Targeting the Spleen Tyrosine Kinase with Fostamatinib as a Strategy against Waldenström Macroglobulinemia

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Abstract

Purpose: Waldenström macroglobulinemia (WMG) is a lymphoproliferative disorder characterized by good initial responses to standard therapeutics, but only a minority of patients achieve complete remissions, and most inevitably relapse, indicating a need for novel agents. B-cell receptor signaling has been linked to clonal evolution in WMG, and Spleen tyrosine kinase (Syk) is overexpressed in primary cells, suggesting that it could be a novel and rational target.

Experimental Design: We studied the impact of the Syk inhibitor fostamatinib on BCWM.1 and MWCL-1 WMG-derived cell lines both in vitro and in vivo, as well as on primary patient cells.

Results: In WMG-derived cell lines, fostamatinib induced a time- and dose-dependent reduction in viability, associated with activation of apoptosis. At the molecular level, fostamatinib reduced activation of Syk and Bruton’s tyrosine kinase, and also downstream signaling through MAPK kinase (MEK), p44/42 MAPK, and protein kinase B/Akt. As a single agent, fostamatinib induced tumor growth delay in an in vivo model of WMG, and reduced viability of primary WMG cells, along with inhibition of p44/42 MAPK signaling. Finally, fostamatinib in combination with other agents, including dexamethasone, bortezomib, and rituximab, showed enhanced activity.

Conclusions: Taken together, these data support the translation of approaches targeting Syk with fostamatinib to the clinic for patients with relapsed and possibly even newly diagnosed WMG.

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Introduction

Signaling through the B-cell receptor (BCR) occurring through both antigen-dependent and -independent mechanisms appears to play an important role in the pathobiology of several common B-cell malignancies. BCR aggregation results in phosphorylation of the Igα (CD79a) and Igβ (CD79b) immunoreceptor tyrosine-based activation motifs (ITAMs) catalyzed by members of the Src family of kinases (SFK), such as Lyn. Phosphorylated ITAM residues then serve as docking sites for the spleen tyrosine kinase (Syk), and binding results in a conformational change that facilitates exposure of tyrosines 348 and 352 for phosphorylation by SFKs, as well as Syk autophosphorylation. Later association with other signaling intermediates, such as Shc, Bruton’s tyrosine kinase (BTK), phospholipase Cγ2, and PI3K, results in downstream activation of signal transduction pathways crucial to lymphoma pathobiology. Among these are the proliferation-associated MAPKs such as p44/42, and the survival-associated protein kinase B/Akt (1, 2).

Waldenström macroglobulinemia (WMG) is diagnosed in the presence of a lymphoplasmacytic B-cell lymphoma involving the bone marrow and a serum IgM monoclonal protein (3). Though this disease typically has an indolent clinical course, its presenting features can include symptomatic anemia, thrombocytopenia, hepatosplenomegaly, and lymphadenopathy, among others, and currently available therapies are not curative. At the molecular level, recent studies have identified the L265P mutation of myeloid differentiation primary response gene 88 (MYD88) as a commonly recurring abnormality in WMG patients (4–8). This mutation contributes to disease pathobiology through activation of NF-κB signaling (4), as well as of BTK (9), implicating a role for BCR signaling. Indeed, previous studies had linked BCR signaling to clonal evolution in WMG (10). These findings led to translation of the BTK inhibitor ibrutinib (11) to the clinic for patients with relapsed and/or refractory WMG. In this setting, ibrutinib showed significant antitumor activity (12), with a response rate of 81%, though no complete remissions were noted.

With this validation of BCR signaling as a target in WMG, we considered the possibility that other intermediates could be attractive as well. We focused in particular on Syk given the availability of fostamatinib, a specific and clinically relevant (13) Syk inhibitor and previous findings showing that Syk was...
Novel therapies are needed for Waldenström macroglobulinemia (WMG) because the currently available standard and novel agents induce high overall response rates but few patients achieve complete remission, and none are cured of this lymphoproliferative disorder. The current study validates the use of the Spleen tyrosine kinase inhibitor fostamatinib as an attractive strategy using cell lines and primary samples in vitro, as well as a xenograft model in vivo. Since fostamatinib is currently undergoing clinical testing, our data provide a rationale for translation of this drug into trials targeting patients with relapsed and/or refractory, and possibly even newly diagnosed WMG.

Materials and Methods
Reagents
Fostamatinib disodium (FosD; R935788) and bortezomib were purchased from Selleck Chemical, and stock solutions were prepared in PBS and DMS (Fisher Scientific), respectively. Rituximab was purchased from the MD Anderson Pharmacy, while dexamethasone was from Sigma-Aldrich, with stock solutions made in PBS and ethanol (Fisher Scientific), respectively.

