Multiplexed Evaluation of Microdosed Antineoplastic Agents In Situ in the Tumor Microenvironment of Patients with Soft Tissue Sarcoma

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ABSTRACT

Purpose: A persistent issue in cancer drug development is the discordance between robust antitumor drug activity observed in laboratory models and the limited benefit frequently observed when patients are treated with the same agents in clinical trials. Difficulties in accurately modeling the complexities of human tumors may underlie this problem. To address this issue, we developed Comparative In Vivo Oncology (CIVO), which enables in situ investigation of multiple microdosed drugs simultaneously in a patient’s tumor. This study was designed to test CIVO’s safety and feasibility in patients with soft tissue sarcoma (STS).

Patients and Methods: We conducted a single arm, prospective, 13-patient pilot study. Patients scheduled for incisional biopsy or tumor resection were CIVO-injected 1 to 3 days prior to surgery. Saline or microdoses of anticancer agents were percutaneously injected into the tumor in a columnar fashion through each of eight needles. Following excision, drug responses were evaluated in the injected tissue.

Results: The primary objective was met, establishing CIVO’s feasibility and safety. Device-related adverse events were limited to transient grade 1 nonserious events. In addition, biomarker evaluation of localized tumor response to CIVO microinjected drugs by IHC or with NanoString GeoMx Digital Spatial Profiler demonstrated consistency with known mechanisms of action of each drug, impact on the tumor microenvironment, and historic clinical activity.

Conclusions: These results are an advance toward use of CIVO as a translational research tool for early evaluation of investigational agents and drug combinations in a novel approach to phase 0 trials.

Introduction

Most investigational oncology agents entering phase I trials fail to provide significant therapeutic benefit to patients with cancer (1). This is underscored by low (~10%) approval for drugs entering phase I (2). The lack of dependable laboratory models on which to test new agents and accurately predict success in patients is a long-standing and well-recognized challenge (3, 4). The plethora of rodent xenograft models available for preclinical investigation often lack a fully functioning immune system or a completely relevant human tumor microenvironment (TME), and thus fail to accurately capture the complexities and behaviors of human solid tumors (5). New approaches to the evaluation of patient-derived tumor explants, slices, organoids, and spheroids may partially address this issue (6, 7). However, all of these require some degree of laboratory manipulation and all lack access to physiological influences only present in the patient with cancer including, but not limited to, human circulation and metabolism. Novel research tools and innovative approaches that enable safe and rapid assessment of the therapeutic potential of early-stage investigational agents could improve the ability to prioritize beneficial new therapies.

The Comparative In Vivo Oncology (CIVO) platform was developed to enable investigation of multiple early-stage investigational agents and drug combinations simultaneously and directly in the intact TME of patients with cancer (8). With FDA phase 0 guidance for early assessment of drugs in human patients in mind, CIVO was designed to introduce microdose quantities of multiple agents to distinct, trackable locations within the tumor. Hours to days following drug insertion, the injected tissue is excised and localized drug-induced events capturing tumor cell and TME responses to drug exposure are assessed. We have previously reported that localized tumor responses to approved drugs, investigational agents, and drug combinations following microinjection correctly predict responses to the same systemically delivered therapies in xenograft models of cancer (8–10). We also established feasibility of detecting drug-specific tumor and immune cell responses in the veterinary clinic in canine patients with soft tissue sarcoma (STS; ref. 11). Building off of these investigations, we initiated a multisite nonsignificant risk clinical feasibility study in human patients with...
Simultaneous Multidrug Evaluation in Cancer Patients

**Translational Relevance**

The success rate of new cancer agents from phase I trials to FDA approval is unacceptably low, hovering around 10%. Thus, new research platforms with potential to improve translational oncology are needed. We developed the CIVO platform to enable investigation of multiple drugs simultaneously and directly in solid tumors while the tumor is still intact and situated in the patient. In this first clinical trial, we demonstrate that use of CIVO for drug evaluation in patients with solid tumors is safe and feasible. Furthermore, tumor cell and microenvironment responses were consistent with known mechanisms of action and clinical effects of the drugs used in this pilot. By enabling safe evaluation and comparison of multiple drugs in patients with cancer, CIVO represents a potential bridge between preclinical experimentation and clinical investigation.

STS. The main objectives were to evaluate: (i) safety and (ii) feasibility of a multidrug investigation using CIVO in patients with STS. STS was chosen as the indication for this investigation primarily due to surface accessibility of lesions, thus facilitating CIVO microinjection. In addition, STS represents a highly heterogenous disease with more than 100 subtypes currently recognized (12–14). The heterogeneous nature of STS along with substantial preexisting clinical response data to antitumor agents enabled early assessment of whether the localized tumor responses induced by CIVO are consistent with clinical responses to systemically delivered drugs. Our intention was to obtain an initial evaluation of whether CIVO can ultimately be used by drug developers to forecast the therapeutic potential of early investigational agents by capturing the impact of localized drug exposure on both tumor cells and the TME following intratumoral drug microdosing.

