Drug Repurposing Identifies a Synergistic Combination Therapy with Imatinib Mesylate for Gastrointestinal Stromal Tumor

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Abstract

Gastrointestinal stromal tumor (GIST) is a rare and therefore often neglected disease. Introduction of the kinase inhibitor imatinib mesylate radically improved the clinical response of patients with GIST; however, its effects are often short-lived, with GISTs demonstrating a median time-to-progression of approximately two years. Although many investigational drugs, approved first for other cancers, have been subsequently evaluated for the management of GIST, few have greatly affected the overall survival of patients with advanced disease. We employed a novel, focused, drug-repurposing effort for GIST, including imatinib mesylate–resistant GIST, evaluating a large library of FDA-approved drugs regardless of current indication. As a result of the drug-repurposing screen, we identified eight FDA-approved drugs, including fludarabine phosphate (F-AMP), that showed synergy with and/or overcame resistance to imatinib mesylate. F-AMP induces DNA damage, Annexin V, and caspase-3/7 activities as the cytotoxic effects on GIST cells, including imatinib mesylate–resistant GIST cells. F-AMP and imatinib mesylate combination treatment showed greater inhibition of GIST cell proliferation when compared with imatinib mesylate and F-AMP alone. Successful in vivo experiments confirmed the combination of imatinib mesylate with F-AMP enhanced the antitumor effects compared with imatinib mesylate alone. Our results identified F-AMP as a promising, repurposed drug therapy for the treatment of GISTs, with potential to be administered in combination with imatinib mesylate or for treatment of imatinib mesylate–refractory tumors. Mol Cancer Ther; 13(10); 2276–87. ©2014 AACR.

Introduction

Rare or orphan diseases are defined in the United States as diseases affecting less than 200,000 patients. Although orphan diseases individually affect small groups of patients, collectively 25 to 30 million Americans suffer from rare diseases (1). Given the costs associated with the discovery, development, registration, and commercialization of new drug treatments, it has traditionally been difficult for pharmaceutical companies to achieve an adequate return on investment for orphan diseases. As a result, today, we have more than 6,000 rare diseases that lack effective treatments. Therefore, recent attention has been paid to the merits of exploring new potential uses of FDA-approved drugs, a drug development strategy termed drug repurposing. Repurposing FDA-approved drugs create opportunities to advance promising new treatments to patients suffering from rare diseases much more quickly and at substantially lower development costs (2). Most importantly, repurposing FDA-approved drugs provides treatment options for patients whose disease progresses at a rate that is incompatible with the 12- to 17-year time period required to discover, develop, and commercialize new drugs.

Gastrointestinal stromal tumor (GIST), although considered rare, is the most common mesenchymal malignancy of the digestive tract with an estimated annual incidence of 6,000 cases in the United States (3). Management of the disease has been transformed by the identification of activating mutations in the tyrosine kinase receptors, KIT and PDGFRA, in approximately 85% of GISTs (4–6). These discoveries have led to the unprecedented disease control of advanced GIST with the introduction of the kinase inhibitors imatinib mesylate (IM), sunitinib malate, and regorafenib (6–22). However, the success of imatinib mesylate in GIST has been tempered by the fact that the treatment only increases the median time to tumor progression by approximately two years (6, 11–23). Therefore, it is clear that additional therapeutic approaches are needed.
We previously reported through a quantitative drug screen of FDA-approved drugs in GIST cells that four drugs, auranofin, bortezomib, idarubicin HCl, and F-AMP, demonstrated selective anticancer activity as single agents (1). Auranofin was prioritized for validation, and as a result of our previous studies, we obtained FDA clearance to proceed with a phase I study in patients with recurrent GIST (1). This drug-repurposing trial will soon be underway to translate these findings to GIST patients with recurrent and metastatic disease (1). In this report, we describe how we have leveraged our previous work, focusing on identifying repurposed drugs that might work in combination with imatinib mesylate.

Materials and Methods

General methods

All cell proliferation and caspase-3/7 activity measurements were made on 384-well, black μClear microplates (Greiner bio-one). Cell proliferation experiments were assessed using the CellTiter-blue or CellTiter-glo reagent according to the manufacturer’s protocol (Promega). Fluorescence or luminescence measurements were made using the Infinite M200 Pro plate reader (Tecan). Data were normalized to percentage inhibition.

