Unbiased compound screening identifies unexpected drug sensitivities and novel treatment options for gastrointestinal stromal tumors

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Running title: Unexpected novel treatment options for GISTs

Key words: gastrointestinal stromal tumor (GIST), imatinib mesylate, mithramycin A, mitoxantrone, leiomyosarcoma


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The authors declare that they have no competing financial interests.

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Notes: 5701 words, 5 Figures, 3 Supplementary Figures, 1 Supplementary Table
Abstract

Most gastrointestinal stromal tumors (GISTs) are caused by oncogenic KIT or PDGFRA activation, and the small molecule kinase inhibitor imatinib mesylate is an effective first-line therapy for metastatic or unresectable GIST. However, complete remissions are rare and most patients ultimately develop resistance, mostly due to secondary mutations in the driver oncogenic kinase. Hence there is a need for novel treatment options to delay failure of primary treatment and restore tumor control in patients who progress under therapy with targeted agents. Historic data suggest that GISTs do not respond to classical chemotherapy, but systematic unbiased screening has not been performed. In screening a compound library enriched for FDA-approved chemotherapeutic agents (NCI Approved Oncology Drugs Set II), we discovered that GIST cells display high sensitivity to transcriptional inhibitors and topoisomerase II inhibitors. Mechanistically, these compounds exploited the cells' dependency on continuous KIT expression and/or intrinsic DNA damage response defects, explaining their activity in GIST. Mithramycin A, an indirect inhibitor of the SP1 transcription factor, and mitoxantrone, a topoisomerase II inhibitor, exerted significant antitumor effects in mouse xenograft models of human GIST. Moreover, these compounds were active in patient-derived imatinib-resistant primary GIST cells, achieving efficacy at clinically relevant concentrations. Taken together, our findings reveal that GIST cells have an unexpectedly high and specific sensitivity to certain types of FDA-approved chemotherapeutic agents, with immediate implications for encouraging their clinical exploration.
Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and the most common sarcomas in some geographic regions. Most GISTs are caused by activating mutations of the KIT or PDGFRA (platelet-derived growth factor receptor alpha) receptor tyrosine kinase genes (1-4) and can be effectively treated with the small molecule tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec®) (1-4). However, complete remissions are rare, and up to 50% of GIST patients develop resistance during the course of the first two years of systemic treatment (5). Because the approved multi-kinase inhibitors sunitinib malate (Sutent®) (6) and regorafenib (Stivarga®) (7) offer only limited additional benefit for the majority of patients (8), novel therapeutic options are needed to prolong disease stabilization, achieve symptomatic benefit and delay the occurrence of therapy resistance. It is known from a number of studies that resistance to kinase inhibitors is mainly caused by secondary mutations of the driver oncogenic kinase (9,10). Treatment strategies that do not focus on kinase inhibitors may therefore be advantageous, but have remained largely unexplored in GISTs.

It is widely believed that GISTs respond poorly to chemotherapeutic agents commonly used to treat mesenchymal malignancies (5,11). This notion, however, is mainly based on clinical studies that were carried out before the characterization of the KIT/PDGFRA driver mutations in GIST and the introduction of specific diagnostic markers, such as KIT immunohistochemistry. Taking into account that GISTs and intra-abdominal leiomyosarcomas (LMS) are histopathologically very similar, it is possible that earlier clinical trials included gastrointestinal LMS (11), a tumor entity that is known to be highly resistant to chemotherapy. Therefore, a reassessment of the response of GISTs to chemotherapeutic agents is warranted. Additional support for this notion stems from the fact that histone H2AX, a component of the DNA damage and repair machinery, has recently been found to play a role in GIST cell viability and apoptosis (12,13).
In the present study, we performed a compound screen of FDA-approved chemotherapeutic agents (National Cancer Institute Approved Oncology Drugs Set II) (14) in GIST cell lines. Unexpectedly, GIST cells were highly sensitive to drugs targeting gene transcription or inhibiting topoisomerase II. Two compounds, mithramycin A and mitoxantrone, were chosen for further investigation and proved to be active in both imatinib-sensitive and imatinib-resistant GIST cell lines, patient-derived primary GIST cells and two xenograft mouse models. Mechanistically, our results are explained by cellular dependence on oncogenically activated KIT, which is substantially downregulated on the transcriptional level by mithramycin A, as well as high expression levels of topoisomerase II and/or downregulation of topoisomerase I, which sensitize GIST cells to mitoxantrone-induced DNA damage.

Taken together, our results show that unbiased drug screens can identify novel and unexpected drug sensitivities in GISTs caused by the underlying biological alterations of the tumor (15). This approach will facilitate the development of novel treatment options and biomarkers of response with a goal towards a personalized approach even for patients with TKI-resistant GIST.

Materials and Methods

Cell culture

The imatinib-sensitive human GIST cell lines GIST882 (kindly provided by Jonathan A. Fletcher, Brigham and Women’s Hospital, Boston, MA, USA) and GIST-T1 (16) were derived from untreated metastatic GISTs (12,17). GIST882 cells carry a homozygous mutation in KIT exon 13 (K642E) (18), whereas GIST-T1 cells carry a heterozygous KIT exon 11 deletion (V560_Y578del) (16). Imatinib-resistant GIST cell lines GIST430, GIST48 and GIST48B (kindly provided by Jonathan A. Fletcher) (19) were grown as previously described (13). These cells were derived from human GISTs that developed clinical resistance to imatinib therapy. GIST430 carries a heterozygous primary KIT exon 11 deletion (V560_L576del) and a secondary KIT exon
13 point mutation (V654A), whereas GIST48 cells are characterized by a homozygous primary KIT exon 11 mutation (V560D) and a secondary KIT exon 17 mutation (D820A). GIST48B cells are derived from GIST48 cells, with which they share the KIT mutational status, but have no detectable KIT protein expression (19).