**Patients and Methods**

**Study design**

The trial was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines, Declaration of Helsinki, and all applicable regulatory requirements. All patients gave written informed consent. After receiving a Non-Declaration of Helsinki, and all applicable regulatory requirements. Conference on Harmonisation Good Clinical Practice Guidelines, than 100 subtypes currently recognized (12–14). The heterogenous nature of STS along with substantial preexisting clinical response data to antitumor agents enabled early assessment of whether the localized tumor responses induced by CIVO are consistent with clinical responses to systemically delivered drugs. Our intention was to obtain an initial evaluation of whether CIVO can ultimately be used by drug developers to forecast the therapeutic potential of early investigational agents by capturing the impact of localized drug exposure on both tumor cells and the TME following intratumoral drug microdosing.

The study Medical Director provided overall study and pharmacovigilance program oversight. An External Review Committee was charged with monitoring any conflicts of interest and providing additional oversight for identification of unexpected safety issues. The study monitor performed 100% source data verification to ensure quality of the data. Data validation and integrity checks were conducted for all data entered.

All patients 18 years or older with at least one suspected STS tumor compatible with CIVO injection, for which a surgical procedure to remove tumor tissue was planned, were eligible. Additional inclusion criteria included Eastern Cooperative Oncology Group clinical performance status of 0 to 2, absolute neutrophil count >1,000/μm³, platelet count >50,000/μm³, hematocrit >25%, creatinine <3.0 mg/dL, total bilirubin <4.0 mg/dL, bilirubin <4.0 mg/dL, serum glutamicoxaloacetic transaminase, prothrombin time, and partial thromboplastin time ≤1.5 times the upper limit of normal. Patients with active fungal, viral, or bacterial infections, pregnant women, patients unable to give informed consent, patients on anticoagulants, and patients with tumors located near critical structures were excluded.

After screening and enrollment (visit 1), microinjection occurred on Day 1 (visit 2), 1 to 3 days prior to the scheduled surgery (visit 3). Follow-up visits (visits 4 and 5) were scheduled at approximately Day 7 and Day 28 (Supplementary Fig. S1). Where available, clinical outcomes were collected for up to 1-year post-microinjection through routine clinical follow-up. Safety was assessed and concomitant medications were reviewed at visits 2, 3, 4, and 5.

**CIVO loading and injection**

The handheld CIVO device is designed to deliver multiple drugs simultaneously in an arrayed format. The device contains eight 25-gauge needles designed to deliver drug along a 4 to 10 mm column, adjustable to the target tumor’s size. Drugs were mixed with fluorescent tracking marker (FTM) and loaded into the device under aseptic conditions. Immediately prior to the microinjection, ultrasonography was used to measure the mass and evaluate its internal architecture. When available, preoperative imaging was also reviewed to help identify the optimal injection area. After imaging, the device depth and injection length settings were adjusted and the injection was performed.

**Sample collection and processing**

The excised tumor tissue was processed using standard surgical pathology procedures (diagnostic samples and margins, as applicable). Injection columns within the research sample were visualized via gross examination using custom filter glasses and a flashlight. The research sample was sectioned transverse to the injection columns to obtain 3 to 4 mm transverse sections. Each transverse section was then placed into prechilled 10% buffered formalin containing 0.92 mg/mL sodium orthovanadate, 1.5 mg/mL sodium glycerophosphate, 1 mg/mL sodium fluoride, and 2.2 mg/mL sodium pyrophosphate for 2 hours at 4 °C before fixation at room temperature for >48 hours. Sections were paraffin embedded and cut into 4 μm for staining with Harris hematoxylin and eosin Y reagents (H&E) or specific biomarkers via IHC.

**IHC and high-plex protein biomarker analysis**

Sections were subjected to IHC staining with antibodies to detect effects on both cancer and immune cells. A list of antibodies used is included in Table 1. For fluorescent IHC, sections were antigen retrieved and incubated overnight in primary antibody and for 1 hour in Alexa Fluor conjugated secondary antibody. Sections were...
counterstained with DAPI. Whole slide images were acquired at 40×20 magnification using a 3DHistech Pannoramic 250 Flash digital slide scanner (3DHistech).