All images were taken under an Eclipse 80i fluorescence microscope (Nikon) equipped with a QIClick digital CCD camera (QImaging). Images were acquired and analyzed by the MetaMorph Imaging System (Molecular Devices).

Chemicals and antibodies

The FDA-approved drug library contains 796 drugs with known bioavailability and safety profiles in humans were provided by the Lead Development and Optimization Shared Resource within the NCI-designated Cancer Center at the University of Kansas Medical Center (Kansas City, KS). Imatinib mesylate and F-AMP were purchased from Selleckchem and dissolved in sterile water and dimethyl sulfoxide (DMSO) before study, respectively. The following antibodies were used: anti-c-KIT, anti-p-c-KIT (Tyr719), anti-AKT, anti-p-AKT (Ser473) anti-p-AKT (Thr308), anti-ERK1/2, anti-p-ERK1/2 (Thr202/Tyr204), anti-cleaved caspase 3 (Cell Signaling Technology), anti-β-actin (Sigma), and anti-Ki67 (Dako).

Cell culture

Cells were cultured as described previously (1). Briefly, GIST-T1 cells (c-KIT exon 11 heterozygous mutation, obtained in 2006) were kindly provided by Takahiro Taguchi (Department of Human Health and Medical Science, Graduate School of Kuroshio Science, Kochi University, Kochi, Japan) and maintained in DMEM containing 10% FBS and supplied with 10 μmol/L imatinib mesylate to maintain drug resistance. Imatinib mesylate was removed from the culture for indicated hours in experiments with GIST T1-10R cells. Hs 919.T cells were purchased from ATCC in 2012 and maintained in DMEM containing 10% FBS. All cells were supplied with 1% penicillin/streptomycin and were maintained in a 5% CO2 atmosphere at 37°C. Authentication for cell lines that were not purchased from ATCC was carried out by the University of Kansas Cancer Center Clinical Molecular Oncology Laboratory.

High-throughput synergy screening

Screening conditions were optimized as described previously (1). Briefly, drugs or vehicle (DMSO) were pre-loaded by the University of Kansas High-Throughput Screen Laboratory to each well to give a final dose ranging from 10 to 0.078 μmol/L (serial two-fold dilutions). Cells were aliquoted into plates with or without imatinib mesylate. The imatinib mesylate concentrations used for the combination study were: 10, 10, and 40 nmol/L for GIST-T1, GIST T1-10R, and GIST 882 cells, respectively.

Drug combination studies

Cells were seeded and allowed to attach overnight. Imatinib mesylate and the FDA-approved drugs were archived in robotically accessible vials, to which media were added in preparation for addition to master plates by a Nimbus 96 liquid-dispensing workstation (Hamilton). Liquid transfers to dilution and assay plates were handled using the same workstation adapted for the combination study procedure. Each 384-well master plate contained four 9 × 9 dose-matrix blocks, with eight serial two-fold dilutions (concentrations ranging from 7.8 nmol/L to 1 μmol/L) of the top concentration for each agent. Additional wells were reserved for untreated and vehicle-treated control wells. The cytotoxicity of each drug and their combinations was assessed by drug response curve, median-effect plot, and normalized isobologram using the CalcuSyn Software (now replaced by CompuSyn). The median-dose effect is defined using the following equation:

\[
\frac{f_a}{f_u} = \left( \frac{D}{D_{m}} \right)^m
\]

where \(f_a\) is the fraction of cells affected by drug, \(f_u\) is the fraction of cells unaffected by drug, \(D\) is the drug concentration, \(D_{m}\) is the median-effect dose, and \(m\) is the slope or kinetic order (26). The combination index (CI) for each two-drug interactions is defined using the following equation:

\[
CI = \frac{(D_{1})_{1} + (D_{2})_{2}}{(D_{1})_{1} + (D_{2})_{2}}\left(\frac{D_{1}}{D_{m1}}\right)^{f_a/f_u} + \left(\frac{D_{2}}{D_{m2}}\right)^{f_a/f_u}
\]

where CI(1, 1, and 1) indicate synergism, additive effect, and antagonism, respectively. \((D_{1})_{1} \sigma (D_{2})_{2}\) is for \((D_{1})_{1} \sigma (D_{2})_{2}\) “alone” that inhibits a system x% (26–28).
Comet assay

