UNC569, a Novel Small-Molecule Mer Inhibitor with Efficacy against Acute Lymphoblastic Leukemia In Vitro and In Vivo

Sandra Christoph1,2, Deborah DeRyckere1, Jennifer Schlegel1, J. Kimble Frazer3, Lance A. Batchelor3, Alesia Y. Trakhimets4, Susan Sather1, Debra M. Hunter7, Christopher T. Cummings1, Jing Liu5, Chao Yang5, Sandra Christoph1,2, Deborah DeRyckere1, Jennifer Schlegel1, J. Kimble Frazer3, Lance A. Batchelor3, Alesia Y. Trakhimets4, Susan Sather1, Debra M. Hunter7, Christopher T. Cummings1, Jing Liu5, Chao Yang5, Catherine Simpson6, Jacqueline Norris-Drouin6, Emily A. Hull-Ryde6, William P. Janzen5,6, Gary L. Johnson6,7, Xiaodong Wang5, Stephen V. Frye5,7, H. Shelton Earp III6,7, and Douglas K. Graham1

Abstract

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. Although survival rates have improved, patients with certain biologic subtypes still have suboptimal outcomes. Current chemotherapeutic regimens are associated with short- and long-term toxicities and novel, less toxic therapeutic strategies are needed. Mer receptor tyrosine kinase is ectopically expressed in ALL patient samples and cell lines. Inhibition of Mer expression reduces prosurvival signaling, increases chemosensitivity, and delays development of leukemia in vivo, suggesting that Mer tyrosine kinase inhibitors are excellent candidates for targeted therapies. Brain and spinal tumors are the second most common malignancies in childhood. Multiple chemotherapy approaches and radiotherapies have been attempted, yet overall survival remains dismal. Mer is also abnormally expressed in atypical teratoid/rhabdoid tumors (AT/RT), providing a rationale for targeting Mer as a therapeutic strategy. We have previously described UNC569, the first small-molecule Mer inhibitor. This article describes the biochemical and biologic effects of UNC569 in ALL and AT/RT. UNC569 inhibited Mer activation and downstream signaling through ERK1/2 and AKT, determined by Western blot analysis. Treatment with UNC569 reduced proliferation/survival in liquid culture, decreased colony formation in methylcellulose/soft agar, and increased sensitivity to cytotoxic chemotherapies. MYC transgenic zebrafish with T-ALL were treated with UNC569 (4 μmol/L for two weeks). Fluorescence was quantified as indicator of the distribution of lymphoblasts, which express Mer and enhanced GFP. UNC569 induced more than 50% reduction in tumor burden compared with vehicle- and mock-treated fish. These data support further development of Mer inhibitors as effective therapies in ALL and AT/RT. Mol Cancer Ther; 12(11); 2367–77. ©2013 AACR.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children and represents nearly 30% of all pediatric cancers (1, 2). Although the overall survival (OS) rate for pediatric B-cell ALL is approximately 85%, specific biologic subtypes, including T-ALL, have a poorer prognosis even with current therapeutic protocols and treatment of relapsed ALL remains a challenge (3). In addition, the intensified therapy used in current protocols is associated with a significant and increased risk for short- and long-term toxicities (slowed growth, organ damage, and secondary malignancy; refs. 4, 5). Therefore, novel, more effective, and less toxic therapies are needed.

Tyrosine kinases are frequently abnormally regulated in cancer cells. Overexpression of Mer receptor tyrosine kinase (RTK), a member of the Tyro3/Axl/Mer family of RTKs, has been reported in a variety of human cancers, including B- and T-ALL (6–8). Ectopic expression of Mer in lymphocytes in transgenic mice promotes the development of leukemia/lymphoma (9, 10). In
humans, Mer is ectopically expressed in pediatric T-cell ALL and pre-B-cell ALL patient samples. In contrast, Mer is not expressed in normal mouse and human T- and B-lymphocytes at any stage of development (11). Inhibition of Mer expression by short hairpin RNA (shRNA) has been shown to reduce prosurvival signaling, dramatically increase the sensitivity of leukemia cells to cytotoxic agents, and significantly delay development of leukemia in a mouse model (12, 13). Taken together, these data provide a rationale for targeting Mer as a therapeutic strategy in ALL.