NanoString’s DSP was also utilized, allowing for simultaneous analysis of multiple antibodies within defined regions of tissue sections. Tissue sections were incubated with a cocktail of 32 unique oligonucleotide-conjugated antibodies and counterstained with a nuclear stain. 600 and 100 μm analysis regions of interest (ROI) were defined using the nuclear counterstain and FTM present within the tissue at the drug injection sites. Antibody oligos were photocleaved within the selected ROIs and collected for nCounter analysis as described previously (17). nCounter digital barcode counts for each antibody and ROI sample were first normalized using internal spike-in controls and subsequently normalized to the area (μm²) of their ROI compartment.

Image analysis

Custom segmentation and biomarker-specific algorithms was performed using previously reported (8) custom image analysis software (CIVOAnalyzer). Microinjection zones were identified using signal from the FTM co-microinjected at each position. Cells within the defined ROI around each injection site were classified as biomarker-positive or biomarker-negative in each available section for each injection site.

The fraction of CC3⁺ cells was measured within concentric rings (0.1 mm spacing) centered on the injection site. Plotting percent CC3⁺ versus radial distance from the injection site reveals a dose–response curve (drug concentration and response decreases with increasing radial distance). Overall CC3 response was quantified by integrating the area under the radial response curve (AUC) extending from 200 to 1,700 μm from the center of each injection. Values were background adjusted by subtracting the local background response level observed at radial distances between 1.9 and 2.0 mm from the injection site. For any tumor where drug exposure clearly expanded beyond a 2.0 mm radius, representative tissue neighboring the ROI acted as the background correction control. The AUC scales with −log ED₅₀ (the dose at which 50% of cells are affected). A resistant tumor would therefore have a low value of AUC, implying a high ED₅₀, whereas a susceptible tumor would have a high value of AUC, implying a low ED₅₀.

Statistical analysis

To investigate individual tumors for susceptibility to individual drugs, the AUC of the sterile saline vehicle response curve was subtracted, when available, from the AUC of the drug response to control for any effects of the sterile saline injection. We then graded this response by comparing the difference in AUC to a set of thresholds derived from the SD of the measurement difference. Each response was color-coded according to Table 2.

To determine the SD values for these thresholds, a separate analysis was conducted for each biomarker. We modeled the measurements as consisting of a fixed but unknown response to each drug in each tumor plus a random sampling error. The coefficients and residuals for the model were estimated using linear mixed effects models (18). The SD of the residual was then multiplied by √2 to estimate the SD of the difference between two measurements.

Quantification of myeloid cell infiltrate

For quantification of myeloid cell infiltrates in CIVO-microinjected sites, intensity thresholds were used to identify nuclear areas positive for DAPI, which were then dilated to capture the membrane and cytoplasm. Areas positive or negative for the biomarkers of interest (i.e., CD206, CD68, CC3) were identified within those dilated areas, expressed as a percent of total cellular area within the respective ROIs, and graphed using Graphpad Prism software.

Table 1. Antibody information.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>Agilent Technologies</td>
<td>Clone 124</td>
</tr>
<tr>
<td>Cleaved caspase-3 (CC3)</td>
<td>Cell Signaling Technologies</td>
<td>AB_2341188</td>
</tr>
<tr>
<td>CD3</td>
<td>Leica Biosystems</td>
<td>Clone LN10</td>
</tr>
<tr>
<td>CD8</td>
<td>Abcam</td>
<td>Clone 144B</td>
</tr>
<tr>
<td>CD44</td>
<td>Abcam</td>
<td>Cat #ab157107</td>
</tr>
<tr>
<td>CD68</td>
<td>Agilent Technologies</td>
<td>Clone KPI</td>
</tr>
<tr>
<td>CD206</td>
<td>Abcam</td>
<td>AB_1523910</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Cell Signaling Technologies</td>
<td>Clone D2WBE</td>
</tr>
<tr>
<td>Phospho-p4A4/42 MAPK (pERK)</td>
<td>Cell Signaling Technologies</td>
<td>Clone D15.14E</td>
</tr>
<tr>
<td>Phospho-PDGFR alpha(pPDGFRα)</td>
<td>Abcam</td>
<td>AB_304906</td>
</tr>
<tr>
<td>Phospho-S6 ribosomal protein (pS6)</td>
<td>Cell Signaling Technologies</td>
<td>Clone D57.2.2E</td>
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<tr>
<td>Phospho-Stat3 (pSTAT3)</td>
<td>Cell Signaling Technologies</td>
<td>Clone D3A7</td>
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</tbody>
</table>

Table 2. Tumor response color coding.