GIST-T1 cells were treated for 3 hours with imatinib mesylate and F-AMP at 2 and 10 μmol/L, respectively. The comet assay was performed under alkaline conditions as described by Singh and colleagues with minor modifications (29). Briefly, cells in ice-cold PBS were mixed with 0.5% low melting point agarose (Promega). Then, the suspensions were cast onto microscope slides precoated with 1% normal melting point agarose and allowed to solidify at 4°C. Solidified slides were immersed in lysis solution, followed by DNA denaturation solution, and prechilled TBE electrophoresis solution. Electrophoresis was conducted at 4°C at an electric field strength of 1 volt/cm. After electrophoresis and rinse, gels were then dehydrated in cold 70% ethanol, air-dried, and stained with ethidium bromide. At least 150 cells in 20 randomly selected fields were evaluated for each sample. DNA damage was graded by Tail DNA%, which is defined as follows: Tail DNA intensity/cell DNA intensity × 100%. Grade 0,0%; grade 1, 0%–10%; grade 2, 10%–30%; grade 3, 30%–50%; grade 4, >50%. Three independent experiments were performed.

Early apoptosis assay

Cells were seeded and allowed to attach overnight. Cells were then treated with drugs for 48 hours. Early apoptosis cells were detected and analyzed by the Guava Nexin Annexin V assay Kit using Guava easyCyte sampling flow cytomter (Millipore).

Cell viability and caspase-3/7 activity assay

Cells were plated and treated similar to the combination screening experiments at indicated concentrations. Cell viability was evaluated using CellTiter-blue reagent (Promega). After reading fluorescence, Caspase-Glo 3/7 Assay Reagent (Promega) was used according to the manufacturer’s instructions. After background subtraction, the caspase-3/7 activities were normalized with cell viability and expressed as fold changes to the relative vehicle controls.

Western blot analysis

Cells were seeded and allowed to attach overnight. Then, cells were treated with drugs at indicated doses for 6 hours. Whole-cell extracts were prepared as described previously (18). Briefly, for each specimen, 50 μg of whole-cell extract was electrophoresed on 10% precast polyacrylamide gel (Bio-Rad) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies overnight at 4°C. After incubation with HRP-conjugated secondary antibody at room temperature, development was carried out using Immun-Star HRP Chemiluminescence Kits (Bio-Rad).

In vivo xenograft mouse model

All procedures were performed following the guidelines adopted by the Animal Care and Use Committee of the University of Kansas Medical Center. Female athymic nude mice (NCr-nu/nu, 5-week-old) were purchased from the NCI (Frederick, MD). Three million GIST-T1 cells in PBS, mixed with an equal volume of cold BD Matrigel Matrix High Concentration (BD Biosciences), were inoculated subcutaneously into the right flank of each mouse. When tumor volume reached 316 mm³ on average, mice were randomly assigned to 6 treatment groups and were treated as follows: (i) control (n = 8), saline, and 5% DMSO, oral gavage and intraperitoneal (i.p.) injection, respectively, once daily; (ii) imatinib mesylate 50 (n = 8), imatinib mesylate at 50 mg/kg, oral gavage, once daily; (iii) F-AMP 60 (n = 8), F-AMP at 60 mg/kg, i.p. injection, once daily in the first week, thereafter 5 days on and 2 days off per week; (iv) F-AMP 120 (n = 8), F-AMP at 120 mg/kg; (v) IM 50 + F-AMP 60 (n = 9); (vi) imatinib mesylate 50 + F-AMP 120 (n = 8). For combination treatments, the dosing schedules are the same as monotherapies. F-AMP was injected immediately after imatinib mesylate administration. Tumor volume and body weight were measured every 2 days. Tumor volume in mm³ was calculated by the following formula: volume = length × width)/2. The day after the last treatment administration, all mice were euthanized and gross necropsies were performed. Tumor tissues were either snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin and paraffin embedded. Tumor tissues were then subjected to hematoxylin and eosin (H&E) staining and immunohistochemical staining. Three major organs (livers, kidneys, and spleens, 6 mice per group) were collected and then subjected to H&E staining for histologic analysis.

Immunohistochemical staining

Sections (4 μm) from formalin-fixed, paraffin-embedded tumor xenografts were subjected to immunohistochemical staining of Ki67, c-KIT, and cleaved caspase-3. Briefly, after deparaffinization and rehydration, tissue sections were treated using citrate buffer (pH 6.0) and then hydrogen peroxide (3%). Sections were then incubated with primary antibodies and then with HRP-labeled polymer antibodies. The staining was visualized by DAB+ (Dako) and nuclei were counterstained with hematoxylin. Photos were captured. Ki67 and cleaved caspase-3 were quantified as the percentage of positive cells in 20 random fields.