Brain and spinal tumors are the second most common malignancies in childhood after leukemia (14). Atypical teratoid/rhabdoid tumors (AT/RT) are rare tumors of the central nervous system. However, in patients less than 3 years of age this tumor accounts for up to 20% of cases. Multiple chemotherapy approaches and radiotherapies have been attempted, yet OS remains dismal (15). This aggressive tumor remains a significant challenge in pediatric neurooncology and new therapeutic approaches are needed. The abnormal expression of Mer in AT/RT cells provides a rationale for targeting Mer as a therapeutic strategy in this aggressive cancer type.

In the past several years, tyrosine kinase inhibitors (TKI) have taken on an increasingly important role in the treatment of cancer. Imatinib, a selective inhibitor of the BCR–ABL tyrosine kinase most impressively validated the concept of designing a small-molecule TKI to treat a defined patient population. The improvement in survival has been dramatic in chronic myelogenous leukemia, which is driven by the BCR–ABL translocation (16). Because some patients experienced relapse due to resistance-conferring point mutations within BCR–ABL, the second-generation ABL kinase inhibitors nilotinib and dasatinib were developed and other ABL kinase inhibitors with activity against BCR–ABL (T315I) including ponatinib are being investigated (17, 18). Other examples of small-molecule TKI under investigation are vandetanib with activity against BCR–ABL (T315I) including ponatinib are being investigated (17, 18).

Immunoprecipitation and detection of Mer in ALL cell lines, in a transgenic zebrafish T-ALL model and in AT/RTs.

Materials and Methods

Tissue culture

Jurkat (T-ALL) and 697 (B-ALL) human leukemia cell lines were obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). These cell lines were cultured in RPMI-1640 media (HyClone Laboratories) supplemented with 10% FBS (Atlanta Biologicals) and penicillin/streptomycin (100 U/mL and 100 μg/mL; HyClone Laboratories). The AT/RT cell line, BT12, was obtained from Bernard Weissman (UNC Lineberger) and was grown in RPMI-1640 media supplemented with 15% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. The 697 and the Jurkat cell lines were authenticated. The identities of 697 (6/2012) and Jurkat (12/2012) cell lines were confirmed by short-tandem repeat (STR) analysis (28) and matched with the information in the DSMZ database. At this time, there is no STR profile publicly available for the BT12 cell line. However, the STR profiles (12/2012) are singular and do not match any in the ATCC or DSMZ collections. All cell cultures were tested negative for Mycoplasma contamination. Recombinant human growth arrest-specific protein 6 (Gas6; #885-GS; R&D Systems) was reconstituted in PBS. Inhibitor UNC569 was synthesized as previously described (27) and stock solutions were prepared in dimethyl sulfoxide (DMSO) at 3 mmol/L.

Immunoprecipitation and detection of phosphorylated Mer

ALL cell cultures were treated with 0.12 mmol/L pervanadate for 3 minutes to stabilize the phosphorylated form of Mer. Cells were lysed in 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mmol/L sodium molybdate, and protease inhibitors (Complete mini; Roche Molecular Biochemicals) and Mer was immunoprecipitated by incubating...
with mouse monoclonal anti-Mer antibody (MAB8912; R&D Systems) and rec- Protein G- sepharose 4B beads (#10-1242; Invitrogen). Immune complexes were collected by centrifugation and washed with lysis buffer. Beads were resuspended in Laemmli buffer (6.25 mmol/L Tris–HCl pH 6.8, 25% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.01% bromophenol blue) and boiled to elute bound protein. Phosphorylated and total Mer proteins were detected by Western blot analysis using a proprietary α-phospho Mer antibody (Phospho Solutions; ref. 29) and anti-human Mer antibody (#1633-1; Epitomics). To determine the relative changes in phosphorylation between UNC569-treated and -untreated cells, the densities of individual bands were measured using ImageJ software (NIH, Bethesda, MD). IC_{50} values were determined by nonlinear regression using GraphPad Prism software.

