Inhibition of phosphorylation of the colony-stimulating factor-1 receptor (c-Fms) tyrosine kinase in transfected cells by ABT-869 and other tyrosine kinase inhibitors


Introduction

Mammary carcinoma has the highest incidence of all cancer types in American women, and its incidence rates have continued to increase since 1986 (1). The mechanism by which mammary epithelial cells undergo genetic changes that result in acquisition of the ability to invade and colonize distant sites is complex (2). Normal and malignant mammary epithelium and the surrounding stromal cells produce and respond to various growth factors, including transforming growth factors α and β (3) and fibroblast growth factors (4). Among the stromal cells, macrophages play a unique role because they are recruited into mammary gland carcinomas (5), and, in the absence of such tumor-associated macrophages, metastatic progression of mammary gland tumors is profoundly reduced (6). Macrophages enhance tumor progression through paracrine circuits involving the production of colony-stimulating factor (M-CSF) by tumor cells (7) or other host-derived stromal cells and also by extracellular matrix–modulating functions mediated by matrix metalloproteinases (8). A recent study has shown that a selective CSF-1R inhibitor inhibits monocyte growth and bone degradation (9), and a multitargeted inhibitor with potent activity against CSF-1R is being evaluated in patients with bone metastases (10).

Overexpression of M-CSF or its receptor (CSF-1R) occurs in a significant percentage of breast, ovarian, prostate, and endometrial cancers (6, 11), and studies using antisense oligonucleotides to suppress M-CSF expression and small-interfering RNA directed against M-CSF or CSF-1R expression have shown that blockade of this pathway inhibits tumor growth in xenograft models (12). M-CSF null mice (ob/ob) are resistant to the development of invasive, metastatic carcinomas. Measurements of circulating levels of M-CSF have proven useful in patients with ovarian, endometrial, and breast carcinoma, both for disease detection and monitoring of response to therapy (13). For all of these reasons, the extent of inhibition of CSF-1R signaling is an important consideration in the evaluation of the properties of receptor tyrosine kinase inhibitors.

ABT-869 is a structurally novel multitargeted tyrosine kinase inhibitor (14, 15). This compound is a potent inhibitor of several class III receptor tyrosine kinases but has little activity against other tyrosine kinases or serine/threonine kinases (15). In preclinical s.c. and orthotopic tumor growth models, ABT-869 has been shown to be
Inhibition of Phosphorylation of CSF-1R in Cells

ABT-869 and other compounds either marketed or in clinical development (imatinib, AG013736, BAY 43-9006, CHIR258, and SU11248) as inhibitors of the CSF-1R catalytic domain using a homogeneous time-resolved fluorescence (HTRF) assay. In addition, the kinetics of the enzymatic inhibition by imatinib against 20 mmol/L HEPES/NaOH buffer (pH 7.5) with 100 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, and 1% glycerol and applied to a Q2 anion exchange column (Bio-Rad, Hercules, CA) equilibrated with same buffer. Elution of proteins was done with 30 mL of a linear gradient from 0.1 to 1 mol/L NaCl in column buffer at a flow rate of 1 mL/min. Fractions of 1 mL were collected and assayed for CSF-1R or KDR activity by HTRF assay, and the protein purity was analyzed by SDS-PAGE and Western blot. The active form of Abl was purchased from Upstate (Charlottesville, VA).

HTRF Assay of Inhibitors

The CSF-1R and KDR IC_{50} values were determined by assay of CSF-1R and KDR using an ATP concentration of 1 mmol/L. A biotinylated peptide (Biotin-Ahx-AEEEYF-FLFA-amide) substrate containing a single tyrosine was used in a microtiter plate assay using HTRF methodology as described in ref. (21). Each inhibitor was assayed at 11 concentrations prepared by serial dilution of a DMSO stock solution of the compound. The concentration resulting in 50% inhibition of activity (IC_{50}) was calculated with MDL Assay Explorer software using a nonlinear regression analysis of the concentration response data. Abl was assayed similarly using a different peptide (Biotin-Ahx-GEAEYIAAFFA) and at an ATP concentration of 10 mmol/L.