Statistical analysis

In vitro data are reported as mean ± SD of 3 to 5 independent experiments. Values were compared using the Student t test or with one-way ANOVA when three or more groups were present using SigmaPlot software (Systat). In vivo data are expressed as mean ± SEM and statistical analyses were carried out with GraphPad Prism 6.0 (GraphPad Software). Two-tailed Student t test was applied for two-group comparison. A P value less than 0.05 was considered as statistically significant.
Results

High-throughput synergy screening identifies F-AMP as combination agents for GIST therapy

Using a robotic screening system, we exposed GIST-T1, GIST 882, GIST T1-10R cells, and the control Hs 919.T cells to the library of FDA-approved drugs in combination with imatinib mesylate. To determine synergistic effects, a CI was calculated for each drug combination. Seventeen drugs (albendazole, amsacrine, mebendazole, paclitaxel, vinorelbine, dactinomycin, digoxin, doxorubicin, mitoxantrone, nilotinib, plicamycin, topotecan, auranofin, digoxin, gentian violet, idarubicin, and ouabain) have synergistic effects with imatinib mesylate on GIST-T1 cell proliferation at the indicated concentrations (Supplementary Fig. S1). Sixteen drugs (chloroquine, cladribine, dazol, etoposide, teniposide, clofarabine, daunorubicin, digitoxin, doxorubicin, nilotinib, topotecan, auranofin, carbimazole, ouabain, gentian violet, and idarubicin) have synergistic effects with imatinib mesylate on GIST 882 cell proliferation at the indicated concentrations (Supplementary Fig. S2). Eight drugs (auranofin, bortezomib, carbimazole, digoxin, gentian violet, idarubicin, ouabain, and F-AMP) have synergistic effects with imatinib mesylate on GIST T1-10R cell proliferation (Supplementary Fig. S3). Ten drugs (bortezomib, doxorubicin, mitoxantrone, idarubicin HCl, digoxin, daunorubicin, vinorelbine, plicamycin, dactinomycin, and mebendazole) failed to show preferential cytotoxicity against GIST cell lines as compared with benign sarcoma Hs 919.T cell line (1). Across all three GIST cell lines, eight drugs showed some degree of synergy with imatinib mesylate, including auranofin, bortezomib, carbimazole, digoxin, gentian violet, idarubicin, ouabain, and F-AMP (FDA-approved drug hits, Supplementary Figs. S1–S3).

Combination studies of FDA-approved drug hits with imatinib mesylate in GIST cell lines

To validate the screening result, GIST or the control Hs 919.T cells were treated with eight FDA-approved drug hits alone or in combination with imatinib mesylate using the checkerboard design (Supplementary Fig. S4). Sixty-four different drug concentration combinations were assessed as indicated in Fig. 1. All drugs showed selectively higher growth inhibition on GIST cells compared with Hs 919.T cells (Fig. 1). We have generated the dose-response curves and median-effect plots of imatinib mesylate and FDA-approved drugs in combination to either agent alone. All drugs, but bortezomib, showed a dose-dependent response on GIST cells at the indicated concentrations. Fludarabine Phosphate Repurposed for GIST