<table>
<thead>
<tr>
<th>Threshold</th>
<th>Interpretation</th>
<th>P value</th>
<th>Color code</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.28 SDs</td>
<td>Complete absence of a response</td>
<td>&gt;0.2</td>
<td>Dark red</td>
</tr>
<tr>
<td>1.28–1.64 SDs</td>
<td>Minor evidence of change</td>
<td>&lt;0.2 and &gt;0.1</td>
<td>Orange</td>
</tr>
<tr>
<td>1.64–1.96 SDs</td>
<td>Response approaching significance</td>
<td>&lt;0.1 and &gt;0.05</td>
<td>Yellow</td>
</tr>
<tr>
<td>1.96–2.58 SDs</td>
<td>Statistically significant response</td>
<td>&lt;0.05 and &gt;0.01</td>
<td>Light green</td>
</tr>
<tr>
<td>2.58–3.29 SDs</td>
<td>Strongly significant response</td>
<td>&lt;0.01 and &gt;0.001</td>
<td>Medium green</td>
</tr>
<tr>
<td>&gt;3.29 SDs</td>
<td>Very strongly significant response</td>
<td>&lt;0.001</td>
<td>Dark green</td>
</tr>
</tbody>
</table>
Table 3A. Patient demographics and tumor characteristics.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age</th>
<th>Race</th>
<th>STS subtype</th>
<th>Grade</th>
<th>Prior therapy</th>
<th>Postoperative therapy</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>31</td>
<td>Caucasian</td>
<td>Fibrosarcoma</td>
<td>2</td>
<td>Neo-adjuvant IRT</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>65</td>
<td>Caucasian</td>
<td>Pleomorphic liposarcoma</td>
<td>3</td>
<td>Prior disease, surgery Neo-adjuvant IRT</td>
<td>Pembrolizumab + doxorubicin IR</td>
<td>PD</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>51</td>
<td>Caucasian</td>
<td>Malignant PNST</td>
<td>N/A</td>
<td>Neo-adjuvant IRT</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>28</td>
<td>Caucasian</td>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>N/A</td>
<td>Neo-adjuvant IRT</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>51</td>
<td>Not reported</td>
<td>Myxoid liposarcoma</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>76</td>
<td>Caucasian</td>
<td>Pleomorphic rhabdomyosarcoma</td>
<td>N/A</td>
<td>None</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>46</td>
<td>African American</td>
<td>Myxoid liposarcoma</td>
<td>3</td>
<td>Neo-adjuvant doxorubicin + olaratumab</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>29</td>
<td>African American</td>
<td>Myxoid liposarcoma</td>
<td>1</td>
<td>None</td>
<td>IRT</td>
<td>Not reported</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>49</td>
<td>Caucasian</td>
<td>Myxoid liposarcoma</td>
<td>1</td>
<td>None</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>54</td>
<td>Caucasian</td>
<td>UPS</td>
<td>3</td>
<td>Prior disease, treated with IRT, surgery, adj. epirubicin + ifosfamide (PD)</td>
<td>Gemcitabine ± pazopanib Trabectedin</td>
<td>SD</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>93</td>
<td>Caucasian</td>
<td>Myxoid sarcoma</td>
<td>2</td>
<td>Prior disease, treated with surgery and adjuvant IRT</td>
<td>None</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>69</td>
<td>Caucasian</td>
<td>UPS</td>
<td>3</td>
<td>Neo-adjuvant IRT</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>44</td>
<td>Not reported</td>
<td>UPS</td>
<td>3</td>
<td>None</td>
<td>Not reported</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3B. Device-related adverse effects reported on this study.

<table>
<thead>
<tr>
<th>Description</th>
<th>Grade</th>
<th>Frequency</th>
<th>Relatedness</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection site pain</td>
<td>1</td>
<td>7/13</td>
<td>Possibly, probably, or definitely related to microinjection</td>
<td>Resolved without sequelae</td>
</tr>
<tr>
<td>Dysgeusia</td>
<td>1</td>
<td>1/13</td>
<td>Possibly related to microinjection contents</td>
<td>Resolved without sequelae</td>
</tr>
<tr>
<td>Injection site bruising</td>
<td>1</td>
<td>1/13</td>
<td>Probably related to microinjection</td>
<td>Resolved without sequelae</td>
</tr>
</tbody>
</table>

Results

Safety and performance of the CIVO platform

Evaluation of the safety and performance of the CIVO platform was conducted in 13 patients with a diversity of prior therapy regimens and STS subtypes, all of which were palpable and amenable to injection (Table 3A). CIVO microinjection of multiple drugs (Supplementary Table S1) was very well tolerated in all 13 patients (Table 3B). Transient grade 1 injection site pain, experienced by 7 of 13 patients, was mitigated or eliminated by the administration of topical anesthetic cream followed by subcutaneous lidocaine. In addition, one patient experienced transient grade 1 injection site bruising and another patient experienced transient grade 1 dysgeusia, possibly related to the microinjection contents (Table 3B). No serious adverse events were reported on this study (Supplementary Table S2).

