

## Drug Repurposing for Gastrointestinal Stromal Tumor

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### Abstract

Despite significant treatment advances over the past decade, metastatic gastrointestinal stromal tumor (GIST) remains largely incurable. Rare diseases, such as GIST, individually affect small groups of patients but collectively are estimated to affect 25 to 30 million people in the United States alone. Given the costs associated with the discovery, development, and registration of new drugs, orphan diseases such as GIST are often not pursued by mainstream pharmaceutical companies. As a result, "drug repurposing" or "repositioning," has emerged as an alternative to the traditional drug development process. In this study, we screened 796 U.S. Food and Drug Administration (FDA)-approved drugs and found that two of these compounds, auranofin (Ridaura) and fludarabine phosphate, effectively and selectively inhibited the proliferation of GISTs, including imatinib-resistant cells. One of the most notable drug hits, auranofin, an oral, gold-containing agent approved by the FDA in 1985 for the treatment of rheumatoid arthritis, was found to inhibit thioredoxin reductase activity and induce reactive oxygen species (ROS) production, leading to dramatic inhibition of GIST cell growth and viability. Importantly, the anticancer activity associated with auranofin was independent of imatinib-resistant status, but was closely related to the endogenous and inducible levels of ROS. Coupled with the fact that auranofin has an established safety profile in patients, these findings suggest for the first time that auranofin may have clinical benefit for patients with GIST, particularly in those suffering from imatinib-resistant and recurrent forms of this disease. *Mol Cancer Ther*; 12(7); 1299–309. ©2013 AACR.

### Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal malignancies of the digestive tract with an estimated annual incidence of approximately 6,000 new cases in the United States (1). Approximately 85% of GISTs contain somatic activating mutations in one of 2 proto-oncogenes: *KIT* or *PDGFRA*, which encode the tyrosine kinase receptors c-KIT and the platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ), respectively (2–4). More recently, mutations in the serine-threonine kinase *BRAF* have been identified in a very small number of GISTs (5, 6).

Historically, there were few safe and effective therapeutic options for long-term GIST treatment and disease

maintenance due to the ineffectiveness of radiation and cytotoxic chemotherapy. The clinical outlook for adults with GIST improved radically following the discovery that this malignancy is most commonly driven by signaling via the c-KIT receptor, which can be inhibited by small-molecule tyrosine kinase inhibitors such as imatinib (7–10). In a series of clinical trials, treatment of advanced-stage patients with imatinib yielded objective response rates of 55% to 78%, with approximately half of patients remaining disease-free for 2 years and median overall survival of approximately 50 to 55 months (11). Imatinib also delayed time to disease progression in the adjuvant setting, with a trend toward improved overall survival. These data led U.S. Food and Drug Administration (FDA) to approve imatinib for treatment of advanced-stage GIST in 2002 and for use in the adjuvant setting in 2008.

Unfortunately, resistance to imatinib has been increasingly observed (8, 11–13). Resistance to initial therapy, so-called "early" or "intrinsic" resistance, occurs in 10% to 20% of patients and late or "acquired" resistance occurs in about 40% of patients. The molecular mechanisms of both categories of resistance are being elucidated including secondary mutations in *c-KIT* or *PDGFR*, leading to the introduction of newer kinase inhibitors targeting these additional pathways into the clinic. However, it is now also clear that GIST cells can use complex, redundant mechanisms to maintain aberrant signaling, which may confound therapy with drugs targeting any particular kinase (4, 8, 14, 15).

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Recently, a putative GIST stem cell (KIT<sup>low</sup>Cd44<sup>+</sup>Cd34<sup>+</sup>) has been identified (16, 17). These GIST stem cells, which have the potential to completely reconstitute tumors and establish metastases, are inherently resistant to inhibitors of c-KIT and PDGFR. Thus, the next generations of therapies for GIST will almost certainly have to include other drugs with independent, potentially complementary mechanisms of action to currently used kinase inhibitors (18).

Costs associated with the discovery, development, and registration of novel, new drugs have been estimated in excess of \$1.5 billion and require 10 to 17 years to complete. As a result, rare or orphan diseases such as GIST are often not pursued by large, for-profit pharmaceutical companies. In recent years, drug repurposing, identifying new therapeutic applications for FDA-approved and abandoned drugs, has been shown as a successful approach in addressing the unmet medical needs of patients suffering from rare diseases. Novel approaches to drug rediscovery, the identification of opportunities to evaluate FDA-approved and abandoned drugs for new therapeutic uses, have emerged. Patients gain access to promising new therapies much more quickly by capitalizing on prior experience, resulting in reduced drug development and registration cycle times and cost (19). By using repurposing and rediscovery strategies, drug development becomes affordable and achievable by non-profit organizations including academia, government, and disease philanthropy organizations, particularly through creative partnerships (20).

We report a drug repurposing approach to identify a potential drug for patients with GIST. Auranofin inhibits thioredoxin reductase (TrxR) enzymatic activity and increases reactive oxygen species (ROS) production. Auranofin treatments lead to early and late apoptosis in GIST cells that result in cell death. In summary, we have shown that inhibition of GIST cell proliferation and induction of apoptosis by auranofin is dependent on drug-induced changes in ROS production.

## Materials and Methods

### General methods

All cell proliferation and caspase activity measurements were made on 384-well, black  $\mu$ Clear microplates (Greiner bio-one). All cell proliferation experiments were assessed using the CellTiter-Blue reagent or CellTiter-Glo reagent according to the manufacturer's protocol (Promega). All fluorescence or luminescence measurements were made using Infinite M200 Pro plate reader (Tecan). The fluorescence values are reported in relative fluorescence units and were measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Data were normalized to percentage inhibition and IC<sub>50</sub> concentrations were determined for each drug over a 72-hour incubation period by the SigmaPlot program (Systat Software).

### Compound library

The FDA-approved drug Library (provided by the Lead Development and Optimization Shared Resource within the NCI Cancer Center at the University of Kansas, Kansas City, Kansas) contains 796 drugs with known bioavailability and safety in humans. Compounds were present at 10 mmol/L in dimethyl sulfoxide (DMSO). The full list of drugs is provided in Supplementary Table S1. For hit validation, imatinib was purchased from the LC Laboratories and dissolved in sterile water. Fludarabine phosphate, idarubicin HCl, and bortezomib were purchased from the Selleck Chemicals. All drugs except imatinib were dissolved in DMSO.