Figure 1. Drug interaction signatures for GIST and benign osteoma cell lines representing all combinatorial data compiled in a 9 × 9 drug matrix. Cells were treated with vehicle, FDA-approved drug, imatinib mesylate (IM) or the drug combination for 72 hours. A, color scale for percentage of the inhibition values of cell proliferation. Synergistic antiproliferative effects of FDA-approved drugs and imatinib mesylate in cells were assessed by CellTiter Glo assay in GIST-T1 (B), GIST 882 (C), GIST T1-10R cells (D), and in Hs 919.T, a benign osteoid osteoma cell line (as a nonmalignant control; E) at 64 different drug ratios (concentrations range from 7.8 nmol/L to 1 μmol/L, half-dilutions for each drug). Right columns represent imatinib mesylate treatment alone and bottom rows represent FDA-approved drug treatment alone. Arrows indicate the increasing concentrations of each drug.
experimental conditions (Supplementary Figs. S5–S12). Median-effect plots linearize all dose-effect curves that followed the mass-action law principle. The slope calculated by the software (eq.1; refs. 26–28, 30) is then used to generate the normalized isobologram (eq. 2, Supplementary Figs. S5–S12). The combination data points in the normalized isobologram figures that fall on the bottom left indicate synergism. Therefore, apparent synergy could be observed in the normalized isobologram plots (Supplementary Fig. S5–S12). To preclude unintended observer bias and to distinguish additive from truly synergism, the combination index (CI) model has been employed to evaluate drug interactions, which was originally developed by Chou and colleagues (26–28, 30). The CI method is based on the multiple drug-effect equation of Chou–Talalay, which has been widely used for drug interaction studies (26–28, 30). The CI values were tabulated in Fig. 2. More than 50% of the CI values of idarubicin and carbimazole in combination with imatinib mesylate were less than 1, which indicates synergism. More than 50% of the CI values of digoxin, bortezomib, auranofin, ouabain, and gentian violet in combination with imatinib mesylate were greater than 1 and indicate these combinations are antagonistic on the GIST cells (Fig. 2). F-AMP in combination with imatinib mesylate was highly synergistic on the representative GIST lines (95%, 77%, and 84% CI values were less than 1 in GIST-T1, GIST 882, and GIST T1-10R cells, respectively) compared with the other seven FDA-approved drug hits (Fig. 2). On the basis of the well-documented F-AMP (31–34), and its antiproliferative effects on the imatinib mesylate–resistant cells, we chose to further characterize the activity of F-AMP in vitro and in vivo alone and in combination with imatinib mesylate.

Combination treatment with imatinib mesylate and F-AMP enhances DNA damage in GIST cells

In a previous study, we found that imatinib mesylate treatment alone led to a transient upregulation of p53 and imatinib mesylate–induced DNA damage (15). We speculated that the synergistic activities of imatinib mesylate and F-AMP could be the result of enhanced DNA damage. Therefore, comet assays were used to detect the effects of the combination treatment of imatinib mesylate and F-AMP on double-strand DNA breaks in GIST cells. After 3 hours, 2 μmol/L of imatinib mesylate or 10 μmol/L of F-AMP treatment led to significant DNA damage compared with control in GIST-T1 cells (Fig. 3A–C). The combination treatment significantly increased DNA damage by 2.1-fold ($P < 0.05$, grades 3–4) and 4.7-fold ($P < 0.01$, grades 3–4) compared with imatinib mesylate or F-AMP treatment alone, respectively (Fig. 3A–C).

Effect of F-AMP on PI3K, AKT, and ERK1/2 pathways in GIST cells

Exposure to imatinib mesylate resulted in dephosphorylation of KIT, AKT, and ERK1/2 in GIST cells (1, 18). Our results indicate that F-AMP treatment does not alter the PI3K, AKT, and ERK signaling pathways in GIST cells (Fig. 3D). Combined with the comet assay results, our data suggest that the imatinib mesylate treatment both inhibits PI3K, AKT, and ERK signaling pathways and induces DNA damage, whereas F-AMP appears to augment DNA damage.

F-AMP alone or in combination with imatinib mesylate induces apoptosis in GIST cells

Given the synergistic interaction we observed between F-AMP and imatinib mesylate in the combination treatment with imatinib mesylate and F-AMP enhances DNA damage in GIST cells

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F-AMP alone or in combination with imatinib mesylate induces apoptosis in GIST cells