**Western blot analysis**

Whole-cell lysates were prepared and resolved by SDS–PAGE. The following antibodies from Cell Signaling Technology were used for Western blot analysis according to the manufacturer’s recommendations: anti-phospho-Akt (Ser473, #9271), anti-Akt (#2922); anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; pERK1/2, Thr202/Tyr204, #9106), anti-p44/42 MAPK (Erk1/2, #9102), anti-PARP (#9542), anti-caspase-3 (#9662), anti-tubulin (#2125), anti-tym3 (#5585), and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; #5863, HRP). Anti-actin antibody (#sc-1616 HRP) was purchased from Santa Cruz Biotechnology and anti-Axl antibody (H00008656-M1; Novus) was used. Secondary antibodies were labeled with horseradish peroxidase (HRP)–conjugated secondary antibodies (donkey-anti-goat, #31430; donkey-anti-mouse, #31410; donkey-anti-rabbit, #31320). HRP-labeled secondary antibodies were detected by chemiluminescence (PerkinElmer). For BT12 experiments, gels with electrophoresed, Mer immunoprecipitates were transferred and blotted with phosphotyrosine antibody (#sc-508 HRP) or back blotted with the NIH anti-Mer polyclonal.

**Colorimetric assay for detection of metabolically active cells**

Jurkat and 697 cells were plated at optimal density in 96-well plates (3 × 10^4 cells/mL) and cultured for 8 hours before addition of UNC569 or an equivalent volume of DMSO control. After 24 hours, cells were harvested and plated in fresh medium containing UNC569 or vehicle only for an additional 24 hours. MTT reagent (#M5655 Sigma) was added to a final concentration of 0.65 mg/mL and the cells were incubated for another 4 hours. Solubilization solution (2 × 10% SDS in 0.01 mol/L HCl) was added and plates were incubated at 37°C overnight. Optical density was determined at 562 nm with a reference wavelength of 650 nm. BT12 cells were plated at optimal density in 96-well plates (5 × 10^4 cells/mL) and cultured for 8 hours before addition of UNC569 or an equivalent volume of DMSO control. After 48 hours, 20 μL of MTS reagent (CellTiter 96 AQueous One Solution Reagent #G3582; Promega) was added to each well. The cells were incubated for 4 hours. Absorbance was recorded at 490 nm using a 96-well plate reader. Relative cell numbers for all cell lines were calculated by subtraction of background absorbance and normalization to untreated controls. IC_{50} values were determined by nonlinear regression using GraphPad Prism software.

**Apoptosis assay**

Jurkat or 697 cells were plated at 3 × 10^5 cells per milliliter and treated with UNC569 and/or cytotoxic agents (methotrexate, #A6770; Sigma; etoposide, #341205; CalBiochem) for 48 hours. Alternatively, cells were treated with UNC569 for 24 hours, then harvested and cultured in fresh medium containing UNC569 or vehicle only for an additional 24 hours. Cells were collected by centrifugation at 240 × g for 5 minutes. Cell pellets were resuspended in PBS containing 1 μmol/L YO-PRO-1 (Y3603; Invitrogen) and 1.5 μmol/L propidium iodide (PI; P3566; Invitrogen). Samples were incubated for 15 minutes and fluorescence was detected using a FC500 flow cytometer and analyzed with CXP data analysis software (Beckman Coulter).

**Methylcellulose assay**

Jurkat or 697 cells were plated in methylcellulose-based medium (#1101; ReachBio) containing UNC569 or DMSO control according to the manufacturer’s protocol (500 cells/mL methylcellulose, 4% agar). After the initial plating, additional UNC569 or DMSO control was added to the top of the methylcellulose in a volume of 100 μL every other day. Colonies were grown for 8 days and then counted. Bright field images were taken with a spinning disk confocal 31 Marianas (Intelligent Imaging Innovations, Inc.) based on an inverted Zeiss Axio Observer Z1 (Carl Zeiss, Inc.). A CoolSnap HQ2 camera featuring 1,392 × 1,040 imaging pixels (Photometrics, Inc.) via a 5× Zeiss air objective was used. As imaging acquisition software Slidebook 5.0 (Intelligent Imaging Innovations, Inc.) was used. Single colony pictures were generated using a Nikon Eclipse TS100 microscope (Nikon, Inc.) with Nikon Plan 10X, numerical aperture 0.25 objective lens and with a Nikon Digital Sight DS-L2 camera equipped with Firmware DS-L2 Ver4.60.