Leiomyosarcoma cell lines (SK-UT1, SK-LMS; ATCC) and normal human dermal fibroblasts (Lonza) were maintained according to manufacturer’s recommendations.

A short-term culture was established from a patient with a clinically imatinib- and sunitinib-refractory GIST that underwent surgery for removal of a progressing lesion at UPMC Presbyterian Hospital, Pittsburgh, PA (Institutional Review Board protocol no. 0509050) as previously described (13).

In vitro apoptosis and proliferation assays

Apoptosis and cell viability studies were performed using the Caspase-Glo and CellTiter-Glo luminescence-based assays (Promega) (13). Cells were plated in 96-well flat-bottomed plates (Perkin Elmer), cultured for 24h and then incubated for 48h (Caspase-Glo) or 72h (CellTiter-Glo) with the respective compounds at indicated concentrations or DMSO-only solvent control. Luminescence was measured with a BioTek Synergy 2 Luminometer (BioTek). Data were normalized to the DMSO-only control group.

Compound screen and inhibitor treatments

The National Cancer Institute (NCI) Approved Oncology Drugs Set II compound library was obtained from the NCI under a material transfer agreement. It contains 89 FDA-approved anti-cancer drugs dissolved at 10 mM concentration in DMSO. The full list of drugs is available from the National Cancer Institute (14) and is also shown in Supplemental Table 1. Cells were cultured in 96-well plates and each screen was performed in triplicate at two drug concentrations, 1 μM and 10 μM, to allow for some therapeutic range. Treatment with DMSO,
the KIT inhibitors imatinib and sunitinib (both at 1 μM in DMSO) as well as the proteasome inhibitor bortezomib (0.01 μM in DMSO; all from LC Laboratories) was included in each set of experiments (in triplicate) as negative and positive controls, respectively. Cellular viability and apoptosis were assessed by using luminescence-based assays (see above).

Results from the triplicate screens were averaged and normalized to their respective average DMSO control. These results were then combined into a “response score” using the formula (drug response score=\(\frac{\text{apoptosis} + (1 / \text{viability})}{2}\)) to reflect the fact that luminescence values for increased apoptosis were >1 and values for decreased cell viability were <1. Hits were defined as having a response score ≥2-fold over DMSO-treated cells (i.e., ≥2). Very similar results were obtained when a response score greater than the imatinib (in imatinib-sensitive cells) or sunitinib (in imatinib-resistant cells) score was considered as hit.

For follow-up studies, cells were incubated in imatinib (1 μM), mithramycin A (at various concentrations in DMSO as indicated; Sigma), mitoxantrone (at various concentrations in DMSO as indicated; Thermo Fisher Scientific), α-amanitin (1 μg/ml in dH2O; Sigma) or mock-treated with 0.1 % DMSO or dH2O for up to 72h as indicated.

**Immunological and cell staining methods**

Protein lysates of cells growing as monolayer were prepared as described previously (20). Xenograft specimens were minced on ice in the presence of lysis buffer and homogenized using a Tissue Tearor (BioSpec) (20). 30 μg of protein were loaded on a 4-12% Bis-Tris gel (Invitrogen) and blotted onto a nitrocellulose membrane.

Immunofluorescence analysis for phosphorylated RNA polymerase II and CBP was performed as described previously (12). To assess the cellular localization of pATM S1981, pH2AX S139, MRE11 and 53BP1, Alexa Fluor 488- or Cy3+-conjugated secondary antibodies (Invitrogen and Jackson Immunoresearch, respectively) were used (30 min at room temperature
in the dark). Cells were analyzed using an Olympus AX70 epifluorescence microscope equipped with a SpotRT digital camera.

Immunohistochemical staining of paraffin-embedded mouse xenograft sections was done as described previously (21).

Primary antibodies used for immunoblotting, immunofluorescence and immunohistochemistry were the following: actin (Sigma), ATM and pATM S1981 (both Epitomics), pATM S1981 (Rockland), CBP (Santa CruzA), cyclin A (Novocastra), cleaved caspase 3 (Cell Signaling), H2AX (Bethyl), pH2AX S139 (Millipore), pH3 S10, pKIT Y719 (both Cell Signaling), KIT (DakoCytomation), MRE11 (Novus), PARP (Invitrogen/Zymed Laboratories), RNA polymerase II, pRNA polymerase II S2 and pRNA polymerase II S5 (all Covance), Topoisomerase I (Abcam), Topoisomerase II (Cell Signaling), p27Kip1 (BD Biosciences Pharmingen, San Diego, CA) and 53BP1 (Calbiochem).

Apoptotic cells were visualized using the In situ Cell Death Detection Kit (Roche Applied Sciences) according to manufacturer’s recommendations (12).

Reverse transcriptase (RT)-PCR and quantitative real time RT-PCR

For RT-PCR and quantitative real time RT-PCR (qRT-PCR) were performed as described previously (13,22). Exon-overlapping, mRNA/cDNA-specific primers were used to amplify KIT (forward: 5’-TCATGGTCGGATCACAAAGA-3’, reverse: 5’-AGGGGCTGCTTCCTAAAGAG-3’; Operon) and β-actin (forward: 5’-CCAAGGCCAACCGCGAGAGATGAC-3’, reverse: 5’-AGGTTACATGGTGGTGCCGAGAC-3’). β-Actin served as reference gene for relative quantification in quantitative real time RT-PCR (qRT-PCR) experiments.
Comet assay

DNA double strand breaks were detected by using the CometAssay® kit from Trevigen under neutral conditions (23). In brief, cells were treated with DMSO or mitoxantrone for the indicated amount of time. Apoptotic cells were excluded from the assay by removing the supernatant before trypsinising. Cells were then washed in ice-cold PBS, diluted to 75,000 cells/ml and added to molten LMAgarose. After spotting onto two-well CometSlides™ and solidifying, slides were immersed in lysis solution and electrophoresed in chilled TBE buffer (21 V, 10 min). Slides were then fixed in 70% ethanol and dried. DNA was labeled with SYBR Green and slides were viewed under a Nikon ECLIPSE 50i epifluorescence microscope (Nikon). Comet tails were analyzed using Comet Assay IV v4.2 software (Perceptive Instruments). At least 100 cells were analyzed per sample.