Determination of the K_i of ABT-869 and Imatinib with rCSF-1R Catalytic Domain

The range of ATP concentrations in the final assay was 0.2 to 1 mmol/L. The imatinib concentrations were used 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L. The complete reaction mixture (40 µL) in a black Microfluor 96-well plate contained buffer (0.2 mg/mL bovine serum albumin added), CSF-1R catalytic domain, compound, and ATP and was initiated by the addition of substrate (0.5 mmol/L final). After a 1-hour reaction, a solution (10 µL) of 0.5 mol/L EDTA was added to stop further kinase reaction. A volume of 50 µL of a mixture of streptavidin-allophycocyanin and europium-chelate anti-phosphotyrosine in development buffer was added immediately after the EDTA. The response (ratio of 665 and 615 nm fluorescence) of each well was recorded 1 hour after addition of the HTRF reagents using a Packard Discovery plate reader. The background response from wells prequenched with EDTA was subtracted from all wells, and the data were analyzed using Lineweaver-Burk plots.

Effect of Inhibitors on Levels of Phosphorylated-CSF-1R in Transfected Cells by Western Blot Analysis

NIH3T3 cells stably transfected with the cDNA for full-length human CSF-1R were placed overnight in 100-mm culture dishes at 3,000,000 per dish. The cells were serum-
deprived for 1 hour (DMEM/0.1% fetal bovine serum) and then resuspended in fresh DMEM containing 0.1% fetal bovine serum, with inhibitor compounds diluted from DMSO stock solutions (1% DMSO final). The positive and negative control dishes contained no inhibitor. After 1 hour, the cells (except negative controls) were stimulated for 10 minutes with 200 ng/mL human M-CSF (R&D Systems, Minneapolis, MN). After this treatment, the cells were immediately lysed using radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% IGEPAL, 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.25% sodium deoxycholate] containing protease inhibitors (Complete, Roche, Indianapolis, IN) and NaF (1 mmol/L), and the lysates cleared by centrifugation at 15,000 rpm in a refrigerated microcentrifuge for 15 minutes. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and 600 μg protein from each incubation was immunoprecipitated overnight as described (4), with a rat monoclonal antibody to human CSF-1R (Calbiochem, San Diego, CA). The immunocomplexes were then incubated with 0.1 mL of protein G Sepharose beads (Pharmacia Biotech, Piscataway, NJ), recovered by centrifugation, and washed with PBS/Tween. After treatment with SDS-loading buffer, the proteins were resolved on a 3% to 8% Tris-acetate SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane. For phosphotyrosine analysis, membranes were probed with anti-phosphotyrosine (clone 4G10, Upstate). To determine the total amount of CSF-1R, the blots were reprobed with an antibody to CSF-1R (Santa Cruz Biotechnology). For both determinations, a secondary antibody conjugated to HRPO was used, and detection of the blots was done using enhanced chemiluminescence reagents (Pierce). The total phosphotyrosine and CSF-1R bands were digitized by UN-SCAN-IT-gel software (Silk Scientific, Orem, UT) for quantification. The anti-phosphotyrosine bands were normalized using the corresponding CSF-1R bands, and the percent inhibition was calculated at each concentration of inhibitor. IC<sub>50</sub> values were estimated by plotting the percent inhibition at each concentration versus the logarithm of the inhibitor concentration.

**Cellular KDR Phosphorylation Assay**

NIH3T3 cells stably transfected with the cDNA for full-length human KDR (VEGFR2) were maintained in DMEM with 10% fetal bovine serum and 500 μg/mL geneticin. KDR cells were plated at 20,000 per well into duplicate 96-well tissue culture plates and cultured overnight in an incubator at 37°C with 5% CO<sub>2</sub> and 80% humidity. The growth medium was replaced with serum-free growth medium for 2 hours before compound addition. Compounds in DMSO were diluted in serum-free growth medium (final DMSO concentration 1%) and added to cells for 20 minutes before stimulation for 10 minutes with VEGF (50 ng/mL). Cells were lysed by addition of radioimmunoprecipitation assay buffer containing protease inhibitors and NaF and placed on a microtiter plate shaker for 10 minutes. The lysates from duplicate wells were combined, and 170 μL of the combined lysate was added to the KDR ELISA plate. The KDR ELISA plate was prepared by adding anti-VEGFR2 antibody (1 μg/well; R&D Systems) to an unblocked plate and incubated overnight at 4°C. The plate was then blocked for at least 1 hour with 200 μL/well of 5% dry milk in PBS. The plate was washed twice with PBS containing 0.1% Tween 20 (PBST) before addition of the cell lysates. Cell lysates were incubated in the KDR ELISA plate with constant shaking on a microtiter plate shaker for 2 hours at room temperature. The cell lysate was then removed, and the plate was washed five times with PBST. Detection of phospho-KDR was done using a 1:2,000 dilution of biotinylated 4G10 anti-phosphotyrosine (Upstate), incubated with constant shaking for 1.5 hours at room temperature, and washed five times with PBST; for detection, a 1:2,000 dilution of streptavidin-HRP (Upstate) was added and incubated with constant shaking for 1 hour at room temperature. The wells were then washed five times with PBST and K-Blue HRP ELISA.