Technical evaluation of CIVO performance demonstrated high efficiency from needle loading (100%) through visual detection of microinjection sites in surgically excised tissue (93/104 microinjections, 89%, Fig. 1A inset; Supplementary Table S3). These results are consistent with our previous evaluation in canine patients with STS (11) but represent a significant improvement over our first-in-man study in patients with lymphoma, where, using an early prototype model, only one injection site out of 12 (8%) could be confirmed within tumor tissue (8). As expected, successful observation of response, or lack thereof, to microinjected drugs depended on the quality of the excised tissue sample. Eight of the 13 resected tumors following CIVO microinjection had 4+ sites where response to drug was amenable to evaluation. Although preoperative radiation is generally not associated with high rates of sarcoma necrosis or size change by time of resection, other than for myxoid liposarcoma (19), tumors from patients who were subjected to prior radiation treatment within 1 month of drug microinjection trended toward poorer quality tissue and thus lack of evaluable sites trended higher in patients with either no prior radiation therapy or when treatment was received over 1 month prior to the microinjection procedure. Excluding one tumor, where all injected sites were observable but the excised tissue contained less than 1% tumor cells, 83% (40/48) of potential injection sites across 6 patients consisted of identifiable injection sites with tissue of sufficient quality surrounding the FTM to enable interpretation of response to CIVO microinjection (Fig. 1A: Supplementary Table S3). Retrieval of evaluable sites trended higher in patients with either no prior radiation therapy or when treatment was received over 1 month prior to the microinjection procedure. Excluding one tumor, where all injected sites were observable but the excised tissue contained less than 1% tumor cells, 83% (40/48) of potential injection sites across 6 patients consisted of identifiable injection sites with tissue of sufficient quality surrounding the FTM to enable interpretation of response to CIVO microinjection (Fig. 1A: Supplementary Table S3). These results emphasize the importance of careful planning and execution of microinjection procedures to maximize the likelihood of obtaining evaluable tissue samples for evaluation.
CIVO reveals localized and quantifiable responses to distinct microinjected drugs. A, IHC image of a CC3-stained section (red) from a myxoid liposarcoma (MLS) microinjected with sterile saline (gray arrowhead), doxorubicin (red arrowhead), gemcitabine (yellow arrowhead), and trabectedin (turquoise arrowhead). The FTM (green) identifies each injection site center on a DAPI-stained tissue background (blue). The panel inset shows all eight FTM-labeled injection sites visualized in the corresponding gross tissue specimen using a blue excitation light flashlight and a yellow camera filter. Nonlinear image modifications were applied equally with the sole purpose of enhancing screen and print visualization. B, 40× magnification IHC images depicting H&E staining of the saline, doxorubicin, gemcitabine, and trabectedin injection sites. Black bars represent 50 μm. C, Quantification of the fraction CC3+ cells against the radial distance from the center of each injection site (in μm) for sterile saline, doxorubicin, gemcitabine, and trabectedin in the MLS depicted in A and B. Error bars represent SEM calculated from quantification performed at three tissue levels within the sample.

Figure 1.

preoperative imaging and positioning of the CIVO injection, whether the patient is treated with preoperative radiation or not, so that patients can be excluded if imaging reveals poor quality tissue.

CIVO induces localized and quantifiable drug-specific responses

As an initial evaluation of CIVO’s potential to differentiate the activity of distinct anticancer drugs, we examined CIVO-induced tumor responses to chemotherapy agents such as doxorubicin, gemcitabine, docetaxel, and trabectedin, all commonly used to treat patients with STS. Doxorubicin frequently induced visible localized regions of tumor cell apoptosis with 50% of tumors (four of eight) exhibiting enrichment of CC3+ cells (Fig. 1A; Supplementary Fig. S3). Drug-induced morphologic changes were also observed surrounding the injection sites (Fig. 1B; Supplementary Fig. S3). The more recently approved agent trabectedin induced similar effects in five of six tumors where identifiable sites of microinjection were observed (Fig. 1; Supplementary Fig. S3). Microinjection of docetaxel rarely induced tumor response with an increase in apoptosis observed in only one of eight tumors (Supplementary Fig. S3). However, consistent with its known mitotic inhibition, docetaxel induced morphologic changes associated with mitotic arrest and localized enrichment of phospho-histone H3 positive cells in the tumor that exhibited a response. This activity was unique to docetaxel and not observed at injection sites of any other agent (Supplementary Fig. S3). Notably, none of the gemcitabine injection sites (0/7) showed increased levels of CC3 above vehicle injected controls and histologic appearance of tumor cells at those sites was similar to those of vehicle controls (Fig. 1A and B). As observed in preclinical contexts, quantification of CC3+ cells revealed a graded response, with highest responses closest to the injection site gradually dissipating outward (Fig. 1C), consistent with the drug gradient set up by diffusion.