Given the synergistic interaction we observed between F-AMP and imatinib mesylate in the combination
screening, we optimized three combinations that gave the lowest three CI values in each cell line as the optimal doses to evaluate the apoptosis by caspase-3/7 activities (Supplementary Table S1). To examine whether the F-AMP and imatinib mesylate–induced apoptosis in GIST is caspase-dependent, cells were incubated with optimal doses of the two drugs alone and in combination for 48 hours (Fig. 4A–C) and 72 hours (Supplementary Fig. S5), respectively. In GIST-T1 cell lines, each agent individually as well as combined induced significant changes in caspase-3/7 activity after 48-hour or 72-hour treatment compared with the vehicle control group (Fig. 4A and Supplementary Fig. S13A). F-AMP and imatinib mesylate, alone and in combination, at a higher concentration (0.625 μmol/L), induced caspase-3/7 activity in GIST 882 cells after 48-hour or 72-hour treatment compared with the control group (Fig. 4B and Supplementary Fig. S13B). Imatinib mesylate alone at the concentrations of 0.078 and 0.313 μmol/L failed to induce caspase-3/7 activity after 48-hour treatment (Fig. 3C). Imatinib mesylate treatments did not induce caspase-3/7 activity at the indicated concentrations (0.078 or 0.313 μmol/L) after 48 hours or 72 hours in GIST T1-10R cells; F-AMP treatments and the drug combinations did induce significant differences in caspase-3/7 activities compared with imatinib mesylate alone as well as the vehicle control groups (Fig. 4C and Supplementary Fig. S13C). The results confirm that F-AMP and imatinib mesylate induces apoptosis via caspase-dependent signaling pathways in GIST cells, including the imatinib mesylate–resistant cell line. Imatinib mesylate does not alter caspase-3/7 activities in imatinib mesylate–resistant GIST T1-10R cells. The apoptotic activities of imatinib mesylate, F-AMP, or the drug combination were also evaluated in GIST cells using Annexin V staining. The fraction of early apoptotic cells increased significantly in GIST cells after 48-hour treatment at the indicated concentrations (Fig. 4D–F). After treatment, the percentage of Annexin V–positive GIST-T1 cells increased by an average of 2.8-fold in imatinib mesylate...
treatment group (0.313 μmol/L), 3.6 fold in F-AMP treatment group (1.25 μmol/L), and 7.4 fold in the combination treatment group, each relative to the vehicle control group (Fig. 4D). After treatment, the percentage of Annexin V–positive GIST 882 cells increased by an average of 2.3-fold in imatinib mesylate treatment group (0.625 μmol/L), 2.4-fold in F-AMP treatment group (2.5 μmol/L), and 6.4-fold in the combination treatment group, each relative to the vehicle control group (Fig. 4E). After treatment, the percentage of Annexin V–positive GIST T1-10R cells increased by an average of 2.8-fold in imatinib mesylate treatment group (0.313 μmol/L), 3.6-fold in F-AMP treatment group (0.078 μmol/L), and 4.5-fold in the combination treatment group, each relative to the vehicle control group (Fig. 4F).

**F-AMP alone and in combination with imatinib mesylate induces tumor regression in a GIST in vivo**

To translate the *in vitro* findings described above, we carried out *in vivo* proof-of-principle studies in a validated mouse model. A xenograft nude mouse model generated by subcutaneous inoculation of GIST-T1 cells was used to examine the antitumor effects of imatinib mesylate, F-AMP treatment, each alone and in combination. On the basis of the previous studies demonstrating that i.p. administrations of F-AMP at the dose of 125 mg/kg (once daily, 5 days) and 250 mg/kg (once daily, 3 days) were well-tolerated and led to significant tumor growth suppression in murine models, we chose 60 mg/kg and 120 mg/kg regimens of F-AMP in our study (35–39). F-AMP was administered by i.p. injections once daily in the first week, and then 5 days on and 2 days off per week after we observed the body weight loss in the high-dose combination group. After we changed the dosing schedule, the mice in the high dose combination group gained and maintained their body weight throughout the rest of the study (Supplementary Fig. S14). After 73-day treatment, GIST tumor volume decreased by an average of 62% (P < 0.05) in imatinib mesylate treatment group (50 mg/kg), 32.1% and 66.3% (P < 0.01) in the F-AMP treatment groups (60 or 120 mg/kg respectively), and 76.3% (P < 0.001) and 84.8% (P < 0.001) in the imatinib mesylate (50 mg/kg) + F-AMP (60 or 120 mg/kg) groups, each relative to the vehicle-treated control group (Fig. 5A). The paired two-tailed *t* test showed a significant difference between F-AMP (120 mg/kg) alone and imatinib mesylate + F-AMP (120 mg/kg) treatment groups (P < 0.05). Imatinib mesylate and high dose of F-AMP (120 mg/kg) alone as well as the two agents in combination significantly reduced tumor weights compared with the control group. As shown in Fig. 5B, GIST tumor weight was decreased by an average of 78.6% (P < 0.05) in the F-AMP treatment group, each relative to the vehicle control group (Fig. 5A).
mice were tumor-free in imatinib mesylate (50 mg/kg) alone treatment groups, whereas two and three treatment, one mouse was tumor-free in each of the groups treated with vehicle (saline and 5% DMSO), imatinib mesylate (50 mg/kg), F-AMP (60 or 120 mg/kg), or imatinib mesylate (50 mg/kg) + F-AMP (60 or 120 mg/kg) for 73 days. Tumor volumes were measured every 2 days. After 73-day treatment, mice were euthanized and the tumors were weighed.