GIST xenograft models

GIST882 or GIST-T1 cells were bilaterally injected in the flank of female adult athymic nude mice (NMRI, nu/nu; Janvier Laboratories) (21,24). When tumors were 1 cm in diameter, mice were randomized into groups of four animals each for each treatment regimen. Mithramycin A (0.5 mg/kg in PBS) or mitoxantrone (1.5 mg/kg in 0.9% NaCl) was administered intraperitoneally (i.p.) three times a week or every four days, respectively, for two weeks (25,26). Imatinib-treated mice (50 mg/kg twice a day, p.o.) served as positive control, while mice receiving PBS (150 μl, i.p.) were used as negative control. Tumor volume, weight and general health of the mice were recorded (21). After the mice were sacrificed, tumors were excised and divided for fresh frozen samples and histopathologic examination. The animal experiment was approved by the Ethics Committee of KU Leuven (Leuven, Belgium).

Histopathologic grading of the response to the compounds was performed (21,27) and was based on the microscopic amount of necrosis, myxoid degeneration or fibrosis with grade 1 representing a minimal response (0-10%) and grade 4 representing a maximal response.
Apoptotic and mitotic cells were visualized by immunohistochemical staining for cleaved caspase 3 and phosphorylated histone H3 (S10), respectively (21). The number of positive cells was counted per 10 high power fields (HFP) at 400-fold magnification.

**Statistics**

Statistical significance was assessed using Student's t test for independent samples and the Mann-Whitney U test for not normally distributed samples (VassarStats, http://vassarstats.net). P values ≤0.05 were considered significant. Unsupervised hierarchical clustering analysis was performed using Cluster 3.0 (28) and visualized using MapleTree (http://www.eisenlab.org/eisen).

**Results**

A compound screen identifies inhibitors of transcription and of topoisomerase II as active agents against GIST cells

To determine the sensitivity of GIST cells to chemotherapeutic agents, we performed a screen using the NCI/NIH Approved Oncology Drugs Set II (14), which consists of 89 FDA-approved compounds (Suppl. Table 1). Two imatinib-sensitive (GIST882, GIST-T1) and three imatinib-resistant (GIST430, GIST48, GIST48B) human GIST cell lines were tested. Because it is conceivable that historically GISTs were frequently misclassified as abdominal leiomyosarcoma (LMS), two LMS cell lines (SK-LMS, SK-UT-1) were analyzed in direct comparison.

A total of thirty-seven compounds were identified that had anti-tumor activity in at least one GIST cell line for at least one of the concentrations tested (Suppl. Table 1). Many of the active compounds had response scores that were substantially higher than the response scores of imatinib or sunitinib. Importantly, a two-way unsupervised hierarchical clustering analysis separated GIST and LMS cells (Fig. 1). Although one GIST cell line (GIST430) clustered close
to the SK-LMS and SK-UT-1 cells, this unbiased analysis underscores a trend towards the different drug sensitivities of these two tumor entities. Compounds that showed activity in GIST cells clustered according to their mode of action (Fig. 1). The most effective compound classes were transcriptional inhibitors and inhibitors of topoisomerase II, especially those with intercalating properties (see below).

The most effective compound in our screen was the proteasome inhibitor bortezomib with strong activity in all GIST cell lines tested. This confirms our previous study that had identified bortezomib as an effective compound against GIST cells (13). The compounds that ranked second and third in antineoplastic efficacy were the transcriptional inhibitors mithramycin A and dactinomycin, which showed activity in all GIST lines tested (Fig. 1, Suppl. Table 1). Remarkably, bortezomib has previously been shown to function in part through blocking ongoing gene transcription in GIST (13) and these three compounds, mithramycin A, dactinomycin and bortezomib, were found to cluster (Fig. 1).

Additional agents with high anti-tumor activity in GIST cells were topoisomerase II inhibitors, especially those with an additional intercalating property, such as mitoxantrone, daunorubicin, doxorubicin and valrubicin (Fig. 1, Suppl. Table 1). GIST cells were resistant to most alkylating agents as well as estrogen receptor modulators, aromatase inhibitors, photo-activated drugs and immunomodulatory agents (Suppl. Table 1). Microtubule poisons showed some sensitivity in GIST48 and its derivative GIST48B (Suppl. Table 1) but not in other GIST cell lines.

Taken together, our screen of a library of FDA-approved compounds in GIST cells showed significant antitumor activity of two main drug classes, inhibitors of gene transcription and inhibitors of topoisomerase II.
Mithramycin A and mitoxantrone are effective inducers of apoptosis and cell cycle arrest in imatinib-sensitive and imatinib-resistant GIST cells

Having identified inhibitors of gene transcription and topoisomerase II inhibitors as two main drug classes with activity in GIST cells, we selected one compound of each class, mithramycin A and mitoxantrone, for further validation. These studies were carried out in one imatinib-sensitive (GIST882) and one imatinib-resistant (GIST430) cell line.

