor tyrosine kinase that belongs to a nine-member family of kinases including Src, Yes, Fyn, Lyn, Lck, Hick, Fgr, Blk, and Yrk (6, 7). Src has been implicated in cell proliferation, survival, adhesion, migration, invasion, inflammation, and angiogenesis. Src is activated by growth factor receptors, cytokine receptors, and focal adhesion kinase (8). Src interacts with a network of intracellular signaling pathways, including the integrin/focal adhesion kinase, β-catenin/Wnt, RAS-MAP/ERK kinase, phosphoinositide 3-kinases–AKT, and Janus-activated kinase–signal transducers and activators of transcription pathways (7).

c-Src is overexpressed and activated in a large number of human malignancies. It has been linked to the development of cancer and progression to distant metastases, specifically in gastrointestinal malignancies (5). However, the precise functions of c-Src in cancer remain unclear. In addition to increasing cell proliferation, the key role played by c-Src in cancer seems to be to regulate adhesion, invasion, and motility, particularly in cancer cells during the later stages of cancer progression (9, 10). Ablation of Src family kinases inhibits tumor growth, as well as invasiveness and motility of cancer cells (11, 12). This observation prompted the advancements in a number of small-molecule Src kinase inhibitors that reduced cancer invasion and metastasis in preclinical models, leading to the development of clinical trials using these agents. AZD0530, a potent and selective small-molecule inhibitor of Src kinase, is currently being tested in phase II clinical trials in patients with different malignancies (13, 14).

Waldenstrom macroglobulinemia is a low-grade B-cell malignancy characterized by the presence of lymphoplasmacytic cells...
in the bone marrow (15, 16). The specific tropism of these cells to the bone marrow niches indicates their dependence on adhesion and critical interaction with the bone marrow microenvironment (17). We previously showed that the CXCR4/stromal cell-derived factor (SDF1) axis induces migration and adhesion in Waldenstrom macroglobulinemia, and that SDF1 is highly expressed in the bone marrow of Waldenstrom macroglobulinemia patients (17).

In this study, we characterized the role of Src tyrosine kinase in migration, adhesion, cytoskeletal signaling, cell cycle, and survival of Waldenstrom macroglobulinemia cells. We further examined the role of Src in SDF1-induced signaling and functions in Waldenstrom macroglobulinemia.

**Materials and Methods**

**Reagents.** The Src inhibitor AZD0530 (AZD) was provided by AstraZeneca. AZD0530 was diluted in DMSO and stored at -20°C, and diluted in culture medium immediately before use. SDF1 was purchased from R&D Systems.

**Cells.** The Waldenstrom macroglobulinemia cell line BCWM1 was a kind gift from Dr. Steve Treon, Dana Farber Cancer Institute, Boston, MA, and was developed from a patient with untreated IgM κ Waldenstrom macroglobulinemia (18). Cells were cultured in RPMI-1640 containing 10% fetal bovine serum (Sigma Chemical), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). The umbilical vein endothelial cells (HUVEC; Cambrex) were cultured in EGM-2 MV media (Cambrex) reconstituted according to the manufacturer’s instructions.

Patient samples were obtained after approval from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients according to the declaration of Helsinki. Primary Waldenstrom macroglobulinemia cells were obtained from bone marrow samples using CD19-positive microbead selection (Milteny Biotech).

**Translational Relevance**

In this study, we tested the role of a specific Src kinase inhibitor on the adhesion, migration, and survival of Waldenstrom macroglobulinemia cells. The use of the Src inhibitor AZD0530 led to significant inhibition of adhesion, migration, and cytoskeletal signaling induced by SDF1, as well as cell cycle. These results lend insights into the role of Src kinase activity in Waldenstrom macroglobulinemia, which could serve as a framework for future clinical trials using Src inhibitors in combination with other therapeutic agents to improve the outcomes of Waldenstrom macroglubolinemia patients.
with >90% purity as previously described (19). Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by Ficoll-Hypaque density sedimentation, and in some cases CD19-positive cells were selected using microbead selection.

**Immunoblotting.** Waldenstrom macroglobulinemia cells were harvested and lysed using lysis buffer (Cell Signaling Technology) complemented with 5 μmol/L NaF, 2 μmol/L Na3VO4, 1 μmol/L phenylmethylsulfonylfluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin, following the manufacturer's instructions. For determination of the time-dependent-activation of Src by SDF1, the effect of AZD0530 on SDF1-induced activation of Src, cells were prestarved for 3 h. Whole-cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) as previously described (19). All antibodies used for immune-blotting were purchased from Sigma-Aldrich, except the anti-α-actin and anti-tubulin, which were purchased from Sigma.