**Soft agar assay**

BT12 cells were plated in 0.7% agarose (#CA3510-8 Denville Scientific) over a higher percentage agar base layer. Agarose was overlaid with medium [RPMI-1640 media supplemented with 15% FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL)] with UNC569.
Medium and UNC569 were renewed twice weekly. Colonies were grown for 14 days before staining with 2 mg/mL MTT (#M5655; Sigma). Representative pictures are scans made with Perfection V700 Scanner (Epson) and subsequent counting using ImageJ Software.

**Leukemic zebrafish experiments**

Transgenic zebrafish expressing human MYC in lymphocytes (31) were crossed to lck:EGFP zebrafish (32). Leukemic fish were identified via fluorescence microscopy and images were captured using an Olympus MVX10 Microscope with a SPOT Insight Camera and software package. Zebrafish with cancer were then treated by "immersion assay," as described previously (33). Briefly, fish were housed in 4 μmol/L UNC569 plus DMSO, equimolar DMSO vehicle alone, or water only. Daily water changes with fresh drug were conducted for a treatment duration of 14 days. Fluorescent images were captured at day 14 for comparison with pretreatment images. Images were normalized to an internal reference standard. Fluorescence intensity was determined using ImageJ software and plotted as a three-dimensional spatial representation for each image. Plots were integrated to generate volumetric quantifications of the area under the curve. GFP fluorescence was compared in the same fish pre- and posttreatment to assess responses, reflected as percentage gain or loss in GFP intensity (termed the "GFP score").

**Statistical analyses**

Statistically significant differences between means were determined using a two-tailed, unpaired Student t tests for zebrafish and cell culture experiments, respectively. The level of significance for all statistical analyses was chosen a priori to be $P < 0.05$. Statistical analyses were conducted using GraphPad Prism software (Version 5.0; GraphPad Software).

**Results**

**UNC569 inhibits Mer and downstream oncogenic signaling pathways in ALL cells**

Activation of Mer stimulates proliferative and antiapoptotic signaling, including the PI3K/AKT and MAPK/ERK pathways (12, 29). Western blot analysis was used to determine inhibition of Mer (phospho-Mer) in response to treatment with UNC569 and effects on downstream oncogenic signaling pathways in ALL cells. ALL cells were treated with UNC569 and a dose-dependent decrease in the levels of the active, phosphorylated form of Mer was observed in both 697 B-cell ALL (IC$_{50}$ = 141 ± 15 nmol/L) and Jurkat T-cell ALL (IC$_{50}$ = 193 ± 56 nmol/L) cells (Fig. 2A and B). Mer inhibition was achieved with lower UNC569 doses in the 697 cell line compared with the Jurkat cell line (Fig. 2A). The Jurkat cell line expressed Mer and Tyro3, but no Axl (Supplementary Fig. S1). To show specificity for Mer, we tested whether UNC569 inhibited the activation of Tyro3 (phospho-Tyro3) in addition to Mer. Jurkat cells were treated with UNC569 and no decrease in the levels of the active, phosphorylated form of Tyro3 was detected (Supplementary Fig. S2), suggesting that any phenotypic effects noted from UNC569 were not mediated via inhibition of the closely related Axl or Tyro3 tyrosine kinases.
Decreased Gas6 ligand-stimulated phosphorylation of ERK was observed in Jurkat cells treated with UNC569 relative to cells treated with vehicle only (Fig. 2C). In the 697 cell line, Gas6-stimulated AKT phosphorylation was decreased after treatment with UNC569. We did not anticipate significant changes in AKT activation in the presence of Gas6 or after treatment with UNC569 in Jurkat cells as in these cells AKT is constitutively active due to a mutation in PTEN, a phosphatase that regulates AKT activity (34). These data show that UNC569 can effectively inhibit the activation of Mer and downstream signaling, including the PI3K/AKT and MAPK/ERK pathways and indicate a biochemical mechanism by which UNC569 may influence oncogenic processes including cell growth, proliferation, and survival of ALL cells.