A, inhibition of tumor growth in mice by imatinib mesylate (IM), F-AMP treatment alone or in combination. Data, mean ± SEM. *, P < 0.05; **, P < 0.01; ***; P < 0.001, versus control; †, P < 0.05, versus F-AMP 120 mg/kg. B, dot plot of tumor weights from each treatment group. The number of tumor-free mice was measured in controls (n = 3) and imatinib mesylate 50 (n = 3), F-AMP 60 (n = 3), F-AMP 120 (n = 3), imatinib mesylate 50 + F-AMP 60 (n = 2), and imatinib mesylate 50 + F-AMP 120 (n = 3) treated groups. Data, mean ± SEM. *, P < 0.05; **, P < 0.01.

The safety profiles of imatinib mesylate, F-AMP alone, and in combination were assessed by monitoring body weight and survival of mice in each group. The doses and schedules in the study did not cause discernible adverse effects for monotherapy or combination therapy, as shown by no significant loss of body weight (Supplementary Fig. S14), and no noticeable pathologic changes in the livers, kidneys, and spleens among all groups were seen (Supplementary Fig. S15).

**Imatinib mesylate and F-AMP-mediated tumor growth inhibition correlates with decreased Ki67 and c-KIT expression**

To assess the effects of imatinib mesylate and/or F-AMP on tumor morphology, we performed H&E staining and immunohistochemical staining for Ki67, a proliferation marker, for c-KIT (CD117), a marker for GIST cells, and cleaved caspase-3, an apoptosis marker on tumor sections. H&E staining of tumor sections exhibited enhanced histologic response in treated mice than control mice, except for F-AMP (60 mg/kg) group, as shown by the decrease in cellularity, an increase in stromal fibrosis, and the focal areas of necrosis (Fig. 6A). The enhanced histologic response is more evident in combination treatments (Fig. 6A).

Except for the lower F-AMP (60 mg/kg) alone group, GIST-T1 tumors treated with imatinib mesylate and F-AMP alone or in combination, showed a significant decrease in cell proliferation as demonstrated by the significant reduction (23.8%, 28.3%, 24.9%, and 58.8%, P < 0.01) in Ki67-positive cells in tumors isolated from imatinib mesylate (50 mg/kg), F-AMP (120 mg/kg) and the imatinib mesylate (50 mg/kg) + F-AMP (60 or 120 mg/kg) groups compared with control, respectively (Fig. 5B). In addition, a significant reduction in Ki67-positive cells (29.6%) was observed in imatinib mesylate + F-AMP (60 mg/kg) group when compared with 60 mg/kg F-AMP alone. A decrease in Ki67-positive cells was also observed in imatinib mesylate + F-AMP (120 mg/kg) as compared with the treatment of either drug alone (Fig. 6B).

To further evaluate the therapeutic response to imatinib mesylate and F-AMP, we stained tumor sections for c-KIT expression to assess the number of residual tumor cells present in the specimens. Consistent with the Ki67 staining, the numbers of c-KIT-positive cells were lower in imatinib mesylate (50 mg/kg), F-AMP (120 mg/kg), and the imatinib mesylate (50 mg/kg) + F-AMP (60 or 120 mg/kg) groups, as compared with control group (Fig. 6A). The decrease in c-KIT staining, including percentage of positive cells and staining...
intensity, was substantially more pronounced in the combination treatment group compared with single-agent treatment groups (Fig. 6A).

Tumor apoptosis was examined by staining for the presence of cleaved caspase-3. After the treatment of imatinib mesylate and F-AMP, alone and in combination, tumor tissues showed decreased positive staining for cleaved caspase-3 as compared with control tumors (Fig. 6A and B).