Cellular viability assays
Proliferation and viability assays were performed using the WST-1 reagent as previously described (18, 19). Briefly, cells were plated in triplicate and exposed to the conditions noted in the text, and premixed WST-1 cell proliferation reagent (Clontech Laboratories) was added and used according to the manufacturer’s specifications. Conversion of WST-1 to the formazan dye was measured at 650 nm using a Victor 3V plate-reader (PerkinElmer Life Science), and viability data were prepared using GraphPad Prism version 6 showing the mean ±

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Translational Relevance
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Tissue culture and patient samples
The BCWM.1 cell line derived from a patient with WMG (15) was from Dr. Steven Treon (Dana Farber Cancer Institute; Boston, MA). These cells are CD5 , CD10 , CD19 , CD20 , CD23 , CD27 , CD38 , CD138 , CD40 , CD52 , CD70 , CD117 , clgM , clgG , clgA , ckappa , and clambda (15), and harbor the MYD88 L265P mutation but have a wild-type C-X-C chemokine receptor type 4 (CXCR4; ref. 16). MWCL-1 WMG cells were from Dr. Stephen Ansell (The Mayo Clinic; ref. 17), and are CD3 , CD19 , CD20 , CD27 , CD38 , CD49D , CD138 , clgM , and k+. They also harbor the MYD88 L265P mutation and a wild-type CXCR4 (16), but have a TP53 missense mutation at exon 5 (V143A, GTG; ref. 17). BWCM-1 cells were propagated in RPMI-1640 media (Life Technologies) supplemented with 2 mmol/L L-glutamine (Invitrogen) and 10% FCS (Hyclone), while MWCL-1 cells were grown in similarly supplemented IMDM (Life Technologies). All cultures also contained 100 U/mL penicillin and 100 µg/mL streptomycin (Mediatech). Cell line authentication was performed by the MD Anderson Characterized Cell Line Core Facility using short tandem repeat DNA microbeads (Miltenyi Biotec).
Experiments were performed in duplicate on different days, and results from one representative dataset are presented in the text.

**Apoptosis and cell-cycle analysis**
Induction of apoptosis in cells treated with fostamatinib was detected by staining with an antibody to Annexin V and with ToPro3 (both from Life Technologies). FACS was then performed using a Fortessa FACS machine (Applied Biosystems). Cell-cycle analysis was similarly performed after staining with 1 μmol/L propidium iodide (Sigma-Aldrich).

**Western blotting**
Cells were harvested, washed, and lysed with 1× lysis buffer (Cell Signaling Technology) containing 1× Protease and 1× Phosphatase inhibitor mixtures (Roche Diagnostics). Lysates were then sonicated and clarified by centrifugation, and protein concentrations were determined using the DC Assay Kit (Bio-Rad). A total of 50 μg of protein boiled in denaturing buffer (3% dithiothreitol, 0.1 mol/L Tris-HCl, pH 6.8, 4% sodium dodecylsulfate, 0.2% bromophenol blue, 20% glycerol) was separated by gel electrophoresis, and transferred onto nitrocellulose membranes. These were blocked with Tris-buffered saline + 0.01% Tween 20 containing 5% non-fat milk, and then exposed to primary and secondary antibodies. Protein abundance was visualized using the Enhanced Chemiluminescence Kit according to the manufacturer’s specification (Pierce Scientific) with autoradiography film (Eastman Kodak). The images were scanned and densitometry of the protein bands was performed using ImageJ Software (NIH; Bethesda, MD) with normalization to β-actin as a loading control. Primary antibodies used included those against phospho-Tyr525/526 Syk (#12081), phospho-Tyr323 Syk (#2715), and total Syk (#2712), phospho-Tyr323 BTK (#2715) and total BTK (#3533), phospho-Ser117/Ser221 MAPK kinase (MEK; #9154) and total-MEK (#9122), phospho-Thr202/Tyr204 p44/42 MAPK (#4370) and total p44/42 MAPK (#9102), and phospho-Ser473 Akt (#4058) and total AKT (#9272), all of which were from Cell Signaling Technology. Loading controls were provided using an anti-β-actin antibody (A1978) from Sigma-Aldrich.