Clinical response to single-agent doxorubicin has been reported in the 14% to 37% range (20, 21) and in this study, localized apoptotic responses to doxorubicin varied in size and intensity, ranging from no response, to responses extending 4,000 μm from the microinjection site of select tumors (Fig. 2A–F). Visualization of doxorubicin fluorescence around the injection site confirmed that the lack of activity in nonresponding tumors was not due to failed drug delivery and lack of exposure (Fig. 2G–L). Of the tumors with elevated CC3 in response to doxorubicin microinjection, three of four were diagnosed as myxoid liposarcomas, an STS subtype that is particularly responsive to chemotherapy (22). Of the tumors showing no significant elevation of apoptosis in response to doxorubicin, two (subjects 2 and 10) were...
Figure 2.
Tumor subtype specific responses to doxorubicin microinjection are consistent with historical clinical data. IHC images showing CC3 (A–F) and doxorubicin fluorescence (G–L) staining at the sites of microinjection of doxorubicin in 6 different patients. White bars represent 500 μm, yellow bars represent 1,000 μm. Note that the tumor sample in panels F and L came from a patient who failed previous anthracycline-based therapy. MLS, myxoid liposarcoma; FSA, fibrosarcoma; PLS, pleomorphic liposarcoma. Nonlinear image modifications were applied to all images with the sole purpose of enhancing screen and print visualization. M, CC3 response data from all available tumors, quantified at the doxorubicin and trabectedin injection sites. Tumor responses are corrected for background expression levels and baseline vehicle levels (when available). Data were averaged across one to six different levels along the injection column (depending on tissue availability). The different colors represent the statistical significance of a given response with a gradient: dark red cells (P-value < 0.2), light green cells (0.05 > P-value > 0.01), medium green cells (0.01 > P-value > 0.001), and dark green cells (P-value < 0.001). Gray boxes indicate data were not recovered. Black boxes indicate the drug was not included in that patient's microinjection.

Diagnosed as undifferentiated pleomorphic sarcomas (UPS; Table 3A; Fig. 2F, L, and M). One-year posttreatment follow-up on subject #2 was consistent with lack of response to microinjection of doxorubicin, exhibiting progressive disease (PD) on treatment with doxorubicin and pembrolizumab (Table 3A). In addition, prior to the CIVO procedure, subject #10 exhibited tumor recurrence despite receiving prior adjuvant anthracycline therapy, suggesting that preexisting resistance to doxorubicin may underlie lack of observed apoptotic response. Following the CIVO procedure, subject #10 developed lung metastases 3 months postoperatively and received gemcitabine/C6 (Fig. 3B). Standard IHC was used to verify responses (Fig. 3C–L). The most prominent signal induced by exposure to doxorubicin was upregulation of phospho-ERK (pERK; Fig. 3B and C). Upregulation of phospho-S6 (pS6) was also detected suggesting activation of receptor tyrosine kinase (RTK) signaling (Fig. 3B and D). Moreover, a modest increase in expression of CD44, a receptor for hyaluronan and many other extracellular matrix (ECM) components, was also observed (Fig. 3B and E). In contrast, no change in expression of prosurvival proteins such as BCL2 was detected (Fig. 3B and F). We also did not detect consistent perturbations of most immune cell markers (Fig. 3B and G–I). However, a modest but notable enrichment of the myeloid cell marker CD68 was observed and confirmed by IHC (Fig. 3B and J). Further examination by IHC revealed increased phosphorylation of both STAT3 (Fig. 3K), a third effector of RTK signaling, and PDGFRα, a prominent mesenchymal RTK (Fig. 3L). Of note, distinct patterns of elevated phosphorylation were observed between several of the proteins highlighted by our analysis. For instance, increased pSTAT3 appeared as a halo of elevated signal further distal from the FTM than that of pERK which was closely proximal to the FTM (Fig. 3C compared with 3K). The observed increased phosphorylation of the PDGFRα and downstream effectors was not limited to the tumor analyzed by DSP and was also observed in...
multiple tumors exhibiting lack of apoptotic response to doxorubicin (Supplementary Fig. S4). These results are consistent with well-established prosurvival functions of PDGFR signaling and clinical development of an anti-PDGFRα antibody, olaratumab (23–25).