Discussion

Since the FDA approval of imatinib mesylate as a drug treatment for GIST in 2002, the treatment of this sarcoma has radically improved. GIST, resistant to standard
Fludarabine Phosphate Repurposed for GIST

chemotherapy and radiation, can now be controlled, at least initially, with imatinib mesylate in the majority of cases. However, these effects are often short-lived, with GISTs demonstrating a median time to progression of approximately two years (12, 16, 21). Therefore, alternative drugs or drugs in combination with imatinib mesylate should be considered for GIST therapy. To accelerate the development of new therapies for patients with GIST, we employed an in vitro screening approach of FDA-approved drugs to identify drug-repurposing opportunities. From the screens of FDA-approved drugs, we identified eight drugs that inhibit GIST cell proliferation alone or in combination with imatinib mesylate. F-AMP showed the highest degree of synergism with imatinib mesylate on GIST cells and was prioritized for further validation because of its efficacy demonstrated in other cancers, the wealth of clinical experience with this agent, and its single-agent in vitro activity in several GIST cell lines (31–34). Fludara (fludarabine phosphate injection) has been approved by the FDA for the treatment of patients with B-cell chronic lymphocytic leukemia (CLL) who have not responded to or whose disease has progressed during treatment with at least one standard alkylating agent–containing regimen (FDA). These results are documented in the FDA “rare disease research database” (RDRD), with at least one marketing approval for a common disease indication, for a rare disease indication, or for both common and rare disease indications up through June 2010.

F-AMP inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase and is being repurposed for different cancers (31–34). This was particularly intriguing given our previous finding in studies to deduce signaling activity in GIST cell lines treated with imatinib mesylate, in which we applied bioinformatics approaches. We established that imatinib mesylate produced reduced activity in the KIT pathway, as well as unexpected activity in altering the TP53 pathway (15). Pursuing these findings, we determined that imatinib mesylate–induced DNA damage is responsible for the increased activity of p53, thus identifying a novel off-target activity for this drug. We, therefore, speculated that imatinib mesylate and F-AMP might have both complementary and unique activities in GIST, warranting further clinical investigations. Our study of imatinib mesylate and F-AMP combinations has focused mainly on the question of whether a combination is synergistic rather than simply additive. Synergy could help to define the point at which the unique combination of these agents can provide additional benefit (i.e., improved efficacy with equal or less toxicity) over simply increasing the dose of either agent. Demonstrating synergistic proof-of-principle preclinically, would then warrant translating these findings to patients.

Our in vitro cytotoxicity data showed F-AMP alone potently inhibited GIST cells (1). In the present combination studies, we found that the interaction between imatinib mesylate and F-AMP is highly synergistic in GIST cells. Further validation by Western blot analysis showed that F-AMP does not inhibit c-KIT/akt and MAPK pathways in GIST-T1, GIST 882, and GIST T1-10R cells. However, both F-AMP and imatinib mesylate treatment induce DNA damage, suggesting that the combination of F-AMP with imatinib mesylate increases the growth inhibition effect of GIST cells by synergistically enhancing DNA damage. Caspase-3/7 activity analysis and Annexin V staining results suggest that imatinib mesylate and F-AMP in combination and alone induce caspase-3/7 activity and apoptosis in GIST cells. Therefore, the combination of F-AMP and imatinib mesylate represents a promising alternative treatment for patients with imatinib-resistant GIST, and warrants clinical evaluation in recurrent GIST.

In agreement with the excellent therapeutic response to imatinib mesylate therapy of GIST patients with exon 11 KIT mutations (40, 41), our data employing the mouse xenograft model showed imatinib mesylate (50 mg/kg, orally once daily) profoundly inhibited the growth of GIST-T1 tumor, which contains a primary imatinib-sensitive mutation in KIT exon 11. These data are also consistent with the previous reports of the GIST-inhibitory effects of imatinib mesylate in preclinical models (42–45), suggesting our GIST xenograft model was successfully established and reliable. For the first time, our studies demonstrated that F-AMP alone (120 mg/kg) has substantial anti-GIST activity which is comparable with imatinib mesylate (50 mg/kg) and that the combination of imatinib mesylate and F-AMP (either at 60 or 120 mg/kg) synergistically inhibits tumor growth. Furthermore, advanced GISTs progressing on tyrosine kinase therapy frequently have secondary mutations; therefore, there is a rationale for testing combination therapies that target RTK-independent pathways, such as those that block DNA synthesis and lead to enhanced DNA damage. The high degree of synergy observed with the F-AMP/imatinib mesylate combination in vitro and in vivo in this study provide a strong rationale for considering this combination in patients with GIST.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Z.Y. Pessetto, Y. Ma, S.J. Weir, A.K. Godwin
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.Y. Pessetto, Y. Ma, J.J. Hirst, A.K. Godwin
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