**UNC569 induces apoptosis in ALL cell lines**

To determine whether UNC569 inhibits oncogenic phenotypes in ALL cells, 697 and Jurkat cultures were treated with UNC569 or DMSO control only and effects on proliferation and/or survival were examined after 48 hours. Preliminary studies showed efficient Mer inhibition in ALL cells after 24 hours of treatment with UNC569, but phosphorylated Mer levels were increased by 48 hours (Supplementary Fig. S3). Thus, for the phenotypic studies described here, UNC569 and medium were replenished after 24 hours to ensure continuous Mer inhibition throughout the experiments. First, UNC569-mediated effects on cell number in 697 and Jurkat cultures were determined using the MTT assay. Inhibition of Mer by UNC569 decreased the number of metabolically active Jurkat and 697 cells after 48 hours in culture (Fig. 3A). The 697 cell line was more sensitive to UNC569 (IC50 = 0.5 μmol/L; 95% confidence interval (CI), 0.35–0.75 μmol/L) compared with Jurkat cells (IC50 = 1.2 μmol/L; 95% CI, 0.76–1.78 μmol/L). This observation was consistent with the relative sensitivity of these cell lines for Mer inhibition by UNC569 (Fig. 2A). The results of the MTT assay can be
attributed to either a decrease in proliferation or an increase in cell death. To distinguish between these two effects, the incidence of apoptosis in response to treatment with UNC569 was analyzed by flow cytometry after cells were stained with YO-PRO-1 iodide (YoPro) and PI dyes to identify early apoptotic (YoPro⁺) and dead cells (YoPro⁺, PI⁺). Both cell lines exhibited a statistically significant increase in the percentage of apoptotic and dead cells in comparison with the DMSO control in response to treatment with UNC569 (Fig. 3B). The apoptosis signaling effectors caspase-3 and PARP were also analyzed. Jurkat and 697 cells treated with UNC569 exhibited increased levels of cleaved caspase-3 and cleaved PARP compared with cells treated with DMSO control only (Fig. 3C), confirming the induction of apoptosis in response to treatment with UNC569.

**UNC569 increases sensitivity of leukemia cells to standard ALL chemotherapies**

Jurkat and 697 cells were treated with the chemotherapeutic agents methotrexate or etoposide (VP 16) for 48 hours, either alone or in combination with UNC569 to investigate the interaction between UNC569 and cytotoxic chemotherapies that are currently in clinical use. Apoptotic and dead cells were detected by flow cytometry after staining with YoPro and PI. Treatment with the low concentration of UNC569 used here was not sufficient to induce significant cell death in the Jurkat and 697 cell lines, but resulted in a significant increase in the percentage of dead cells in response to treatment with methotrexate or etoposide compared with treatment with the chemotherapeutic agents alone (Fig. 4A and B). More specifically, treatment with UNC569 resulted in a significant increase in apoptotic and dead cells in Jurkat cultures treated with methotrexate relative to cultures treated with methotrexate alone (26.4% ± 2.9% vs. 17.5% ± 1.3%; P = 0.03). Similarly, treatment of 697 cells with UNC569 resulted in a statistically significant increase in apoptotic and dead cells compared with etoposide alone (43.3% ± 2.6% vs. 33.03% ± 0.42%; P = 0.02) and a trend when compared methotrexate alone (28.93% ± 4.96% vs. 18.13% ± 3.63%; P = 0.1). These results show increased sensitivity of ALL cells to cytotoxic chemotherapies in response to Mer inhibition with UNC569.