### Cell culture

GIST-T1, a tumor cell line possessing a heterozygous mutation in *KIT* exon 11, was kindly provided by Takahiro Taguchi (Division of Human Health and Medical Science, Kochi University, Nankoku, Kochi, Japan) and untransformed smooth muscle cell line ULTR cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (14). GIST 882 cells (*c-KIT* exon 13 homozygous mutation) were gifted by Jonathan A. Fletcher (Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and maintained in RPMI containing 15% FBS (21, 22). GIST T1-10R cells, derived in Andrew K. Godwin's laboratory, were maintained in DMEM containing 10% FBS and supplied with 10  $\mu$ mol/L imatinib to maintain drug resistance. Imatinib was deleted from the culture for indicated hours in experiments with GIST T1-10R cells. Hs 919.T cells were purchased from ATCC and maintained in DMEM containing 10% FBS. All cell lines were supplied with 1% penicillin/streptomycin and were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C. Authentication for cell lines that were not purchased from ATCC was carried out by the Clinical Molecular Oncology Laboratory (The University of Kansas Cancer Center, Kansas City, Kansas).

### Quantitative drug screen assay

Drugs or vehicle (DMSO) were preloaded by the KU High Throughput Screen Laboratory (KU HTSL) as 250 nL aliquots on an Echo550 platform to each well to give final doses ranging from 10 to 0.078  $\mu$ mol/L (serial twofold dilutions) in 25  $\mu$ L in duplicates (38 plates for each cell line). Cells were grown to 80% confluence, harvested, and aliquoted into 384-well plates at concentrations of 1,000 to 1,500 cells per well in a total volume of 25  $\mu$ L per well with or without imatinib mesylate using a Matrix Wellmate (Thermo Scientific). The imatinib mesylate concentrations used for the combination study were the IC<sub>30</sub> values: 10, 10, and 40 nmol/L for GIST-T1, GIST T1-10R, and GIST 882 cells, respectively (data not shown). Cells were cultured for 72 hours at 37°C. Aliquots of 5  $\mu$ L CellTiter-Blue Reagent were added directly to each well, the plates were incubated at 37°C for 3 hours, and the fluorescent signal was measured.

Imatinib-treated (at established  $IC_{50}$  value for each cell line) wells and vehicle-only wells on each plate were uniformly distributed throughout the screen and served as intraplate controls. Background plates containing vehicle only were inserted throughout the screen as interplate controls. The controls were used to calculate the background levels of the assay and to evaluate assay response as well as performance. Performance of the assay was calculated and the  $Z'$  factors were  $\geq 0.5$  or more (23).

#### Drug screen assay in Hs 919.T. cell line

Drugs or vehicle (DMSO) were preloaded by the KU HTSL as 250 nL aliquots on an Echo550 platform to each well to give a final dose of 1  $\mu\text{mol/L}$  in 25  $\mu\text{L}$ . Cells were grown to 80% confluence, harvested, and aliquoted into 384-well plates at concentrations of 750 cells per well in a total volume of 25  $\mu\text{L}$  per well using a Matrix Wellmate (Thermo Scientific). The cells were cultured for 72 hours at 37°C. Twenty-five microliters CellTiter-Glo Reagent was added directly to each well, the plates were incubated at room temperature for 15 minutes, and the luminescence signal was measured. Following subtraction of background counts, the percentage of growth inhibition was calculated by dividing the value from drug-treated sample by that from control well incubated in the absence of drug. Samples exhibiting more than 50% growth inhibition in the presence of 1  $\mu\text{mol/L}$  drug were classified as positive hits.

#### TrxR activity measurement

Cells in log phase of growth were grown to 80% confluence, harvested, and aliquoted into 6-well plates at concentrations of 500,000 cells per well in a total volume of 1 mL per well. The cells were allowed to attach overnight and were treated with or without auranofin or imatinib at indicated concentrations for 6 hours. The cells were harvested and 50  $\mu\text{g}$  of total protein was used for each sample assessment. TrxR activity was measured using the TrxR Reductase Assay Kit (Abcam) per manufacturer's instructions.

#### ROS activity measurement

Cells in log phase of growth were grown to 80% confluence, harvested, and aliquoted into 96-well plates at concentrations of 15,000 cells per well in a total volume of 100  $\mu\text{L}$  per well. The cells were allowed to attach overnight. The cells were preloaded with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 minutes and treated with or without auranofin or imatinib at indicated concentrations for 6 hours. ROS activity was measured using the OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, INC) as per manufacturer's instructions.

#### Early apoptosis assay

Cells in log phase of growth were grown to 80% confluence, harvested, and aliquoted into 6-well plates at a concentration of 5,000,000 cells per well in a total volume

of 5 mL. The cells were allowed to attach overnight and then were treated with auranofin at different concentrations (0, 0.2% DMSO; 0.1 and 10  $\mu\text{mol/L}$ ) for 24 hours. Early apoptosis cells were detected by the Guava Nexin Annexin V Assay Kit using Guava easyCyte sampling flow cytometer (Millipore).

#### Cell proliferation and caspase-3/7 activity assay

Cells in log phase of growth were grown to 80% confluence, harvested, and aliquoted into 384-well plates at concentrations of 1,000 to 1,500 cells per well in a total volume of 20  $\mu\text{L}$ /well using a Matix Wellmate (Thermo Scientific). The cells were allowed to attach overnight and 5  $\mu\text{L}$  of culture media containing either vehicles or drug were added to each well. Cell proliferation was evaluated. After reading fluorescence, Caspase-Glo 3/7 Assay Reagent (Promega) was used according to the manufacturer's instructions. After background subtraction, luminescence was measured and quantified by normalizing luminescence to fluorescence. Caspase-3/7 activity values were expressed as percentage of the mean of the relative vehicle controls.