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<tr>
<th>Table 1. Summary of kinase inhibitory activities of ABT-869 (15)</th>
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<td><strong>Structurally related receptor tyrosine kinases</strong></td>
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<sup>*</sup>IC<sub>50</sub> determined at an ATP concentration of 1 mmol/L.

<sup>†</sup>IC<sub>50</sub> determined at an ATP concentration of 5 to 10 μmol/L.
substrate (Neogen, Lexington, KY) was added to each well. Development time was monitored at 650 nm in a SpectraMax Plus plate reader until 0.4 to 0.5 absorbance units were obtained (~10 minutes) in the VEGF only wells. Phosphoric acid (1 mol/L) was added to stop the reaction and the plate was read at 450 nm. Percent inhibition was calculated using the VEGF only wells as 100% controls and wells containing 5 μmol/L pan-kinase inhibitor as 0% controls, and no VEGF wells were used to monitor endogenous phosphorylation state of the cells. IC50 values were calculated by non-linear regression analysis of the concentration response curve.

Results

Specificity of ABT-869 as an Inhibitor of Receptor Tyrosine Kinases

ABT-869 (Fig. 1) is an inhibitor of VEGF and platelet-derived growth factor families of receptor tyrosine kinases, with potent activity against KDR, Flt-1, Flt-3, c-Kit, and CSF-1R. However, this compound is not an inhibitor of fibroblast growth factor receptor, epidermal growth factor receptor, or the soluble tyrosine kinases, such as Src or Abl, nor of the serine/threonine kinases tested. The enzyme inhibition properties of ABT-869 are summarized in Table 1 (15).

HTRF Assays of CSF-1R, KDR, and Abl

The inhibition of the enzymatic activity of CSF-1R and KDR was determined by HTRF assay of CSF-1R and KDR active kinase domains using an ATP concentration of 1 mmol/L. The Abl assays were carried out at 10 μmol/L ATP to provide a more sensitive assay of inhibitor potencies. These data are summarized in Table 2. ABT-869, and the other compounds (excluding imatinib) were found to be potent KDR inhibitors. Although less active than these other compounds, imatinib has activity against CSF-1R at submicromolar concentrations (IC50 = 422 nmol/L). Using a commercial preparation of active recombinant Abl for comparison, we found that imatinib is a moderately potent inhibitor of this enzyme in the HTRF assay, whereas AG013736 was unexpectedly found to be nearly 10-fold more potent than imatinib (IC50 = 52 nmol/L) as an inhibitor of active Abl. ABT-869 and the other compounds have little in vitro activity as inhibitors of Abl.

Kᵢ of Imatinib and ABT-869 with CSF-1R

The kinetics of the reaction of ABT-869 and imatinib with CSF-1R using various concentrations of ATP and inhibitor was analyzed. The Lineweaver-Burk analysis of imatinib inhibition is shown in Fig. 2 and shown to be competitive with ATP with a Kᵢ of 120 nmol/L. The Kᵢ of ABT-869 was similarly analyzed and found to be 3 nmol/L. These experiments were done with the soluble catalytic domain construct, and the competitive nature of the inhibition implies that both compounds bind to the ATP-binding site of CSF-1R.

Inhibition of CSF-1R Phosphorylation in NIH3T3/CSF-1R Cells

The inhibition of phosphorylation in cells was measured using NIH3T3 cells transfected with the full-length

![Figure 2](https://example.com/figure2.png)
human CSF-1R gene and stimulated with M-CSF. CSF-1R was immunoprecipitated from the cells, and the level of phosphorylation was determined by Western blot using an anti-phosphotyrosine antibody. To estimate as precisely as possible the relative potencies of the compounds, incubations were done at five concentrations selected from 1, 3, 10, 30, 100, 300, and 1,000 nmol/L, with the concentration range adjusted for the potency of the inhibitor as shown in Fig. 3. ABT-869 and the five other compounds were evaluated, the pictures of the blots are shown in Fig. 3, and the results are summarized in Table 2. Complete inhibition of phosphorylation was observed at 100 nmol/L ABT-869 (Fig. 3A), and the IC\textsubscript{50} was estimated to be 16 nmol/L from a digital analysis of the intensity of the bands (Fig. 4A). AG013736 and BAY 43-9006 were also potent inhibitors, whereas SU11248 was less potent. CHIR258 was the least potent of the compounds evaluated in this assay, with a cellular IC\textsubscript{50} (535 nmol/L) substantially higher than found in the enzyme assay (IC\textsubscript{50} = 26 nmol/L). Imatinib was found to inhibit the cellular assay at submicromolar concentrations (Fig. 3B), and an IC\textsubscript{50} of 118 nmol/L was calculated upon analysis of the digitized densities of the bands (Fig. 4B).