**Adhesion assays.** To test adhesion to fibronectin we used 96-well plates coated with fibronectin (EMD Biosciences). Moreover, we examined adhesion to both stromal and endothelial cells, in which a confluent monolayer of stromal or HUVEC cells was generated by plating (5 × 10^6) cells in a 96-well plates 24 h before the experiment. HUVEC cells were activated with 10 ng/mL tumor necrosis factor-α for 1 h before the experiment.

Waldenstrom macroglobulinemia cells (BCWM1 and CD19-positive from patient samples) were treated with 0 or 2 mmol/L AZD0530 for 1 h, and cells (2 × 10^5)/well were incubated in the 96-well plates for 1 h at 37°C. In adhesion-to-fibronectin experiments cells were activated with 30 nmol/L during incubation, and nonactivated cells served as control. Moreover, bovine serum albumin and poly-L-lysine-coated wells were used as negative and positive controls, respectively. After incubation, wells were washed with PBS to remove all nonadherent cells, and Calcein-AM was added for 1 h and the degree of fluorescence was measured using a spectrophotometer at excitation wavelength of 485 nm and emission wavelength of 520 nm.

**Transwell and transendothelial migration assays.** Migration was determined using the transwell migration assay (Costar), as previously described (20). Waldenstrom macroglobulinemia cells (BCWM1 and CD19-positive from patient samples) were treated with 0 or 2 μmol/L AZD0530 in 1% FCS medium for 1 h, placed in the upper chambers, and were allowed to migrate to 0 or 30 nmol/L SDF1 in 1% FCS medium, in the lower chamber. After 4 h of incubation, cells that migrated to the lower chambers were counted using a Beckman Coulter cell counter (Beckman).

**Immunofluorescence.** BCWM1 cells were treated with 0 or 2 mmol/L AZD0530 in 1% FCS medium for 1 h, and then with 0 or 30 nmol/L SDF1 for 1 min. Cells were then fixed with 2% formaldehyde in PBS for 30 min at room temperature, permeabilized with Saponin 0.02% in PBS, stained with phallolidin-Alexa-Fuor-568 (5 μg/mL), spun onto slides, mounted, and analyzed by fluorescent microscope using an epifluorescence microscope (Nikon Eclipse E800, Nikon) and a Photometric Cool snap CF color camera (Nikon).

**Cell cycle analysis.** BCWM1 cells were treated overnight with increasing concentrations (0, 0.5, 1, and 2 μmol/L) of AZD0530. Cells were then fixed with methanol, RNA was degraded by RNAase, DNA was stained with 5 μg/mL propidium iodide (Sigma Chemical), and cells were analyzed by flow cytometry.

**Cell viability test.** BCWM1 cells, PBMCs, and CD19-positive cells from healthy volunteers (1 × 10^6 cell/mL) were cultured with 0, 0.5, 1, and 2 μmol/L of AZD0530 for 24 h. Cell growth was assessed by measuring MTT (Chemicon International) dye absorbance.

**Apoptosis.** BCWM1 cells were treated overnight with increasing concentrations (0, 0.5, 1, and 2 μmol/L) of AZD0530. Cells were then stained with Apo-2.7 labeled with Per-CP-Cy5.5 for 1 h on ice. Cells were then washed, resuspended in PBS, and apoptosis was quantified by flow cytometry (Beckman Coulter Inc.).

**Statistical analysis.** Results were reported as the mean ± SD for experiments done in three replicate samples, and statistical significance was determined using Student's t-test. The minimal level of significance was P < 0.05.

**Results**

**Expression of Src tyrosine kinase in Waldenstrom macroglobulinemia and the effect of SDF1 and AZD0530 on its phosphorylation.** We first examined the expression of phosphorylated and total forms of Src kinase in Waldenstrom macroglobulinemia
patient samples and cell lines compared with expression in CD19-positive cells obtained from the bone marrow of healthy donors. Figure 1A shows that Src was overexpressed in Waldenstrom macroglobulinemia patients and in BCWM1 compared with CD19-positive cells from healthy donors, and that the levels of phosphorylation of Src correlated with the expression of total Src. Figure 1B shows that AZD0530 inhibited the basal phosphorylation of Src in BCWM1 cells in a dose-response manner, and that the dose of 2 μmol/L led to a complete inhibition of basal phosphorylation of Src.