#### Western blot analysis

Cells in log phase of growth were grown to 80% confluence, harvested, and aliquoted into 6-well plates at a concentration of 5,000,000 cells per well in a total volume of 5 mL. The cells were allowed to attach overnight and then were treated with drugs at indicated doses for 6 hours. Whole-cell extract was prepared as described previously (14). Briefly, for each specimen, 50  $\mu\text{g}$  whole-cell extract was electrophoresed on 10% precast polyacrylamide gel (Bio-Rad) and transferred onto nitrocellulose membranes. After 1-hour blocking in 5% bovine serum albumin or 5% nonfat milk in TBS/0.1% Tween-20, membranes were incubated with primary antibodies (1:1,000 or 1:500 dilution) overnight at 4°C. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 hour, development was carried out using Immun-Star HRP Chemiluminescence Kits (Bio-Rad). All Western blots were conducted in triplicate. Phosphorylated proteins were normalized to total proteins and quantified using Image J (NIH, Bethesda, MD).

#### Antibodies

The following antibodies were used for Western blot analysis: rabbit anti-c-KIT, rabbit anti-c-KIT XP, rabbit antiphospho-c-KIT (Tyr719), rabbit anti-AKT, rabbit antiphospho-AKT (Ser473) XP, rabbit antiphospho-AKT (Thr308), mouse anti-ERK1/2, mouse antiphospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology); mouse anti- $\beta$ -actin (Sigma).

#### Statistics

Data were reported as mean  $\pm$  SEM of 3 to 5 independent experiments, each treatment was conducted in duplicate or triplicate. Values were compared using

the Student *t* test or with one-way ANOVA when 3 groups were present. Results were deemed statistical significant if  $P < 0.05$ .

## Results

### Screening FDA-approved drugs for activity in GIST

To identify drugs that might be repurposed for patients with GIST, we used a quantitative drug screen experimental approach to assess the activity of 796 FDA-approved drugs in GISTs. The CellTiter-Blue oxidation–reduction dye is used as an indicator of cell viability. Before the screen, the cell viability assay was miniaturized to a 384-well format to accelerate assay throughput. For optimization of assay performance, the number of cells per well, the length of the incubation period, and the volume of CellTiter-Blue reagent were empirically determined (Table 1). To reduce systematic errors such as variation in incubation time and time drift in measuring different plates, DMSO tolerance and edge effect were also considered and tested. Our DMSO tolerance assay indicates that the DMSO used in the screen does not affect cell viability (see Supplementary Material, Supplementary Fig. S1A). In addition, the edge effect assay indicates that after 5 hours, the signal will have significant reduction in wells at edge (see Supplementary Material and Supplementary Fig. S1B). Therefore, we controlled reading time to less than 5 hours for each cell line.

The protocol of the preliminary screening study is summarized in Table 1. A total of 152 plates were screened. The assay was conducted well over the entire course of the screen as shown by the *Z'* values. The averaged signal/background ratio, *Z'* value, and the signal window of the control wells for each cell line were summarized in Supplementary Table S2.

Data were normalized as percentage inhibition relative to vehicle control and plotted in a 3-dimensional plot (Fig. 1A) for each plate. Curves were classified into 3 categories (Supplementary Table S3; Fig. 1B–D) and  $IC_{50}$  values were calculated and both of them were used to define active/inactive drugs. FDA-approved drugs failing to show

activity were ruled out from the following analysis.  $IC_{50}$  values were calculated for drugs showing positive inhibition values ( $IC_{50}$  value  $< 1.0 \mu\text{mol/L}$ ). Typical  $IC_{50}$  curves were shown in Fig. 1E.

On the basis of drug concentration–response curve quality in the primary screen, 29 drugs showed preclinical potent *in vitro* activity ( $IC_{50} \leq 1 \mu\text{mol/L}$ ) against one or more GIST cell lines including 2 mutant GIST cell lines (GIST-T1, exon 11 *KIT* mutation, and GIST 882, exon 13 *KIT* mutation), and an imatinib-resistant variant of T1 cell line (GIST T1-10R, derived in the Andrew K. Godwin's laboratory). The screen results are summarized in Table 2.

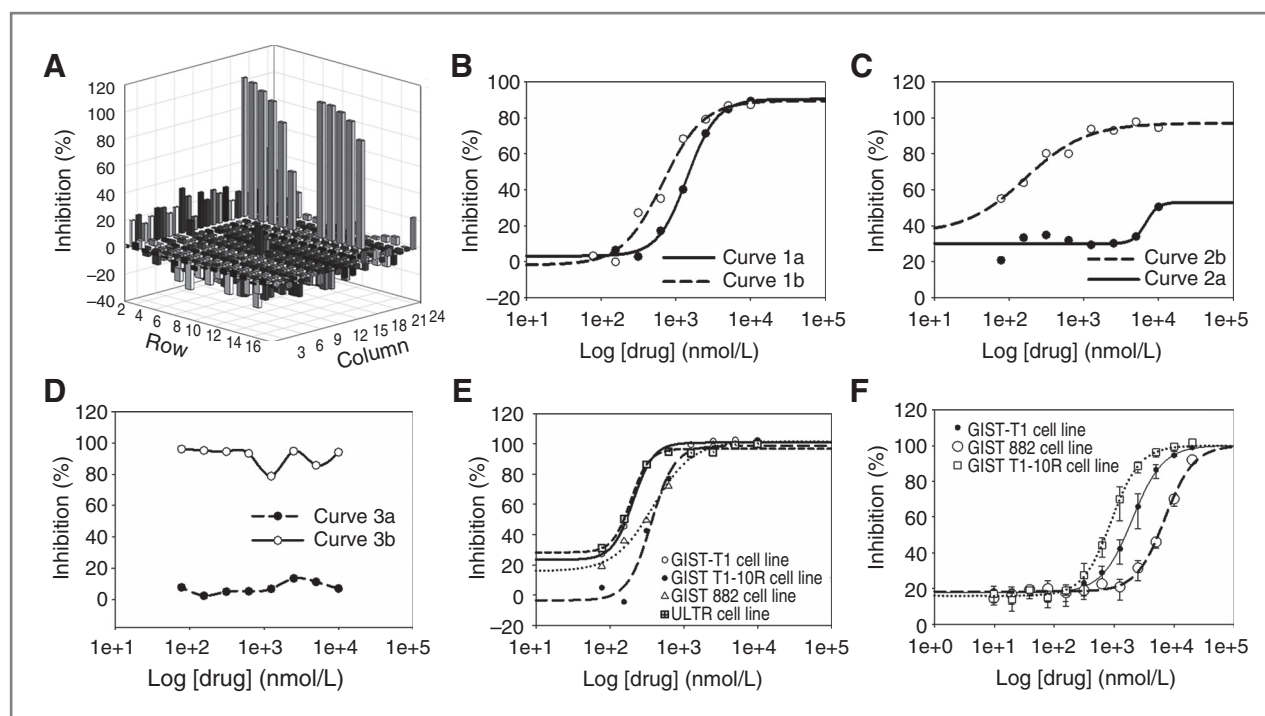
### Validating activity of FDA-approved drugs in GIST