Treatment of GIST882 and GIST430 cells with increasing concentrations of mithramycin A (0.001 μM to 10 μM), induced a statistically significant pro-apoptotic and growth-suppressive effect that was seen in both cell lines beginning at concentrations of 0.1 μM (Fig. 2A, Suppl. Fig. 1A). Likewise, concentration-dependent cleavage of PARP and caspase 3 was detected by immunoblotting in both cell lines starting at 0.1 μM indicating the onset of apoptosis (Suppl. Fig. 1B). At the same time, expression of the S phase marker cyclin A decreased in a concentration-dependent manner. Based on these results, the lowest effective concentration of mithramycin A was defined as 0.1 μM and was used in all further experiments.

For mitoxantrone, a statistically significant increase in apoptosis and reduction of cell viability was seen starting at 0.1 μM in GIST882 (Fig. 2B), whereas these effects were only seen at concentrations of 1.0 μM and 10.0 μM in GIST430 (Fig. 2B). A TUNEL assay confirmed the higher sensitivity of GIST882 to mitoxantrone when compared to GIST430 (Suppl. Fig. 2A). Immunoblot analysis showed strong apoptosis-associated PARP and caspase 3 cleavage after 1.0 μM mitoxantrone in GIST882, whereas the effect was less pronounced in GIST430 at these concentrations (Suppl. Fig. 2B). Protein expression of the S phase marker cyclin A was completely lost at 10 μM in both GIST882 and GIST430 (Suppl. Fig. 2B). Based on these results, the minimum effective concentrations for mitoxantrone was defined as 1 μM for GIST882 and 5 μM in GIST430.

It is important to note that the minimal effective concentrations defined in our study (0.1 μM mithramycin A and 1 to 5 μM mitoxantrone) are clinically achievable in humans.
levels of mithramycin have been reported to reach peak concentrations of 0.30 to 0.35 μM after a 2-hour continuous infusion of the standard dose of 25 μg/kg (29). While the standard dose of mitoxantrone in humans (10-14 mg/m²) produces peak plasma concentrations in the range of 1.0 μM (30-32), high-dose regimens using up to 80 mg/m² have been tested extensively and have been deemed safe (33). In summary, these results demonstrate that mithramycin A and mitoxantrone exert their antineoplastic activities at concentrations that are relevant for the clinic and can be achieved in the sera of cancer patients (32,34).

The response to mithramycin A and mitoxantrone is time-dependent

To ascertain the time-dependency of the effects of mithramycin A and mitoxantrone in GIST cells, we treated GIST882 and GIST430 cells at the minimal effective concentrations for 1 to 72h (Fig. 2C-E).

Mithramycin A caused an apoptotic response starting after 24h in GIST882 and GIST430 cells (Fig. 2C,D). Levels of the S phase marker cyclin A decreased in parallel with apoptosis induction (Fig. 2C).

Mitoxantrone induced a more rapid onset of apoptosis in GIST882 starting after 8h of treatment (Fig. 2E). In GIST430 cells, an apoptotic response was detected after 24h (Fig. 2E). There was a very strong apoptotic effect in GIST430 after 72h as reflected by nearly complete PARP and caspase 3 degradation (Fig. 2E,F). Mitoxantrone also led to an exit of the cell division cycle as shown by increased levels of p27Kip1 in GIST882 and a decrease in cyclin A in GIST430 (Fig. 2E).

Taken together, our results show differences in the timing of apoptosis induction and growth suppression between mithramycin A (24h in GIST882 and GIST430) and mitoxantrone (8h in GIST882 and 24h in GIST430) that may reflect distinct mechanisms of action.
Mithramycin A leads to loss of KIT expression, transcriptional inhibition and activation of a DNA damage response

We next sought to confirm the mechanism of action of the pro-apoptotic and growth inhibitory activities of mithramycin A and mitoxantrone in GIST cells.

Mithramycin A has DNA-binding properties that lead to an inhibition of gene transcription mediated by SP1 (35,36), one of the major transcriptional activators of the KIT gene (37). Because expression of oncogenically activated KIT is a tumor-specific, crucial driver of GIST pathogenesis and survival, GIST cells are critically dependent on the expression of mutant KIT (12,13,38). We therefore tested first whether mithramycin A treatment causes a downregulation of KIT transcription. We found a substantial downregulation of KIT mRNA expression levels in mithramycin A-treated GIST cells by RT-PCR and qRT-PCR (Fig. 3A). The effect was comparable to cells treated with the RNA polymerase (pol) II inhibitor α-amanitin (Fig. 3A). Importantly, reduced KIT mRNA levels after mithramycin A treatment led to a complete abrogation of KIT protein expression and activation starting at 8h after treatment in both, GIST882 and GIST430 cells (Fig. 3B). The effect on KIT transcription and expression was specific for mithramycin A, because it was not seen in cells treated with mitoxantrone (Fig. 3A).

To further characterize the effects of mithramycin A on ongoing gene transcription, we determined expression and activation of the core transcriptional machinery. Mithramycin A led to a loss of active RNA pol II from chromatin both in its initiating form (S5 phosphorylation of the RNA pol II carboxyl-terminal repeat domain, CTD) and elongating form (S2 phosphorylation of the CTD; Fig. 3C) (39). Immunoblotting demonstrated a loss of pRNA pol II S5 and S2 as well as total RNA pol II in a time-dependent manner (Fig. 3D), a finding that has been described to occur during prolonged transcriptional stalling (40). In addition, mithramycin A induced a redistribution of the transcriptional co-activator CREB-binding protein (CBP) into a coarsely speckled pattern in the nucleus (foci) that is associated with inactive gene transcription (Fig. 3E) (39). These results underscore that reduced KIT mRNA levels after mithramycin A may be the
result of two mechanisms: inhibition of SP1-mediated transcription and a more general attenuation of ongoing gene transcription. Furthermore, it is likely that both processes affect additional vital gene products thereby adding to the effectiveness of the drug.