The combination of doxorubicin and a PDGFRα antagonizing antibody does not increase antitumor activity

The observed elevation of multiple markers of PDGFR pathway activation along with prior clinical evidence of olaratumab activity against STS prompted us to test whether the combination of doxorubicin and olaratumab would induce increased tumor cell apoptosis compared with either doxorubicin or olaratumab alone. Preclinical studies of olaratumab alone (24) or in combination with doxorubicin (26) have shown activity in xenograft models of STS and a previously published phase II clinical study demonstrated superior activity of olaratumab and doxorubicin versus doxorubicin alone (27). Most recently, a follow-up phase III trial (ANNOUNCE) showed no additional activity of olaratumab (28).

In this study, we did not observe enhancement of tumor cell apoptosis in three individual CIVO-evaluated patients when comparing microinjection sites exposed to the combination of doxorubicin and olaratumab to those exposed to doxorubicin alone (Fig. 4). Status of sensitivity to doxorubicin did not affect this result as neither sensitive nor resistant tumors appeared impacted by the addition of olaratumab (Fig. 4A–I). Of note, although olaratumab was microdosed at a final concentration sufficient to either equal or potentially
exceed antibody levels achieved by the intravenous dose (15 mg/kg) used in ANNOUNCE and doses used in the in vitro setting; we did not observe inhibition of PDGFRα, ERK, or S6 phosphorylation (Supplementary Fig. S5).

**Enrichment of M2 macrophages is a putative mechanism of resistance to doxorubicin, addressed by exposure to trabectedin**

Non-cell autonomous effects, particularly those mediated by immune cell components of the TME, impact tumor sensitivity to drug therapy. For instance, an increased number of macrophages has been observed following treatment with chemotherapy and this is associated with poor drug response (29). Impediment of response to therapy is associated particularly with M2 or alternatively activated macrophages that secrete factors that increase cancer cell survival (30). An important function of CIVO is investigation of microenvironment influences on tumor response in each individual.

Because enrichment of CD68 was the most prominent immune cell biomarker detected by our analysis, we investigated whether M2 polarized, protumor macrophages accumulated at sites of doxorubicin injection in two tumors showing the least apoptotic response to drug exposure (subjects 10, 11). IHC containing with antibodies specific for CD68 and CD206, a marker for M2 polarization, was employed to detect localized enrichment of a putative M2 macrophage population. CD68 positive granuloma-like structures were observed at both vehicle control and doxorubicin sites. Consistent with a role for M2 macrophages in chemo-resistance, an increase in CD68/CD206 double positive cells was observed in tumors that showed no apoptotic response to doxorubicin (Fig. 5A, B, D, E, and G–I). Trabectedin has previously been shown to exert its activity in STS partially through selective effects on tumor-associated macrophages (TAM; ref. 31). Microinjection of trabectedin in subjects #10 and #11 provided the opportunity to test whether an agent that targets TAMs could induce an apoptotic response. Consistent with this activity, in the same tumors exhibiting resistance to doxorubicin, sites with trabectedin resulted in an apoptotic response and selective ablation of the CD68/CD206 double positive cell population (Fig. 5C–I). Importantly, taken together with the data presented above, these results demonstrate how multidrug assessment in intact tumors may be used to better define drivers of tumor resistance, particularly, the influences of specific, potentially drug targetable cell populations comprising the TME. Although anecdotal, it is notable that subject #10, who progressed on two previous treatment regimens, is currently on treatment with trabectedin with stable disease (Table 3A).

**Discussion**

The CIVO platform was designed to address a fundamental challenge to drug development, namely the well-recognized discordance between results derived in laboratory models of cancer and clinical responses observed in patients exposed to the same investigational agents. Ultimately the intent for this platform is to reduce the time and resources spent on candidate products that are unlikely to succeed by providing a new tool to distinguish drug candidates that hold promise from those that do not (32). Ideally this could be done early in the drug development process when programs are transitioning from research...
to development. We therefore envision technologies such as CIVO as best employed under the FDA’s exploratory IND path and in phase 0 trials. As the next step toward this vision, here we demonstrate that evaluation of multiple drugs following intratumoral microinjection in patients with CIVO is safe and feasible. Furthermore, we have shown that localized responses to different CIVO microdosed agents were consistent with expected drug mechanisms of action and historic tumor responses to these same agents in STS. The data presented here also point to how CIVO can be used to generate hypotheses regarding tumor resistance to drug exposure, highlighting both tumor cell autonomous and TME responses. In summary, the results of this pilot study promote further investigation of CIVO as a research tool for translational oncology, particularly for earlier clinical evaluation of novel drug candidates and combinations in the phase 0 setting.