The active compounds from the screen were triaged into 3 groups: active in (i) one of the GIST cell lines, (ii) two of the GIST cell lines, or (iii) three of the GIST cell lines (Table 2). The ideal candidate should have a low  $IC_{50}$  value of less than  $1.0 \mu\text{mol/L}$  against 3 GIST cell lines and a high  $IC_{50}$  value of more than  $1.0 \mu\text{mol/L}$  against the ULTR cell line. However, later we found the data generated from ULTR cell line are not informative, as these cells were also transformed (data not shown). Instead, Hs 919.T., a benign osteoid osteoma cell line, was used as the nonsarcoma cell line control. Drugs were screened at the concentration of  $1 \mu\text{mol/L}$ . The data are summarized in Supplementary Table S4 in the Supplementary Materials. Ten drugs (bortezomib, doxorubicin, mitoxantrone, idarubicin HCl, digoxin, daunorubicin, vinorelbine, plicamycin, dactinomycin, and mebendazol) showed inhibition against Hs 919.T. cell proliferation more than 50% indicating that these drugs have no selectivity against sarcoma and nonsarcoma cells. Therefore, based on inhibitory activity against 3 GIST cell lines and subsequent drug development and regulatory science gap analysis of each of the 29 screening hits, 3 drugs (auranofin, bortezomib, and idarubicin) that are active in all GIST cell lines and one drug (fludarabine phosphate) that is active in imatinib-resistant cell line were prioritized for further validation. Pure compounds (>99% purity) were obtained and

**Table 1.** Automation protocol for drug repurposing screen

Step	Event	Parameter	Description	Notes
1	Pre-spot library compounds or DMSO	250 nL	Titration 10–0.7 $\mu\text{mol/L}$ (8 points)	Instrument: Echo 550
2	Add reagent	20 $\mu\text{L}$	GIST-T1 cells: 1,000 cells/well GIST 882 and GIST-T1-10R cells: 1,500 cells/well ULTR cells: 500 cells/well	Instrument: Matrix Wellmate
3	Incubate	72 hours	5% $\text{CO}_2/37^\circ\text{C}$ incubation	
4	Add reagent	5 $\mu\text{L}$	Promega CellTiter-Blue	
5	Incubate	3 hours	5% $\text{CO}_2/37^\circ\text{C}$ incubation	
6	Read	Fluorescence	544 nm Ex/590 nm Em, gain = 70.	Instrument: Infinite M200 Pro





**Figure 1.** Post-HTS analysis. A, data acquisition is shown as a 3-dimensional representation. Plot was generated from GIST T1-10R cell line. B–D, examples of drug concentration–response curves. Detailed information on curves is summarized in Supplementary Table S3. E and F,  $IC_{50}$  curves for auranofin derived from the primary screen and derived from the subsequent validation assays.

prepared as described in Materials and Methods section. *In vitro* activities of the 4 prioritized drugs were evaluated using 12-point dose–response curves ranging from 20  $\mu\text{mol/L}$  to 1  $\text{nmol/L}$ , with triplicate sampling. Typical  $IC_{50}$  curves generated from the validation are shown in Fig. 1F. The structures of the candidate drugs are summarized in Supplementary Table S5. The  $IC_{50}$  values and drug selectivity were confirmed and listed in Table 3. The hit validation of the top candidates was highly reproducible except fludarabine phosphate that was active across the 3 GIST cell lines.

Two drugs, auranofin and fludarabine phosphate, showed selectivity against GIST cell lines. One of them, auranofin, has been shown to inhibit TrxR system and has been proposed as a promising experimental agent for cancer treatment (20, 24, 25). The auranofin pharmacologic data are listed in Supplementary Table S6. Auranofin has an acceptable clinical safety profile (26) and is currently being evaluated in patients with chronic lymphocytic leukemia at the University of Kansas Cancer Center, National Heart, Lung and Blood Institute (Bethesda, MD), and The Ohio State University Comprehensive Cancer Center (Columbus, OH; ClinicalTrials.gov Identifier NCT01419691). Furthermore, we analyzed a microarray dataset GSE31802 (27) for GIST tumors and found that the TrxR (all 3 isoforms TrxR1, TrxR2, and TrxR3) are present in specimens taken from patients with GIST ( $n = 14$ ) with expression of TrxR1 and TrxR3 being 1.2-fold higher ( $P < 0.01$ ) as compared with that of TrxR2 (Supplementary Fig.

S2A and S2B). On the basis of our findings, prior human safety experience, as well as activity of this FDA-approved drug in other cancers, we selected auranofin for further mechanistic studies in GIST.