It has been shown that a prolonged transcriptional block can cause DNA damage (41). In addition, direct binding of mithramycin A to DNA can potentially lead to DNA replication fork stalling (35,36). We therefore asked whether mithramycin A treatment would also induce a measurable DNA damage response. We found that mithramycin A led to an increase of phosphorylated ATM at S1981 after approximately 24 hours (Fig. 3F). This DNA damage response effect was somewhat delayed when compared to the measurable onset of transcriptional inhibition (approximately 8 h) suggesting that the induction of DNA damage is a secondary effect that may be cell cycle-dependent. In line with this notion, a proportion of cells is still expressing S phase markers at 24h after mithramycin A-treatment (Fig. 2C). The fact that pATM S1981 and pH2AX S139 nuclear foci that co-localized with MRE11 and 53BP1, respectively, were detected (Fig. 3G) underscored the induction of a DNA double strand break response.

Taken together, mithramycin A has a dual mechanism of targeting GIST cells for apoptosis by inhibiting ongoing gene transcription and inducing DNA damage.

*Mitoxantrone induces a rapid DNA double strand break response in GIST cells*

As expected based on its known mode of action (42), mitoxantrone rapidly induced phosphorylation of the DNA double strand break (DSB) response protein ATM at S1981 in both GIST882 and GIST430 cells (1 h; Fig. 4A). In addition, mitoxantrone led to the formation of DSB-associated nuclear pH2AX S139 foci in GIST882 and GIST430 (Fig. 4B).

To prove that the rapid ATM phosphorylation induced by mitoxantrone indeed represents the induction of DNA double strand breaks, a Comet assay was performed. Mitoxantrone treatment caused DNA fragmentation as evidenced by so-called comet tails in GIST882 and
GIST430 cells (Fig. 4C). This effect was quantified by analyzing comet tail intensity and tail moment, both of which were significantly altered after mitoxantrone treatment indicating DNA breakage (Fig. 4C, right panels).

Topoisomerase expression levels have been shown to correlate with sensitivity to topoisomerase II inhibitors including mitoxantrone (43). In particular, low expression levels of topoisomerase II have been reported to confer drug resistance whereas high levels can sensitize to topoisomerase II inhibition (44). Moreover, low levels of topoisomerase I were found to correlate with increased sensitivity to topoisomerase II inhibitors (44). We hence analyzed topoisomerase I and II protein expression in GIST882 and GIST430 by immunoblotting (Fig. 4D). We found a markedly decreased expression of topoisomerase I in GIST882 cells in comparison to controls as well as increased levels of topoisomerase II expression in GIST430, which can explain the sensitivity of these cells to mitoxantrone. On the other hand, this finding does not readily explain the lower sensitivity of GIST430 to the drug when compared to GIST882.

To further explore this perplexing finding, we decided to further dissect the DNA double strand break response in GIST430 cells in comparison to GIST882 cells after mitoxantrone treatment. As shown by immunofluorescence microscopy, GIST430 cells had a severe defect in the proper formation of DNA damage-associated nuclear foci that contain pATM S1981 and 53BP1 (Fig. 4E). This finding correlated with the inability of GIST430 cells to maintain ATM phosphorylation (Fig. 4A) despite the presence of broken DNA (Fig. 4C).

Taken together, our results show that mitoxantrone induces DNA breakage in both GIST882 and GIST430 cells. Whereas GIST882 cells mount a rapid DNA damage response and start to undergo apoptosis after 8h of drug treatment, GIST430 show a defective DNA damage response and a delayed but massive apoptosis induction (Fig. 2F). Apoptosis in these cells is therefore most likely not due to a coordinated DNA damage-induced activation of pro-
apoptotic pathways (as in GIST882), but rather due to a mechanism that involves transition into a different cell cycle stage, such as mitotic catastrophe (45).

Inhibitors of transcription and of topoisomerase II are effective in patient-derived imatinib- and sunitinib-double-resistant GIST cells

Having shown that mithramycin A and mitoxantrone have significant activity in imatinib-sensitive and imatinib-resistant GIST cells, we aimed to corroborate our results in patient-derived primary GIST cells. As expected, treatment of clinically imatinib- and sunitinib-resistant primary GIST cells with imatinib or sunitinib had no effect on tumor cell viability (Suppl. Fig. 3A). Of a total of five compounds tested in these primary cells, only treatment with mithramycin A led to significant caspase 3 cleavage and almost complete degradation of PARP indicating a strong apoptotic response. Mithramycin A and treatment with the topoisomerase II inhibitor etoposide (5 μM), led to a substantial reduction of cell proliferation as measured by decreased levels of cyclin A and an increase in p27Kip1 levels. Of note, the proteasome inhibitor bortezomib also induced apoptosis as detected by a PARP cleavage fragment in these cells.

Mithramycin A and mitoxantrone are effective in GIST xenograft models

Lastly, we tested the anti-tumor activity of mithramycin A and mitoxantrone in vivo using GIST xenograft models (21,24).

Both mithramycin A and mitoxantrone had a significant effect on apoptosis induction and cell cycle inhibition as detected by immunoblotting of proteins extracted from excised GIST882 xenografts (Suppl. Fig. 3B). Both compounds also induced major histopathological changes in this model (Fig. 5A). Mithramycin A treatment led to a dramatic response according to a previously defined histopathologic response score (21,27). Importantly, these morphological findings, which included a central necrosis surrounded by a zone of myxoid degeneration, phenocopy the in vivo response to imatinib treatment (21). The histopathological response to
mitoxantrone was remarkably different and resulted in extensive intratumoral hemorrhage and single cell death (Fig. 5A).