**UNC569 decreases colony formation in ALL cell lines**

To further investigate whether Mer inhibition by UNC569 resulted in changes in the oncogenic properties of ALL cells, colony-forming potential in methylcellulose-based medium was assessed. Treatment with UNC569 (400 nmol/L) significantly inhibited the ability of the 697 (Fig. 5A; 95.9 ± 16.8 vs. 14.8 ± 12.8 colonies; P = 0.02) and Jurkat (Fig. 5B; 100.1 ± 23.4 vs. 25.6 ± 6.4 colonies, P = 0.04) cell lines to form colonies compared with cells treated with DMSO control only. Both the size (Fig. 5C) and the number (Fig. 5D) of the colonies were decreased with
increasing doses of UNC569. In contrast, Jurkat cells treated with the control compound UNC1653 did not show any reduction of colony formation in methylcellulose (Supplementary Fig. S4). UNC1653 is a structurally to UNC569-related negative control compound, which does not inhibit Mer (data not shown).

**UNC569 inhibits Mer phosphorylation, reduces proliferation and/or survival, and reduces colony formation in BT12 pediatric rhabdoid tumor cells**

We repeated similar analysis in the BT12 adherent AT/RT cell line with the aim to test whether UNC569 had antioncogenic effects in a pediatric solid tumor. Western blot analysis was used to determine inhibition of phospho-Mer in response to treatment with UNC569 in AT/RT cells. BT12 cells were treated with UNC569 and a dose-dependent inhibition Mer phosphorylation was observed (Fig. 6A). To investigate whether UNC569 inhibits oncogenic phenotypes in BT12 cells, proliferation and/or survival were examined using a MTT assay after 48 hours of treatment with UNC569 or vehicle only. Inhibition of Mer by UNC569 significantly decreased the number of metabolically active BT12 cells after 48 hours in culture (Fig. 6B; IC$_{50}$ = 0.85 µmol/L; 95% CI, 0.4–1.7 µmol/L). UNC569
inhibited colony formation of 697 and Jurkat cells in methycellulose. Furthermore, colony-forming potential in soft agar was assessed to examine whether Mer inhibition by UNC569 resulted in changes in the oncogenic properties of BT12 cells. Treatment with UNC569 (1 μmol/L) significantly inhibited the ability of the BT12 cell line (294.7 ± 16.6 vs. 175.1 ± 28.3 colonies; \( P = 0.02 \)) to form colonies compared with cells treated with vehicle only (Fig. 6C and D).

UNC569 induces disease regression in a transgenic zebrafish model

The effect of UNC569 in an in vivo model of T-ALL was examined using a transgenic zebrafish expressing lymphocyte-specific human MYC controlled by the native zebrafish rag2 promoter (Fig. 7A). These fish develop highly penetrant T-cell malignancies (31). This line was bred to a second transgenic zebrafish line with T cell–specific enhanced GFP expression (32). Consequently, T-cell cancers in these animals are GFP-positive (GFP⁺). Leukemic zebrafish were treated continuously for 2 weeks by immersion in 4 μmol/L UNC569, DMSO alone, or mock-treated in water only. Images were captured before and after treatment with UNC569, and GFP fluorescence intensity was determined as an indicator of tumor burden. UNC569-treated animals showed ≥50% disease regression in 10 of 18 fish, compared with more than 50% responses in only 2 of 14 DMSO-treated and 0 to 10 mock-treated animals, respectively (Fig. 7 and Supplementary Fig. S5). Tumor burden decreased by an average of 47.8% (Fig. 7B), with only 2 of 18 animals having progression during treatment. DMSO-treated fish typically had either progressive (6 of 14 fish) or stable (≤20% regression; 4 of 14 fish) disease, leading to an average tumor increase of 13.0%. Likewise, mock-treated fish exhibited an average increase in tumor burden of 9.9% (Fig. 7B), with 4 of 10 animals having outright progression and 5 of 10 having stable disease. The 47.8% reduction in tumor burden in UNC569-treated animals was statistically superior to the 13.0% and 9.9% increases observed in the two control groups (\( P = 0.0013 \) and 0.0003, respectively). Even in short-treatment courses against already-established cancers, UNC569 clearly shows potent in vivo efficacy as a single-agent against T-ALL, with no apparent toxicity.