#### Auranofin inhibits TrxR enzymatic activity in GIST cells

Gold(I) and gold(III) compounds have been reported to be highly specific inhibitors of mitochondrial TrxR. It has been shown that gold compounds preferentially interact with a selenol group in the active site at the C terminus of TrxR, as selenol displays a greater affinity toward heavy metals (28). Mammalian TrxR is a key enzyme for maintenance of the intracellular-reduced environment. Impaired TrxR system will lead to increased levels of oxidized thioredoxin that will render its ability to protect cells from a variety of oxidative stresses (28). As such, we hypothesized that TrxR might be a molecular target of auranofin treatment in GIST cells. To examine whether auranofin treatment affects the TrxR/Trx system in GIST cells, we compared levels of TrxR activity after auranofin and imatinib treatments. Fig. 2A shows the effect of a 6-hour auranofin treatment on GIST cells. Only GIST T1-10R cells showed a significant decrease of TrxR activity at the lower concentration treatment (0.01 and 0.1  $\mu\text{mol/L}$ ). At higher concentrations (1.0 and 10  $\mu\text{mol/L}$ ), auranofin dramatically reduces the TrxR activity in all 3 GIST cell lines. At auranofin exposures in cell culture up to 24 hours at 1  $\mu\text{mol/L}$ , TrxR

**Table 2.** Drug repurposing for GISTs hits summary

Group	Drug	Therapeutic use	IC <sub>50</sub> (μmol/L)				
			GIST-T1	GIST 882	GIST T1-10R	ULTR	
1	Albendazole	Anthelmintic	0.30	>1.0	>1.0	0.32	
	Amsacrine	Antineoplastic, immune suppressive	0.31	>1.0	>1.0	>1.0	
	Mebendazole	Anthelmintic	0.81	>1.0	>1.0	0.73	
	Paclitaxel	Antineoplastic	0.21	>1.0	>1.0	0.20	
	Vinorelbine	Antineoplastic	0.23	>1.0	>1.0	0.08	
	Chloroquine	Antimalaria	>1.0	0.46	>1.0	>1.0	
	Cladribine	Antineoplastic	>1.0	0.76	>1.0	0.54	
	Danazol	Anterior pituitary suppressant	>1.0	0.67	>1.0	>1.0	
	Etoposide	Antineoplastic	>1.0	0.03	>1.0	>1.0	
	Teniposide	Antineoplastic	>1.0	0.09	>1.0	0.19	
	Fludarabine phosphate	Antineoplastic	>1.0	>1.0	0.81	>1.0	
	2	Clofarabine	Antineoplastic, alkylating agent	>1.0	0.99	>1.0	0.18
		Dactinomycin	Antineoplastic, alkylating agent	0.07	<0.07 <sup>a</sup>	>1.0	<0.07 <sup>a</sup>
		Daunorubicin	Antineoplastic	<0.07	0.17	>1.0	<0.07 <sup>a</sup>
Digitoxin		Inotropic, cardiotoxic	<0.16	0.16	>1.0	<0.07 <sup>a</sup>	
Doxorubicin		Antineoplastic	0.31	0.08	>1.0	0.16	
Imatinib		Antineoplastic	0.18	0.13	>1.0	>1.0	
Mitoxantrone		Antineoplastic	0.42	0.08	>1.0	<0.07 <sup>a</sup>	
Nilotinib		Antineoplastic	0.38	0.10	>1.0	>1.0	
Plicamycin		Antineoplastic antibiotic	0.35	<0.07 <sup>a</sup>	>1.0	0.15	
Topotecan		Antineoplastic	0.08	0.44	>1.0	0.09	
3	Auranofin	Antirheumatic	0.23	0.38	0.16	0.19	
	Bortezomib	Antimyeloma	<0.07 <sup>a</sup>	<0.07 <sup>a</sup>	0.50	<0.07 <sup>a</sup>	
	Carbimazole	Antithyroid	<0.07 <sup>a</sup>	0.05	0.18	<0.07	
	Digoxin	Cardiac stimulant	0.16	0.79	0.93	0.16	
	Gentian Violet	Antibacterial, anthelmintic	0.52	0.25	0.96	0.16	
	Idarubicin	Antileukemic	0.10	0.05	0.69	<0.07 <sup>a</sup>	
	Ouabain	Antiarrhythmic, cardiotoxic, hypertensive, Na/K ATPase inhibitor	0.21	0.03	0.24	<0.07 <sup>a</sup>	

NOTE: Compounds are listed alphabetically and not in order of activity. Group 1 indicates drug activity (<1 μmol/L) in a single GIST cell lines, Group 2 in 2 GIST cell lines, and Group 3 in all 3 GIST cell lines.

<sup>a</sup>IC<sub>50</sub> less than 0.07 μmol/L and drug showed no dose response.

enzymatic activity is inhibited (Supplementary Fig. S3A–S3C). Compared with auranofin treatments, imatinib treatments did not change the TrxR activity level in all 3 GIST cell lines (Fig. 2B).

### Auranofin increases intracellular ROS levels in GIST cells

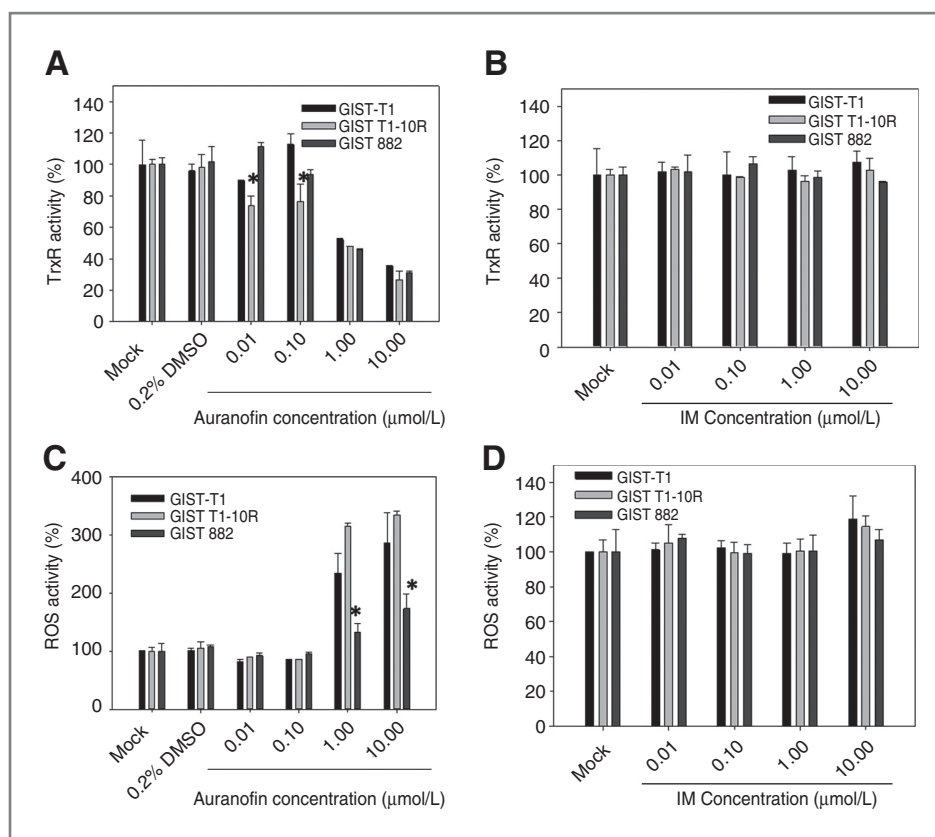
It is known that all types of cells generate low but detectable amounts of ROS under different circumstances