Figure 1C shows that activation of BCWM1 cells with SDF1 induced phosphorylation of Src in a time-dependent manner, and that the highest level of activation was achieved after 1 minute of activation. Figure 1D shows that AZD0530 inhibited the SDF1-induced phosphorylation of Src and other cytoskeletal signaling–related proteins, such as extracellular signal-regulated kinase, AKT, focal adhesion kinase, Crkl, and PAK.

**The role of Src in regulating adhesion and migration induced by SDF1.** Figure 2A and B show the adhesion to fibronectin of BCWM1 cells and cells from three Waldenstrom macroglobulinemia patients, respectively. SDF1 increased the adhesion of Waldenstrom macroglobulinemia cells to fibronectin, and AZD0530 inhibited the SDF1-induced adhesion to fibronectin. Moreover, Fig. 2C shows that AZD0530 inhibited the adhesion of BCWM1 cells to both stromal and endothelial cells (HUVEC cells).

Similarly, Fig. 3A and B show chemotaxis of BCWM1 and cells from three Waldenstrom macroglobulinemia patients, respectively. SDF1 induced chemotaxis of Waldenstrom macroglobulinemia cells, and AZD0530 inhibited the SDF1-induced chemotaxis in Waldenstrom macroglobulinemia cells. To further confirm that the effect of the CXCR4/SDF1 axis was regulated by Src, we tested the effect of AZD0530 in the presence or absence of the CXCR4 inhibitor AMD3100. Figure 3C shows that AMD3100 and AZD0530 had similar inhibitory activity on migration of Waldenstrom macroglobulinemia cells, and their combination had no additive effect.

Both adhesion and chemotaxis are known to depend on actin metabolism and polymerization. Figure 4 shows that SDF1 (30 nmol/L for 1 minute) induced a significant increase in the polymerization of actin (F-actin) in BCWM1; this effect was significantly inhibited by AZD0530.

**Src regulates cell cycle in Waldenstrom macroglobulinemia.** Src is implicated in inducing proliferation in many malignancies (1, 12). We therefore investigated the role of AZD0530 on the cell cycle of Waldenstrom macroglobulinemia. Figure 5A shows that AZD0530 induced G1 arrest in a dose-dependent manner. Moreover, Fig. 5B shows that inhibition of Src with AZD0530 induced inhibition of other kinases important for cell proliferation such as extracellular signal-regulated kinase, AKT, and S6K. Moreover, it inhibited proteins involved in cell cycle transmission from G1 to S phase such as cyclin-dependent kinases CDK2 and CDK6, and increased the activity of the cyclin-dependent kinase inhibitor p27.

**Cytotoxicity of AZD0530.** Supplementary Fig. S1A shows that AZD0530 did not significantly inhibit proliferation or induce apoptosis at the concentrations used to inhibit Src activity. Treatment with 2 μmol/L AZD0530 for 24 hours induced only 30% of inhibition of proliferation and 20% of apoptosis in BCWM1 cells. Moreover, Supplementary Fig. S1B and C show that no cytotoxic effect of AZD0530 was observed on either PBMCs or CD19-positive cells from healthy donors.
Waldenstrom macroglobulinemia is characterized by widespread involvement of the bone marrow in all patients, indicating homing and adhesion of the malignant cells to specific niches in the bone marrow, which provides a protective environment for the survival and proliferation of these cells (21). We have previously shown that the migration and adhesion of Waldenstrom macroglobulinemia cells to the bone marrow niches is dependent on the SDF1/CXCR4 axis and that inhibition of this axis regulates adhesion through a direct interaction of the CXCR4 and VLA-4 receptors (17). Src tyrosine kinase is known to regulate cell adhesion and migration, but its role in SDF1-induced migration and adhesion has not been previously studied in B-cell malignancies. We therefore sought to investigate the role of the tyrosine kinase Src in regulating adhesion and migration in Waldenstrom macroglobulinemia, and to delineate the pathways regulated by Src through the SDF1/CXCR4 axis.

In this study, we first delineated the role of Src kinase and downstream pathways through the differential response of cell lines and patient samples. We found that Src was overexpressed in Waldenstrom macroglobulinemia patient samples and in the Waldenstrom macroglobulinemia cell line BCWM1 compared with CD19-positive cells from healthy donors. This indicates a potential role of Src in the invasiveness and metastasis of Waldenstrom macrosecondary malignancies.