Both mithramycin A or mitoxantrone treatment caused a significantly increased intratumoral apoptosis as detected by immunohistochemical staining for cleaved caspase 3 (464 and 327 apoptotic cells per ten high power fields (HPF) in mithramycin A- and mitoxantrone-treated cells, respectively, compared to 66 apoptotic cells/10 HPF in placebo-treated controls; p<0.0003 and p<0.0001, respectively; Fig. 5B). Furthermore, treatment with mithramycin A or mitoxantrone significantly reduced the number of mitotic cells as measured by positivity for phosphorylated histone H3 S10 (from 15/10 HFP in controls to 100 and 82/10 HPF in mithramycin A- and mitoxantrone-treated cells, respectively; p<0.023 and p<0.009; Fig. 5C). These results were confirmed in a second xenograft model (GIST-T1) (24), in which mithramycin A and mitoxantrone also significantly reduced the number of histone H3 S10-positive mitotic cells (from 381/10 HPF in controls to 158 and 132/10 HPF in mithramycin A- and mitoxantrone-treated cells, respectively; both p<0.0001; Suppl. Fig. 3C).

Although there was no objective reduction in tumor size after mithramycin A or mitoxantrone, the growth of the xenografts was attenuated when compared to control-treated animals (Suppl. Fig. 3D,E). Importantly however, mithramycin A or mitoxantrone treatments did lead to significant intratumoral changes as described above, thereby clearly demonstrating the effectiveness of the treatment (Fig. 5A). It is of note that such changes would not necessarily result in a reduction of tumor size, which is in line with the typical clinical response to imatinib as well as imatinib-treated xenografts (21).

Taken together, both mithramycin A and mitoxantrone treatment showed significant anti-tumor activity in vivo using GIST xenograft mouse models.
Discussion

Imatinib mesylate (Gleevec®) has revolutionized treatment and prognosis of patients with GIST (2). Nevertheless, the majority of patients develops resistance to imatinib as well as the only FDA-approved second- and third-line therapies, sunitinib malate (Sutent®) and regorafenib (Stivarga®), over time (5,7,8). Novel therapeutic strategies are hence urgently needed.

To effectively identify active compounds and to be able to rapidly move candidate drugs into the clinic, we screened compounds of the NCI Approved Oncology Drugs Set II (14) for their anti-tumor activity in a panel of GIST cell lines. We identified a number of highly active compounds that mostly belonged to two distinct drug classes, inhibitors of gene transcription and topoisomerase II inhibitors.

The notion that GISTs are not sensitive to conventional chemotherapy originates from earlier clinical trials (11) that may have been biased by the inadvertent inclusion of histopathologically similar, but highly chemoresistant tumors, such as abdominal leiomyosarcomas. As a result, the actual response rate of GISTs to chemotherapy may have been underestimated. Neither mithramycin A nor mitoxantrone, the two compounds that were chosen for further follow-up in our study because of their high activity in GIST cells *in vitro* and *in vivo*, have systematically been tested for systemic treatment of GIST.

Nevertheless, it is of note that mitoxantrone yielded favorable results when used intraperitoneally to prevent local recurrence of abdominal sarcomas (46). It is worth mentioning, however, that this study did not record the clinical effect of the treatment by histopathologic tumor entity. In addition, data were obtained during the early 1990s, when an unequivocal identification of GISTs was difficult due to the lack of reliable diagnostic markers. Yet, there is more recent evidence for the clinical activity of topoisomerase II inhibitors in GIST. When treated with doxorubicin in combination with imatinib, 36% of imatinib-resistant patients were reported to have had clinical benefit, especially a subgroup of patients without detectable...
**KIT**/PDGFRA mutations (47). Conversely, our compound screen highlighted a remarkable resistance of GIST cells to alkylating agents. These results are supported by clinical findings from a study that tested the alkylator temozolomide in GIST patients and found a very low response rate to this treatment (48). Taken together, our systematic approach to revisiting chemotherapeutic agents for GIST therapy resulted in the identification of highly effective but also inactive compounds that, to a certain extent, are reflected by anecdotal clinical evidence. Interestingly, a recent study reported activity of another FDA-approved compound, auranofin, an oral, gold-containing agent used to treat rheumatoid arthritis, in GIST cells (49).

Mithramycin A has recently gained attention in the treatment of Ewing sarcoma (25). This compound was the top hit in a 50,000 compound screen to identify inhibitors of the EWS-FLI1 transcription factor, the hallmark of the Ewing sarcoma family of tumors (ESFT) (25). Similar to the results presented here, the concentration of mithramycin A used was relatively low (0.1 μM), an activated DNA damage response was detected and the compound was active in a xenograft model. A study testing the clinical activity of mithramycin in Ewing sarcoma patients has been initiated and is currently recruiting patients (NCT01610570; (50). Thus, mithramycin A seems to be particularly effective in sarcomas and other malignancies including GIST that critically depend on ongoing gene transcription.