**Table 3.** Hit validation, drug structures, and IC<sub>50</sub> values of validated hits

Drug	IC <sub>50</sub>			
	GIST-T1	GIST 882	GIST T1-10R	Hs 919.T.
Imatinib	0.06 ± 0.03	0.13 ± 0.02	>1	ND
Auranofin	0.25 ± 0.15	0.64 ± 0.18	0.02 ± 0.01	ND
Bortezomib	0.006 ± 0.002	0.002 ± 0.0003	0.001 ± 0.001	0.15 ± 0.01
Fludarabine phosphate	0.53 ± 0.04	0.54 ± 0.03	0.84 ± 0.20	ND
Idarubicin HCl	0.09 ± 0.01	0.13 ± 0.04	0.05 ± 0.01	0.07 ± 0.003

Abbreviation: ND, drugs showed no dose response.

**Figure 2.** *In vitro* effects of auranofin or imatinib (IM) treatment on TrxR/Trx system. A and B, auranofin inhibits TrxR activity in GIST cells. Imatinib does not alter TrxR activity. GIST cells were treated for 6 hours with the indicated concentration of auranofin before cells were harvested and lysed. For each sample, 50  $\mu$ g of protein cell lysate was assessed for TrxR activity by measuring NADPH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoate). C and D, auranofin increases ROS activity in GIST cells. Imatinib does not increase the production of ROS. GIST cells in a 96-well plate were pretreated with 1 mmol/L DCFH-DA for 60 minutes at 37°C. Cells were then treated for 6 hours with the indicated concentration of auranofin. ROS activity was measured using 2',7'-dichlorofluorescein fluorescence. (\*,  $P < 0.05$ ).