**Fig. 4.** The role of Src in SDF1-induced actin polymerization. Representative immune-fluorescence images show that AZD0530 inhibited the SDF1-induced polymerization of actin in BCWM1 cells. Left, polymerized actin detected by phalloidin (red); middle, 4',6-diamidino-2-phenylindole staining of cell nuclei (blue); right, actin polymerization and nuclei images merged.
To investigate the role of Src in Waldenstrom macroglobulinemia we used the specific Src/Abl kinase inhibitor AZD0530. Previous studies in solid tumors and hematologic malignancies, such as leukemias, have shown that AZD0530 is a specific Src/Abl tyrosine kinase inhibitor, and that it regulates proliferation, invasion and metastasis in these malignancies (22–26). AZD0530 is currently being tested in phase II clinical trials. AZD0530 was shown to inhibit the basal activation of Src in BCWM1, as well as to inhibit the SDF1-induced activation of Src and other cytoskeletal-related protein and polymerization of actin, indicating an important role of Src in the signaling cascade downstream of CXCR4 leading to an increase of migration and adhesion of Waldenstrom macroglobulinemia cells.

Functionally, both in the BCWM1 cell line and in cells from Waldenstrom macroglobulinemia patients, we found that Src was involved in the adhesion of Waldenstrom macroglobulinemia cells to the different components of the bone marrow such as bone marrow stromal cells, endothelial cells, and fibronectin that represents the extra-cellular matrix. Moreover, it inhibited the chemotactic effect of SDF1, which leads to increased infiltration of Waldenstrom macroglobulinemia to the bone marrow. We further confirmed that the effect of SDF1/CXCR4 is Src-dependent, as we showed that either inhibition of CXCR4 by AMD3100 or inhibition of Src resulted in similar effects of inhibition of the chemotaxis. In addition, the combination of the two inhibitors did not have any additive or synergistic effect, suggesting that both CXCR4 and Src are in the same signaling cascade, and inhibiting either of them will have similar activity.

Moreover, we showed that Src was involved in controlling cell-cycle in Waldenstrom macroglobulinemia, in which AZD0530 induced G1 arrest and partially inhibited signaling cascades involved in cell proliferation such as extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK). AZD0530 also inhibited the phosphorylation of Akt, which is a downstream target of Src.

The effect of increasing concentrations of AZD0530 on the cell cycle of BCWM1 cells is shown in Fig. 5A. The cell cycle analysis revealed a dose-dependent G1 arrest, with a significant decrease in the percentage of cells in the S phase. The inhibition of Src was further confirmed by Western blot analysis, where the phosphorylation of Src, Akt, and ERK was reduced in BCWM1 cells treated with AZD0530 (Fig. 5B).

Moreover, Src was found to be involved in the regulation of cell cycle checkpoints, as AZD0530 induced G1 arrest and partially inhibited signaling cascades involved in cell proliferation such as extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK). AZD0530 also inhibited the phosphorylation of Akt, which is a downstream target of Src.

Fig. 5. The role of Src in cell cycle regulation. A, the effect of increasing concentrations of AZD0530 on the cell cycle of BCWM1 cells, showing dose-dependent G1 arrest. B, the effect of AZD0530 on protein related to cell proliferation and cell cycle in BCWM1 cells detected by immune-blotting, showing a decrease of the activation of proliferative and cell cycle–promoting proteins, and an increase of cell cycle inhibitory proteins.
kinase and AKT, which led to inhibition of cell-cycle proteins. However, complete inhibition of Src by AZD0530 did not inhibit cell survival in Waldenstrom macroglobulinemia cells or in normal PBMCs and CD19-positive cells.

Together, these studies indicate that the Src tyrosine kinase regulates SDF1-induced adhesion and migration of Waldenstrom macroglobulinemia cells, and its inhibition by AZD0530 reduces those effects. These studies indicate that AZD0530 can be used in the future to inhibit dissemination of Waldenstrom macroglobulinemia cells. Moreover, the lack of cytotoxicity of AZD0530 on normal cells suggests it as a safe drug to be used in combination with other chemotherapies to improve patient outcome in Waldenstrom macroglobulinemia.

References

Disclosure of Potential Conflicts of Interest
K. Anderson and I. Ghobrial are members of the Millenium, Celgene, and Novartis speakers’ bureaus.

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