Mithramycin A competitively binds to GC-rich promoter regions thereby replacing the transcription factor SP1 (35,36). Through this mechanism, mithramycin A inhibits the transcription of many SP1-regulated genes, such as MYC and SRC (51,52). Interestingly, SP1 is also a major transcriptional activator of the KIT gene (37) and its inhibition in GIST cells by mithramycin A thus led to a substantial decrease of KIT mRNA and protein expression and hence reduced KIT activation. Due to the essential role that expression of oncogenic KIT plays for GIST cell survival, these changes provide a compelling mechanistic explanation for the induction of apoptosis in these cells. Moreover, inhibition of SP1-mediated transcription of SRC family kinase (SFK) expression could have an additive effect of mithramycin A treatment in
GIST as SFKs have been shown to play important roles in GIST cell biology (52,53). Because of its role as inhibitor of SP1, it has been postulated that mithramycin A leads to a relatively gene specific downregulation of transcription. However, in our study we detected displacement and loss of active RNA polymerase II and the transcriptional coactivator CBP from chromatin after treatment with mithramycin A indicating a more global inhibition of cellular transcription that may affect additional vital gene products. In addition to the direct effect on gene transcription, we could show that mithramycin A triggered a DNA damage response in GISTs as indicated by the formation of DNA damage-associated nuclear foci. This response was delayed when compared to DNA double strand break inducers (such as mitoxantrone) as well as the onset of downregulation of KIT expression by mithramycin A indicating that it is a secondary effect that occurs after prolonged transcriptional stalling and/or when a stalled DNA replication fork at the site of mithramycin A binding to DNA is converted to a double strand break. Taken together, mithramycin A may be specifically effective in GIST through several mechanisms: Competitive displacement of SP1 from the KIT promoter leading to rapid downregulation of KIT expression and activation, transcriptional downregulation of cellular oncoproteins other than KIT, such as SRC, global inhibition of cellular transcription and the induction of a DNA double strand break response. All mechanisms are independent of direct KIT kinase inhibition and therefore irrespective of a secondary mutation as mechanism of resistance meaning that mithramycin A is sensitive in imatinib-sensitive and imatinib-resistant GIST.

Topoisomerase II inhibitors, such as mitoxantrone, are known to induce DNA breakage by stabilizing a usually transient double strand break that is catalyzed by topoisomerase II in order to relieve conformational and topological changes DNA during replication. Mitoxantrone has been shown to rapidly induce these breaks in vitro (54), a finding that we confirmed in GIST cells by showing a rapid activation of ATM as well as by the detection of fragmented DNA in a Comet assay. Although protein expression levels of topoisomerase I (low in GIST882) and II (high in GIST430) could explain the sensitivity of GIST882 cells, they failed to explain the lower
sensitivity and delayed apoptosis induction in GIST430 cells. However, we discovered that GIST430 cells have a defective DNA damage response and fail to assemble proper DNA damage foci and maintain ATM activation despite DNA damage. It is hence possible that GIST882 cells rapidly undergo apoptosis in response to DNA damage by activating pro-apoptotic signaling pathways. In contrast, GIST430 cells appear to contain damaged DNA without a proper DNA damage response, which could trigger alternative forms of cell death that require a longer time interval, such as mitotic catastrophe, where cells have to move to a different cell cycle stage before they undergo cell death.

One possibility of achieving an enhanced therapeutic effect is to use drugs in combination. In an attempt to do so, we have tested several combinations of imatinib with either mithramycin A or mitoxantrone in both imatinib-sensitive and imatinib-resistant GIST cells *in vitro*. These preliminary experiments did not show evidence for synergy (data not shown). Interestingly, similar results were obtained in a study by Lee et al. when simultaneously treating breast cancer cells with the EGFR inhibitor erlotinib and the topoisomerase II inhibitor doxorubicin (55). Time-staggered, sequential treatments, however, significantly sensitized these cells to undergo apoptosis, presumably due to extensive network rewiring. Addressing this question in GIST cells will be subject of future studies.

In summary, we have shown that an unbiased compound screen can identify novel drug sensitivities in GIST that can be exploited clinically. Importantly, we were able to link drug activities to intrinsic molecular requirements and defects, such as continued KIT transcription, differences in topoisomerase expression or defects in the cellular DNA damage response. Our study provides a framework for the future development of mono- or combination therapies and biomarkers predicting the individual response of GIST patients with an aim towards more complete remissions and improved long-term disease control.
Disclosure of potential conflicts of interest

None.

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Acknowledgments

The authors would like to thank J. A. Fletcher for sharing important reagents, C. Bakkenist and R. Sobol for help with the comet assay and S. Duensing for critically reading the manuscript and valuable discussions.
Grant Support

This work was supported by a Research Scholar Grant from the American Cancer Society (RSG-08-092-01-CCG; to A.D.), The Life Raft Group (to A.D. and M.D.R.), the GIST Cancer Research Fund (to A.D.) and an Undergraduate Research Grant of the Howard Hughes Medical Institute (to D.L.). A.D. is supported by the University of Pittsburgh Cancer Institute and in part by a grant from the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.
References


Figure Legends

Figure 1. Enhanced chemosensitivity distinguishes gastrointestinal stromal tumor (GIST) from leiomyosarcoma (LMS) cells.

Two-way unsupervised hierarchical clustering of the response scores of five GIST and two LMS cell lines to 89 FDA-approved anticancer drugs (NCI Approved Oncology Drugs Set II) at a concentration of 1 µM (mean values of triplicate experiments). Clustering was done using Cluster 3.0 (28). The height of the “tree” indicates the quality of the correlation. Each row represents a tested drug; cell lines are arranged in columns. Drugs are color-coded according to their mechanism of action (also see Suppl. Table 1). Response scores of >1 (effective drugs) are shown in red, response scores of <1 (ineffective drugs) are shown in green. Note the clustering according to cell type as well as compound class within the group of effective compounds for GIST cells. Control compounds are shown in capital letters.

Figure 2. The transcriptional inhibitor mithramycin A (MMA) and the topoisomerase II inhibitor mitoxantrone (MXN) effectively induce time-dependent GIST cell apoptosis and cell cycle arrest.