Discussion

Mer contributes to leukemogenesis and progression of B- and T-cell ALL (10, 11). In this study, we have extended our previous finding of ectopic Mer expression in human ALL and evaluated Mer inhibition as a novel therapeutic strategy for treatment of ALL. In addition, as a critical step toward development of translational agents targeting Mer for oncology applications, we investigated the use of a novel, first in class Mer-selective small-molecule TKI, UNC569, for treatment of ALL using cell culture and zebrafish models.

![Figure 6](image-url)

Figure 6. UNC569 inhibits Mer phosphorylation, reduces proliferation and/or survival and reduces colony formation in BT12 pediatric rhabdoid tumor cells. A, p-Mer and Mer levels were evaluated by Western blot analysis following treatment with UNC569. BT12 cell cultures were treated with the indicated concentrations of UNC569. Pervanadate was added to cultures for 3 minutes to stabilize the phosphorylated form of Mer. Cells were lysed, immunoprecipitated with polyclonal C-terminal Mer antibody N-14, and subjected to SDS-PAGE. Gels with electrophoresed, Mer immunoprecipitates were transferred and blotted with phosphotyrosine antibody or back blotted with the NIH anti-Mer polyclonal. B, UNC569 reduces proliferation and/or survival of BT12 cells. These data were obtained using the MTT assay. Cell cultures were treated with the indicated concentrations of UNC569 for 48 hours and relative cell numbers were determined. Mean values ± SEs derived from three independent experiments are shown. C, UNC569 reduces long-term colony growth of BT12 cells in soft agar assay. Cells were plated in soft agar in the presence of the indicated concentrations of UNC569. Mean values ± SEs derived from three independent experiments are shown (\( ^* \), \( P < 0.05 \)). D, representative pictures from BT12 soft agar cultures treated with the indicated concentrations of UNC569 or DMSO control only. Pictures are scans made with Perfection V700 Scanner.
The studies described here show potent inhibition of Mer by UNC569 in both B-ALL and T-ALL cell lines. Previous studies have shown activation of MAPK/ERK and PI3K/AKT signaling pathways downstream of Mer in tumor cells and roles for these proteins in cell survival and proliferation are well established (12, 29, 35, 36). Consistent with these observations, we found activation of these pathways downstream of Gas6 in the 697 and Jurkat ALL cell lines. Furthermore, treatment with UNC569 resulted in decreased activation of downstream signaling molecules, including phospho-ERK and phospho-AKT (Fig. 2C). UNC569-mediated inhibition of Mer-dependent prosurvival pathways resulted in dose-dependent induction of apoptosis (Fig. 3B and C) and decreased colony-forming potential in methylcellulose (Fig. 5). Thus, UNC569 inhibits oncogenic signaling and oncogenic properties in leukemia cells. In addition, inhibition of Mer with UNC569 resulted in increased cell death in response to treatment with methotrexate and etoposide (Fig. 4A and B). These data suggest that Mer inhibition may be particularly effective in combination with cytotoxic chemotherapies, allowing for increased therapeutic response and/or reduction of chemotherapy dose and associated potential side effects. Of note, MTX is used in standard pediatric ALL chemotherapy regimens and etoposide is a major component of relapse therapy. Similar results were recently reported for the 697 and Jurkat shRNA Mer knockdown (12, 36). A shRNA Mer knockdown led to decreased ERK/AKT activation, increased apoptosis, a chemosensitization with commonly used chemotherapeutic agents, and a decreased colony-forming potential in methylcellulose or soft agar. Thus, these data provide rationale for introduction of Mer TKIs into the clinic in combination with agents that are already known to be clinically effective.

In addition, in an effort to expand upon potential clinical uses of UNC569, we investigated the potential efficacy of this ligand for the treatment of AT/RT. Despite
some development in the treatment of AT/RT (37, 38). long-term outcomes remain not optimal. Furthermore, current multimodality therapy regimes for AT/RT are toxic and associated with short- and long-term toxicities. AT/RT is a malignancy that requires the development of more effective and less toxic therapies to improve survival. We have shown in this study that the treatment of the AT/RT cell-line BT12 with UNC569 resulted in an inhibition of Mer activation (Fig. 6A), in a decrease of metabolically active cells measured by MTT (Fig. 6B) and in a reduction of the ability of colony formation in soft agar (Fig. 6C and D).