**Xenograft model**
Experiments were performed in accordance with procedures and protocols approved by the MD Anderson Cancer Center Animal Care and Use Committee. Six-week-old non-obese diabetic mice with severe combined immunodeficiency (NOD.Cg-Prkdc<sup>s ascendendo</sup> il2rg<sup>tm1Wjl/SzJ</sup>) from Jackson Laboratories were injected subcutaneously with 1 × 10<sup>7</sup> MWCL-1 cells with Matrigel (BD Biosciences). Seven days after tumor formation, they were injected intraperitoneally with either PBS, or this...
Fostamatinib induces apoptosis of WMG-derived cell lines

To determine whether Syk inhibition could be valuable as an approach against WMG, we examined the activity of fostamatinib against WMG-derived cell lines. Using an assay that measures the metabolism of WST-1 by mitochondrial succinate-tetrazolium reductase, we found that BCWM.1 cells exposed to fostamatinib showed a time- and concentration-dependent decrease in viability (Fig. 1A). This was especially notable at 1 μmol/L of fostamatinib, which reduced viability by up to 45% after three days. To confirm these findings, we studied MWCL-1 cells, which were also sensitive to fostamatinib at 1 μmol/L, with a 53% to 60% reduction in viability after 1 to 3 days of exposure (Fig. 1B), though 0.25 μmol/L showed activity as well after 3 days. Notably, these concentrations are well within those achieved by patients in phase I and pharmacokinetics studies of fostamatinib (22), where peak concentrations as high as 6 μmol/L were observed, suggesting that they are physiologically relevant.

Cell-cycle analysis of BCWM.1 cells exposed to fostamatinib compared with vehicle controls revealed an increase in cells at G0–G1 from 67% to 76% (Fig. 2A, left), and fewer cells were seen in S phase (27%–21%). Similarly, MWCL-1 cells treated with fostamatinib tended to accumulate at G0–G1 (63%–77%; Fig. 2A, right), with a reduction in the fraction in S phase (37%–16%). In both models, fostamatinib increased the proportion of cells with a sub-G0 DNA content, which rose from 12% to 22% in BCWM.1 cells, and from 8% to 13% in MWCL-1 cells, suggesting the activation of programmed cell death. To examine for the latter, cells were stained with Annexin V and with the nuclear dye ToPro3, and analyzed by cell sorting. Compared with vehicle-treated BCWM.1 cells (Fig. 2B, top), fostamatinib increased the proportion that were Annexin V+ from 4.6% to 26.9%, whereas in MWCL-1 cells, this increased from 7.9% to 31.6% (Fig. 2B, bottom). At higher drug concentrations, fostamatinib induced increased levels of apoptosis. Fostamatinib concentrations of 0.1, 1, 5, and 10 μmol/L induced staining with Annexin V in 18.5%, 24.1%, 26.2%, and 34.8% of BCWM.1 cells, respectively (Supplementary Fig. S1A), and in 17.5%, 21.4%, 30.4%, and 57.0% of MWCL-1 cells, respectively (Supplementary Fig. S1B). These findings indicate that Syk inhibition reduced viability in WMG-derived cell lines through a combination of cell-cycle accumulation at G0–G1, and activation of apoptosis.

Activation of Syk is reduced by fostamatinib

To determine the impact of fostamatinib on Syk, we used Western blotting with antibodies specific for phospho-Tyr525/526, which are residues in the activation loop of the Syk kinase domain (23). In BCWM.1 cells (Fig. 3A, left), fostamatinib reduced Syk activation in a concentration-dependent manner, with a 41% decrease in phospho-Syk after 1 μmol/L of this drug. A similar effect was seen in MWCL-1 cells, where fostamatinib reduced levels of phospho-Tyr525/526-Syk by up to 51% (Fig. 3A, right). Also, we examined the impact on phosphorylation of Syk at Tyr326, which provides a binding site for the Cbl ubiquitin ligase (24, 25), and is therefore thought to be inhibitory of Syk activity. Interestingly, fostamatinib also reduced levels of phospho-Tyr323-Syk in BCWM.1 cells (Fig. 3B, left) by up to 98% or more, and in MWCL-1 cells (Fig. 3B, right) by up to 75%. Finally, to determine the net effect on Syk activity of fostamatinib in these models, we examined the levels of phospho-Tyr223-BTK, a modification that occurs as a result of auto-phosphorylation after trans-phosphorylation at Tyr551 in the BTK activation loop (26, 27). Although both BCWM.1 (Fig. 3C, left) and MWCL-1 cells (Fig. 3C, right) had substantial phospho-Tyr223-BTK levels at baseline, fostamatinib reduced these dramatically by up to 95% and 59%, respectively. These findings indicate that fostamatinib...
did reduce Syk activity and the activation status of the downstream intermediate BTK, which is itself a key contributor to the pathobiology of WMG.