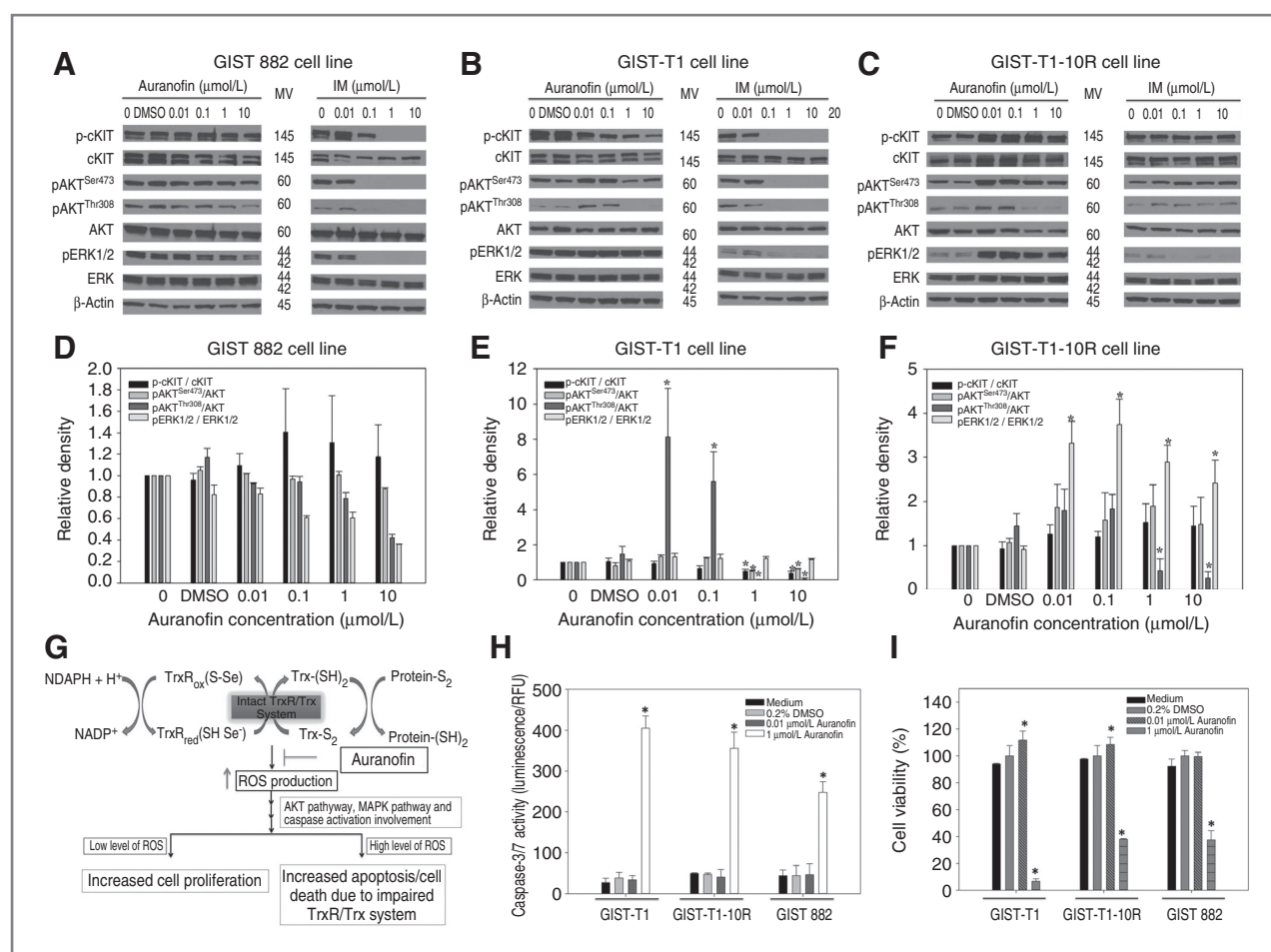


and that ROS serve as important mediators of redox homeostasis under physiologic conditions. The production of ROS is tightly regulated by antioxidant systems, which maintain redox homeostasis within the cellular environment. Overproduction of ROS and impaired antioxidant systems (e.g., TrxR system) may lead to oxidative stress and it is known to contribute to the pathogenesis of several diseases including cancers (24, 25). It is also known that ROS have distinct functional effects, which are dependent on a number of factors such as the type of cell within which ROS are generated and the local concentration of ROS at subcellular sites where they may modulate enzyme activity and influence gene expression (29, 30).

The effect of auranofin on the production of ROS in GIST cells is shown in Fig. 2C. The addition of auranofin (1 and 10  $\mu$ mol/L) strongly stimulates the formation of ROS in GIST-T1 and GIST T1-10R cells (2.4- and 2.6-fold changes, respectively; Fig. 2C). Compared with the other 2 GIST cell lines, GIST 882 cell line did respond to auranofin treatment at the concentration of 1 and 10  $\mu$ mol/L but the decrease in the ROS production was relatively lower (1.5-fold change; Fig. 2C). At lower concentrations (0.01 and 0.1  $\mu$ mol/L auranofin treatment), ROS remained relatively stable in all 3 GIST cell lines. As we expected, the longer treatment with auranofin induces higher production of ROS in all 3 GIST cell lines (Supplementary Fig. S3D–S3F), whereas imatinib treatment had little effect on ROS (Fig. 2D).

### Effect of auranofin on PI3K, AKT, and ERK1/2 pathways in GIST cells

GIST-T1 and GIST 882 cell lines show constitutive activation of the AKT and ERK1/2 pathways and exposure to imatinib resulted in dephosphorylation of KIT, AKT, and ERK1/2 (14). It has been shown that oxidation of thioredoxin or increased ROS may trigger the activation of ERK pathway depending on the transient or prolonged activation (31, 32). Under basal or low oxidative stress, ROS may promote cell survival and proliferation that leads to cancer development (33). Excessive ROS levels, however, can cause cellular damage, and even cell death (34). It is also known that the effect of ROS is cell type-dependent (35–38). To understand the mechanism of action of auranofin in GIST cells, it is important to identify these molecular components involved in the cascade of events that finally trigger cell death. Therefore, we examined whether phosphoinositide 3-kinase (PI3K), AKT, and extracellular signal-regulated kinase (ERK) signaling pathways were involved in auranofin treatment in GIST cells. Our results indicate the effect of auranofin on PI3K, AKT, and ERK signaling pathways in GIST cells is dose- and cell type-dependent. These observations are consistent with our TrxR and ROS activity results. Treatments of 0.01 or 0.1  $\mu$ mol/L auranofin for 6 hours resulted in a basal or low level of ROS that leads to increased pAKT<sup>Thr308</sup> levels (~8-fold and ~5-fold, respectively; Fig. 3B and E) in GIST-T1 cells. When cells



**Figure 3.** Effect of auranofin or imatinib (IM) treatments on signaling pathways and cell viability. GIST 882 (A), GIST-T1 (B), and GIST T1-10R cells (C) were treated with auranofin or imatinib at indicated concentration for 6 hours. For each sample, 50 μg of total protein cell lysate was loaded for Western blots. D–F, bar graph shows combined densitometric quantification (Image J) of Western blot results. Each bar represents results from 2 independent experiments. G, proposed mechanism of auranofin action in GIST cells. Auranofin inhibits TrxR system and results in increased ROS production, which finally leads to apoptosis and cell growth inhibition. H, auranofin induces apoptosis in GIST cells. GIST cells in a 384-well plate were treated for 72 hours with or without 0.01 or 1 μmol/L of auranofin. Caspase-3/7 activity was measured by luminescence. F, auranofin inhibits proliferation of GIST cells. GIST cells in a 384-well plate were treated for 72 hours with or without 0.01 or 1 μmol/L of auranofin. \*,  $P < 0.05$ . RFU, relative fluorescence units.

were treated with 1 or 10 μmol/L auranofin for 6 hours, which resulted in higher fold changes of ROS production (~2.0-fold change; Fig. 2C) in GIST-T1 cells, p-cKIT, pAKT<sup>Ser473</sup>, and pAKT<sup>Thr308</sup> levels are all decreased (~0.5-fold change, ~0.5-fold change, and ~0.06-fold change, respectively; Fig. 3B and E). Similar to GIST-T1 cells, GIST T1-10R cells respond to auranofin in a manner related to ROS levels. Treatments of 0.01 or 0.1 μmol/L auranofin for 6 hours resulted in the increasing pERK1/2 levels (~3.0-fold and ~3.6-fold changes; Fig. 3C and F). Treatments of 1 or 0.10 μmol/L auranofin for 6 hours resulted in the decreasing of pAKT<sup>Thr308</sup> levels (~0.6-fold and ~0.4-fold changes; Fig. 3C and F) and the increasing pERK1/2 levels (~2.7-fold and ~2.4-fold changes; Fig. 3C and F). GIST 882 cell line generates different levels of ROS and is more stable compared with vehicle-treated cells across all auranofin treatments

(~1.5-fold change; Fig. 2C), therefore, auranofin does not noticeably alter phosphorylated levels of cKIT, AKT, and ERK (Fig. 3A and D).