Finally, UNC569 showed potent efficacy as a single-agent in a zebrafish model of MYC-driven T-ALL, with many animals showing significant regression of established tumors after only 2 weeks of treatment (Fig. 7 and Supplementary Fig. S5). Notably, toxicity was not observed in these in vivo assays. Taken together these results suggest that Mer inhibition may have use as a single-agent and may also synergize with standard chemotherapeutics to enhance cancer cell death in both solid tumors and hematopoietic malignancies.

Disclosure of Potential Conflicts of Interest

X. Wang, J. Liu, C. Yang, S.V. Frye, and D. Kireev have ownership interest in a patent for Pyrazolopyrimidine Compounds for the Treatment of Cancer (WO Patent 2011146313, 2011). S.V. Frye is President acting CEO of a consultant for, and has ownership interest in Meryx, Inc. X. Wang is co-founder of Meryx, Inc. No potential conflicts of interest were disclosed by the other authors.

disclaimer

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Authors’ Contributions

Conception and design: S. Christoph, D. DeRyckere, J. Liu, C. Yang, G.L. Johnson, S.V. Frye, H.S. Earp III, D.K. Graham

Development of methodology: S. Christoph, D. DeRyckere, J.K. Frazer, C. Yang, E.A. Hull-Ryde, W.P. Janzen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Christoph, D. DeRyckere, J. Schlegel, J.K. Frazer, A.Y. Trakhmets, S. Sather, D.M. Hunter, C. Cummings, C. Yang, E.A. Hull-Ryde, H.S. Earp III


Writing, and/or revision of the manuscript: S. Christoph, D. DeRyckere, J. Schlegel, J.K. Frazer, J. Liu, D. Kireev, J. Norris-Drouin, E. A. Hull-Ryde, W.P. Janzen, X. Wang, S.V. Frye, H.S. Earp III, D.K. Graham

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Christoph, D. DeRyckere, S.V. Frye, D.K. Graham

Acknowledgments

The authors thank Karen Helm, Christine Childs, and Lester Acosta at the University of Colorado Colorado Center Flow Cytometry Core for their expert technical assistance (supported by grant P30CA046934) and Randall Wong of the University of Colorado Denver Diabetes & Endocrinology Research Center Molecular Biology Core Facility (supported by NIH P30 DK57516) for cell line authentication services. The authors also thank Weike Zhang for the permission to use the patent "pyrazolopyrimidine compounds for the treatment of cancer. WO Patent 2011146313." In vivo imaging was conducted using the IVIS shared resource at the University of Colorado Cancer Center (supported by grant P30-CA046934). In vitro imaging experiments were carried out at the University of Colorado Anschutz Medical Campus Advance Light Microscopy Core supported in parts by NIH/NCRR Colorado CTSI Grant Number UL1-RR025780.

Grant Support

This work was supported in parts by the NIH (ROICAI35708; D.K. Graham) and by a CureSearch Research Fellowship Award (#020660; J.K. Frazer). D.K. Graham is a Damon Runyon-Novartis Clinical Investigator supported in parts by the Damon Runyon Cancer Research Foundation (CI-39-07). Funding was also provided by the National Cancer Institute, NIH, under contract no. HHSN26120080001E.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 15, 2013; revised July 22, 2013; accepted July 26, 2013; published OnlineFirst August 30, 2013.

References


Molecular Cancer Therapeutics

UNC569, a Novel Small-Molecule Mer Inhibitor with Efficacy against Acute Lymphoblastic Leukemia *In Vitro* and *In Vivo*

Sandra Christoph, Deborah DeRyckere, Jennifer Schlegel, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0040

Supplementary Material  Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/09/03/1535-7163.MCT-13-0040.DC1

Cited articles  This article cites 37 articles, 15 of which you can access for free at: http://mct.aacrjournals.org/content/12/11/2367.full.html#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at: /content/12/11/2367.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.