(A, B) Dose-dependent effect of MMA (A) and MXN (B) on apoptosis (left panels) and cell viability (right panels) of GIST882 and GIST430 cells as measured by luminescence-based assays (mean ± standard error; SE). Asterisk denotes p<0.05 in comparison to control. (C, D) Immunoblot analysis (C) for markers of apoptosis and cell cycle regulation and TUNEL assay (D) in GIST cells after treatment with DMSO or 0.1 µM MMA for the indicated times. (E) Immunoblot analysis for markers of apoptosis and cell cycle regulation in GIST cells after treatment with DMSO or MXN at indicated concentrations for 72 h or with MXN at the indicated times. (F) Brightfield image of GIST430 cells treated with DMSO or MXN (5 µM) for 72 h.
**Figure 3.** *Mithramycin A stimulates GIST cell death by transcriptional inhibition and induction of a cellular DNA damage response.*

(A) RT-PCR (left panel) and quantitative RT-PCR (qRT-PCR; right panel) amplification of KIT mRNA in GIST cells after treatment with DMSO, mithramycin A (MMA; 0.1 μM), mitoxantrone (MXN; 1 μM, GIST882; 5 μM, GIST430) or α-amanitin (1 μg/ml) for 48 h. Amplification of β-actin mRNA is shown to demonstrate equal loading (RT-PCR, left panel). qRT-PCR values are normalized against β-actin mRNA values. Columns, mean ± standard error (SE); asterisk denotes p≤0.05 in comparison to DMSO controls. (B) Immunoblot analysis of GIST cells treated with DMSO or MMA for the indicated times and probed for phosphorylated KIT (Y719) and KIT. Actin is shown as a loading control. (C) Quantification of the percentage of GIST882 and GIST430 cells showing a normal, fine-speckled nuclear staining pattern for pRNA polymerase (pol) II (S5, left graph) or pRNA pol II (S2, right graph) as examined by immunofluorescence microscopy after treatment with DMSO, MMA or α-amanitin for 48 h. Columns, mean ± standard error (SE); asterisk denotes p≤0.05 when compared to controls. (D) Immunoblot analysis of GIST882 and GIST430 cells treated with DMSO or MMA for up to 72 h and probed for pRNA pol II (S5), pRNA II pol (S2) and total RNA pol II. For loading control, see actin stains in (B). (E) Immunofluorescence microscopic analysis of GIST882 cells treated with DMSO, MMA or α-amanitin for 48 h and stained for CREB binding protein (CBP, left panels). Nuclei were stained with DAPI (blue). Note the diffuse nuclear staining in DMSO controls and the redistribution into nuclear foci in the treated cells indicating transcriptional inactivation. Quantification of the percentage of GIST882 or GIST430 cells showing a normal nuclear staining pattern for CBP (right panel) after treatment with DMSO, MMA or α-amanitin for 48 h. Columns, mean ± standard error (SE); asterisk denotes p≤0.05 when compared to controls. (F) Immunoblot analysis of GIST882 and GIST430 cells treated with DMSO or MMA for up to 72 h and probed for pATM S1981 and total ATM. Actin is shown as a loading control. (G) Immunofluorescence microscopic analysis of GIST882 for DNA damage response-associated nuclear foci (pATM S1981 and
MRE11, upper panel; γ-H2AX and 53BP1, lower panel) after MMA treatment for 48h. Nuclei in stained with DAPI (blue), 100x magnification.

**Figure 4.** Response of GIST cells to mitoxantrone involves topoisomerase expression and robustness of the cellular DNA damage machinery. (A) Immunoblot analysis of GIST882 and GIST430 cells treated with DMSO or MXN (1 μM, GIST882; 5 μM, GIST430) for up to 72h and probed for pATM S1981 and total ATM. Actin is shown as a loading control. (B) Immunofluorescence microscopic analysis of GIST882 (upper panel) and GIST430 cells (lower panel) for γ-H2AX and 53BP1 after MXN treatment (1 μM, GIST882; 5 μM, GIST430) for 48 h. Nuclei in stained with DAPI (blue), 100x magnification. (C) Detection of DNA double strand breaks in a Comet assay performed under neutral conditions after treating GIST882 and GIST430 cells with DMSO (24 h) or MXN (72 h; GIST8821 μM, GIST4305 μM; left panels). Quantification of tail intensity (% of DNA in comet tail, left graph) and tail moment (right graph). Asterisk denotes p<0.0001 compared to control (Mann-Whitney U test). (D) Immunoblot analysis of topoisomerase I and topoisomerase II (α isoform) expression levels in GIST882 and GIST430 cells in comparison to normal human fibroblasts (NHF). Actin is shown as a loading control. (E) Quantification of the kinetics of the appearance of γ-H2AX (dashed line), pATM S1981 (solid line) and 53BP1 (punctate line) nuclear foci in GIST882 and GIST430 cells as assessed by immunofluorescence staining after MXN treatment (GIST882, 1 μM; GIST430, 5 μM) at the indicated time-points. Cells treated with DMSO were used as a negative control.

**Figure 5.** In vivo activity of mithramycin A and mitoxantrone in a murine GIST xenograft model. (A) Histopathological response of GIST xenografts to treatment with MMA and MXN in comparison to placebo (H&E, 10x magnification; upper panels). Data shown in graph represent
the average of at least eight tumors per group. Columns, mean ± standard error (SE); asterisk denotes p≤0.05. Dashed lines in MMA-treated histograms separate viable tumor (tu), necrosis (nec) and myxoid degeneration (myx). (B, C) Immunohisto-chemical staining for cleaved caspase 3 (B) or phosphorylated histone H3 S10 (C) in GIST xenografts treated with MMA or MXN in comparison to placebo (40x magnification; upper panel). Quantification of apoptotic (B) or mitotic cells (C; brown staining, respectively) per 10 high power fields (HPF; lower panel) represents the average of at least eight tumors per group. Columns, mean ± SE; asterisk denotes p≤0.05.
Boichuk et al., Figure 5
Unbiased compound screening identifies unexpected drug sensitivities and novel treatment options for gastrointestinal stromal tumors

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Cancer Res  Published OnlineFirst January 2, 2014.