### Auranofin stimulates early apoptosis and caspase-3/7 activity, which causes reduction in cell viability in GIST cells

To test our hypothesis that auranofin treatment in GIST cells inhibits TrxR enzymatic activity and increases ROS production leading to apoptosis (Fig. 3G), we carried out early apoptosis detection assay using Annexin V staining. The fraction of early apoptotic cells increased approximately 4-fold, 2.8-fold, and 2.3-fold in GIST-T1, GIST T1-10R, and GIST 882 cells, respectively, after the 24-hour auranofin treatment (1 μmol/L; Supplementary Fig. S4). To further study the apoptotic affect by auranofin treatment, we carried out caspase-3/7 activity assays and cell



viability assays. The apoptotic activity of auranofin was evaluated on GIST cells with the presence of 0.1 and 1  $\mu\text{mol/L}$  auranofin for 72 hours. Auranofin treatment was able to increase caspase-3/7 activity (Fig. 3H). Caspase-3/7 is responsible for cleavage of cellular proteins that are characteristically proteolysed during apoptosis. After 72 hours of treatment, the percentage of living cells was determined and is shown in Fig. 3I. As described in Fig. 3H, auranofin induced a decrease in cell viability after 1  $\mu\text{mol/L}$  treatment in all 3 GIST cell lines. It is very interesting that low-dose auranofin treatment also induced cell proliferation in GIST-T1 and GIST T1-10R cells ( $\sim 1.1$ – $1.2$  fold, Fig. 3I).

## Discussion

The drug screening assay described in the present study is robust and meets criteria for industry-accepted high-throughput screening standards ( $Z' > 0.5$ ; Supplementary Table S2); therefore, this protocol can easily be applied to higher throughput analysis. Of the 796 FDA-approved compounds screened, 29 were shown to possess inhibitory properties against 1 or more GIST cell lines with an  $\text{IC}_{50}$  value equal to or less than 1  $\mu\text{mol/L}$ . Of these, 4 compounds were selected as those representing the most promising starting points for further validation, as they had activity across both imatinib-sensitive and imatinib-resistant GISTs (Table 3). Validation confirmed the activity and selectivity of these drugs. The different results between the primary screen and validation for fludarabine phosphate suggest that the drug may have been preloaded incorrectly using the Echo550 platform, leading to the initial under appreciation of its activity in the primary screen.

One of the hits, auranofin (Ridaura), is an oral, lipophilic gold-containing compound approved by the FDA in 1985 for the treatment of rheumatoid arthritis. Auranofin has multiple cellular effects that contribute to its efficacy as an antiarthritis agent. It inhibits TrxR and induces a permeability transition resulting in cytochrome *c* release from the mitochondria, leading to apoptotic cell death (29, 38–40). In HL-60 human promyelocytic leukemia cells, ROS-induced apoptosis is dependent on p38-MAPK signaling (41). It also induces  $G_2$  cell-cycle arrest, consistent with activation of a DNA damage-responsive checkpoint (42). In addition, auranofin modulates multiple signaling pathways that are critical to cancer cell survival. It inhibits JAK/STAT signaling, reducing the levels of the antiapoptotic protein Mcl-1 (30), reduces levels of NF- $\kappa$ B-transactivated antiapoptotic proteins (30, 43), antagonizes EGF ligand–receptor interactions (44), and blocks the activity of protein kinase C (45). There is evidence that low levels of oxidants activate several cell signaling pathways and regulate cell survival and proliferation, whereas high levels of oxidants stimulate stress-activated signaling pathways leading to cell death (35–37). However, the effect of ROS is cell type-dependent (46). The results of our experiments show a dose-dependent inhibition of TrxR activity and ROS production in the

auranofin concentration range of 0.01 to 10  $\mu\text{mol/L}$ . The response to auranofin treatment in GIST cell lines is closely related to the endogenous or inducible ROS level. The results clearly indicate that early apoptosis was induced by auranofin treatment and caspase-3/7 proteases are activated in response to apoptosis induced by auranofin in all GIST cell lines. These results provide the preclinical proof-of-principle to explore the use of auranofin in the oncology setting.

Auranofin is effective and well tolerated in rheumatoid arthritis. In dose titration studies, auranofin showed activity at doses as low as 2 mg orally daily, with greater efficacy at 6 and 9 mg daily (47–49). Daily doses more than 6 mg led to a more rapid clinical improvement. The most frequent side effects are diarrhea, rash and pruritis, stomatitis, alopecia, and conjunctivitis (47, 49). Serious, but infrequent, side effects include anemia, leucopenia, thrombocytopenia, eosinophilia, and proteinuria. All are reversible with dose reduction or discontinuation of therapy. Its safety in pregnant and lactating women has not been conclusively established. Results from this work are consistent with other investigators reporting that auranofin has potential anticancer effect through induction of apoptotic cell death in tumor cells (24, 28). Besides its potential anticancer activity, recently auranofin has been reported to have potential amebicidal activity and is being repurposed for the treatment of amebiasis (50).

The results obtained in this study support the idea that auranofin can induce apoptosis in GIST cells as a result of inhibition of TrxR and subsequent increases in intracellular levels of ROS. More importantly, the anticancer activity associated with auranofin may be independent of the mutational status and prior response to imatinib and may be more closely related to the endogenous and inducible levels of ROS (Fig. 2 and data not shown). These preclinical data provide a strong rationale to evaluate auranofin in patients with treatment-refractory GIST.

The evaluation of potential new uses for FDA-approved drugs represents an opportunity to rapidly advance to patients with cancer, promising drug therapies by capitalizing on existing data and experience. The same holds true for abandoned or "shelved" drug candidates. There is growing interest in partnering with innovator firms to potentially evaluate new uses for "shelved" drugs, the development of which have been "abandoned" due to issues other than drug safety. Weir and colleagues (20) reported the rapid translation of auranofin *in vitro* screening results in primary CLL cell lines directly into a multisite, clinical proof-of-concept trial in patients with chronic lymphocytic leukemia (ClinicalTrials.gov Identifier NCT01419691). As a result of the studies described in this publication, a drug repurposing strategy is underway to rapidly translate these findings to patients with GIST with recurrent and metastatic disease.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** Z.Y. Pessetto, 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  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Z.Y. Pessetto, S.J. Weir, G. Sethi, A.K. Godwin  
**Writing, review, and/or revision of the manuscript:** Z.Y. Pessetto, S.J. Weir, G. Sethi, M.A. Broward, A.K. Godwin  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Z.Y. Pessetto, M.A. Broward, A.K. Godwin  
**Study supervision:** A.K. Godwin

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