**Inhibition of KDR in a Cellular Assay**

NIH3T3 cells transfected with the cDNA for KDR were used to examine the activity of ABT-869 and the reference compounds in an ELISA measuring the phosphorylation of KDR in cells as described in Methods. The results are summarized in Table 2. ABT-869 and AG013736 are potent inhibitors of KDR phosphorylation (IC\textsubscript{50} < 10 nmol/L). The other tyrosine kinase inhibitors (excluding imatinib) also showed significant inhibition of both assays (IC\textsubscript{50} < 100 nmol/L). Consistent with its lack of activity in the KDR enzyme assay, imatinib is not an inhibitor of KDR phosphorylation in the cell-based ELISA assay.

**Discussion**

This work describes the characterization of six compounds as inhibitors of the soluble catalytic domain of CSF-1R in an enzymatic activity assay and also as inhibitors of receptor autophosphorylation in cells expressing the full-length protein on the cell surface. For comparison, the assays of these compounds as inhibitors of KDR in corresponding enzyme and cellular systems are included. The enzyme and cellular experiments are complementary, as the enzyme assay measures more precisely the affinity of the compound for the ATP binding site, whereas the cellular assay confirms that the compound is an effective inhibitor of the activation of the full-length protein by its natural ligand.

ABT-869 is a multitargeted inhibitor with potent activity against several class III receptor tyrosine kinases and also has activity when administered orally in tumor models in mice (15). The other compounds tested for comparison have been described in the literature and have been shown to be kinase inhibitors with anticancer activity. Some compounds did better in one assay than the other, and ABT-869 was shown to be a potent inhibitor of CSF-1R and KDR in both the enzymatic and cellular assays.
The CSF-1R cellular assay employs the analysis of phosphorytosine in CSF-1R using Western blots compared with the total immunoprecipitated protein. This is a robust assay system since, as shown in Fig. 3, the band corresponding to CSF-1R protein does not change intensity on stimulation of the cells with M-CSF, and also tyrosine phosphorylation of this protein is negligible without induction. The calculated IC_{50} of the compounds can be influenced by factors such as the intensities of the bands on the scanned image and the uniformity of immunoprecipitation and sample loading on the gels. Within these limitations, the use of multiple inhibitor concentrations and normalization of the phosphorytosine determination with the total amount of CSF-1R in each lane allows a quantitative estimation of the potencies of the compounds.

Although imatinib was designed to be a relatively selective inhibitor of Abl (20), it also has activity against c-Kit and platelet-derived growth factor receptor, which is believed to be important in its therapeutic use (22). We found that this compound is also an ATP-competitive inhibitor of the CSF-1R catalytic domain, with a K_i of 120 nmol/L, and also has submicromolar activity in our cellular assay system. Our results with CSF-1R were unexpected because a previous report stated it does not affect the tyrosine phosphorylation of murine c-fms at concentrations up to 10 μmol/L. This difference in observed activity might be due to the species difference or that, in the previous work (23), the analysis was done using a Western blot of whole-cell lysates without immunoprecipitation, and it was not stated whether specific stimulation of c-fms was employed.

While this work was in progress, the inhibition of CSF-1R by imatinib in cells was reported (24) and shown to inhibit M-CSF-induced proliferation of a cytokine-dependent cell line. Treatment of normal hematopoietic progenitor cells with imatinib has been associated with inhibition of monocyte/macrophage colony formation (25), although this was not shown to be the result of CSF-1R inhibition. Although imatinib is not as potent an inhibitor of CSF-1R as some other kinase inhibitors, the additional effect of inhibition of this protein could be an important consideration in its use (24).

Except for imatinib, the other compounds described in this work are inhibitors of KDR, but each has its own pattern of selectivity of inhibition of other kinases (17–19, 26, 27). Their observed activity against CSF-1R is consistent with previous reports, and AG103736 is the only compound in this study other than imatinib having significant activity against Abl. As all of these molecules inhibit multiple kinases, each compound may have different activity profile upon evaluation as a therapeutic agent.

Our data show that direct assessment of the activation of CSF-1R in cells can be accomplished by monitoring receptor autophosphorylation. The use of a cell-based assay to confirm the inhibitory activity of lead compounds and drug candidates, such as ABT-869, against the CSF-1R protein in situ provides valuable information for their assessment as anticancer agents.

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References


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