Fostamatinib inhibits downstream signaling cascades

Given the role of BCR signaling in activating key downstream signal transduction cascades, we next looked at its impact on p44/42 MAPK. Vehicle-treated BCWM.1 (Fig. 4A, left) and MWCL-1 cells (Fig. 4A, right) had abundant levels of phospho-Ser117/Ser221 MEK. After exposure to fostamatinib, however, a dose-dependent decrease was seen, with a reduction in phospho-MEK levels by up to 96% or more in both model systems. This led to inhibition of signaling through p44/42 MAPK, where phosphorylation at Thr202/Tyr204 was reduced by up to 97% and 74% in BCWM.1 (Fig. 4B, left) and MWCL-1 cells (Fig. 4B, right), respectively. Finally, we looked at the impact of fostamatinib on Akt, a pathway whose activity has been described to influence homing and survival of WMG cells (28). Consistent with a role for BCR signaling in activating Akt, Syk inhibition with fostamatinib dramatically reduced phospho-Ser473 Akt levels by up to 95% to 98% in BCWM.1 (Fig. 4C, left) and MWCL-1 cells (Fig. 4C, right).

Antitumor activity of fostamatinib

Since fostamatinib is clinically relevant in that it has been studied in patients with non-Hodgkin lymphomas (13), and is being evaluated in rheumatologic conditions (29), we next determined whether it could show antitumor activity. We developed an in vivo xenograft based on MWCL-1 cells in immunodeficient mice which grew steadily in the vehicle-treated cohort (Fig. 5A). In the fostamatinib-treated group, however, tumor growth was slower, and the difference between the two groups was different at a significance level of 0.0028 with adjustment of multiple comparisons (0.05/18 comparisons; 1 comparison at each of 18 time points). For example, the mean tumor volume of the control group on day 35 was larger than the one of the treatment group at a significance level of 0.0028 (P value = 0.0002). Also, we examined CD20⁺ cells isolated from bone marrow aspirates of

Figure 4.
Fostamatinib inhibits activation of the MAPK and Akt pathways. BCWM.1 and MWCL-1 cells treated as above were analyzed by Western blotting for the activation status of MEK, p44/42 MAPK, and Akt with phospho-specific antibodies as described earlier.
patients with WMG, and found that fostamatinib was able to reduce viability in all of them (Fig. 5B), and this was associated with a decrease in p44/42 MAPK activation (Fig. 5C) in the one sample where sufficient cells were available to evaluate this by Western blotting.

Combination regimens enhance anti-WMG effects

Treatment of patients with WMG in either the front-line or relapsed and/or refractory setting often involves the use of multitudrug regimens including corticosteroids, proteasome inhibitors, monoclonal antibodies, and alkylating agents (3, 30). To therefore see whether fostamatinib could be considered not just as a stand-alone therapy, but also as part of other regimens, combination studies were performed. Dexamethasone as a single agent showed modest to no significant activity against either BCWM.1 or MWCL-1 cells (Fig. 6A, left and right, respectively). Fostamatinib alone impacted upon viability in both cell lines, with an up to 28% reduction in MWCL-1 cells, for example. When the two were combined, however, enhanced activity was seen, especially in MWCL-1 cells, where an up to 80% decrease in viability was noted ($P<0.01$). Proteasome inhibition with bortezomib showed activity and reduced viability by 36%, whereas fostamatinib with bortezomib reduced this further to 72% of controls in MWCL-1 cells ($P<0.01$). Rituximab alone reduced viability by 25%, whereas an 84% reduction was seen with fostamatinib in combination with rituximab in MWCL-1 cells ($P<0.01$). Finally, bendamustine was examined, and also reduced viability in both cell lines (Supplementary Fig. S2), with a reduction to 33% in MWCL-1 cells, which was enhanced further to 18% in combination with fostamatinib ($P<0.01$).

It was also of interest in the context of combination therapy to determine whether different sequences of addition of some of these agents could provide enhanced activity. To examine this possibility, BCWM.1 and MWCL-1 cells were exposed either first to fostamatinib for 24 hours and then to dexamethasone or bortezomib for another 48 hours, or to dexamethasone or bortezomib for 24 hours, followed by dexamethasone or bortezomib with fostamatinib for 48 hours. In BCWM.1 cells, there was an indication that addition of fostamatinib first led to a greater efficacy for the combination than if...
Fostamatinib is the prodrug of R406, and is an oral ATP-competitive inhibitor which showed activity in several preclinical models, including both in vitro and in vivo against diffuse large B-cell lymphoma (40). Friedberg and colleagues performed a phase I/II study of fostamatinib, which identified a dose of 200 mg twice daily for phase II testing (13), with dose-limiting toxicities of neutropenia, diarrhea, and thrombocytopenia. Interestingly, although the overall response rate (ORR) in relapsed diffuse large B-cell lymphoma was only 24% (4/17), in patients with more indolent disorders, such as chronic lymphocytic leukemia/small lymphocytic lymphoma, the ORR was 55% (6/11), with a median progression-free survival of 6.4 months. These data, along with our current studies, provide a rationale to consider translating fostamatinib to the clinic for patients with WMG either alone, or in combination with other standard agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Development of methodology: I. Kuiatse, R.J. Jones

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