If successful, CIVO and other intratumoral microdosing technologies (33) offer several new benefits to clinical development of anticancer agents including reduced time to biological proof-of-concept and potential to focus resources on early-stage drug candidates with evidence of antitumor activities. An advantage specific to CIVO technology is that the microinjection sites and drug-exposed regions of the tumor are easily trackable, designed to make both positive and negative responses easy to interpret. This could be particularly informative for translational studies where greater mechanistic understanding of why some tumors respond to drug exposure whereas others do not leads to data-driven hypotheses and strategies to overcome resistance.

Like any new technology, CIVO has limitations, some observed here, that can be addressed with increased experience in the clinic and

Figure 5.
Enrichment of M2 macrophages is a putative mechanism of resistance to doxorubicin, addressed by exposure to trabectedin. A–C, IHC images showing CD206, CD68, DAPI, and FTM at sites of vehicle, doxorubicin, and trabectedin in patients with sarcoma. Areas of white stain represent costaining of CD206 and CD68. White bars represent 200 μm in larger image and 50 μm in inset image. D–F, CC3 staining in adjacent sections to above images. G, Quantification of M2 macrophage staining (CD206+CD68+) in patients with sarcoma. H, myeloid cell staining (CD206−CD68+). I, apoptotic cell staining (CC3+) as percentage of DAPI+ cell area at injection sites of vehicle, trabectedin, or doxorubicin in 2 different patients with sarcoma. Error bars represent SEM calculated from 10 tissue levels within the sample.
Further development of the technology. As expected, this study demonstrated that the CIVO platform performs best when microinjection occurs in tumor tissue containing a high density of viable tumor cells. Tumors that have robustly responded to therapy in the weeks prior to evaluation of preoperative imaging prior to intratumoral injection, may address this issue. Inclusion of CIVO in window-of-opportunity trials may also help increase data quality without negatively impacting patient care. Furthermore, analysis of lower quality tissue samples may also be improved via development of techniques that pair CIVO microdosing with single cell analysis including laser capture microdissection and rare cell profiling strategies that are under development in our lab. Furthermore, pairing intratumoral microinjection with techniques such as MRI and Raman spectroscopy, which allow patient assessment at more than a single time point, should be explored (35, 36).

Ultimately the true value of CIVO and other intratumoral microinjection technologies depends on the ability to accurately predict patient response to treatment. Although this study demonstrates feasibility for multidrug investigation in patients, larger and more comprehensive trials will be needed to evaluate the correlation between localized responses induced by CIVO and clinical responses to the same drugs administered systemically. With caveats noted below, several observations made in this pilot offer encouraging preliminary evidence that CIVO-induced responses reflect clinical activity. Although differences in the timeframe of mechanism of action may have impacted our ability to observe certain responses within a 72-hour timeframe, we observed that microinjection of historically effective agents such as doxorubicin and trabectedin induced localized antitumor responses more frequently than less effective agents such as gemcitabine and docetaxel (34–37). Also encouraging was the observation that tumors exhibiting robust response to microinjection of doxorubicin tended to be myxoid liposarcomas that are known to better respond to systemic chemotherapy. In addition, although observed with a limited sample set, the lack of increased tumor response upon adding olaratumab in combination with doxorubicin is in line with the recent results of the phase III ANNOUNCE study (28). Finally, two subjects that received post-CIVO chemotherapy showed results consistent with CIVO responses. In one case, subject #10, prior tumor recurrence following anthracycline therapy was consistent with lack of response to microinjection of doxorubicin. The tumor from this same patient also exhibited no response to microinjection of gemcitabine, but elevation of apoptosis at the site of exposure to trabectedin. Although anecdotal, this is consistent with the subject’s response to treatment where, during their 1-year follow up, subject #10 had PD following treatment with gemcitabine (± pazopanib) and thus far has achieved stable disease on trabectedin. If larger, more comprehensive studies continue to reveal similar findings, CIVO may also serve as a precision diagnostic to guide patient treatment. Along these lines, encouraged by the preliminary findings reported here, we are pursuing studies designed to assess whether CIVO can prospectively predict patient response to systemic therapy and thus provide a functional precision medicine approach, particularly for drugs that do not have an associated genetic biomarker.

The National Cancer Moonshot Initiative’s Blue-Ribbon Panel acknowledged that the complexity and heterogeneity of human tumors present a significant challenge for cancer research (38). It is now widely recognized that new technologies and approaches are needed to improve our ability to evaluate the potential of early cancer drug candidates and move beyond the conventional cell culture and xenograft models that have dominated translational oncology research. Multiple technologies are now under development to address this challenge including CIVO, other microdevices for multiplexed intratumoral investigation (33), patient-derived tumor explants, and other ex vivo tumor organoid and spheroid platforms (39–41). Further evaluation of all approaches designed to more efficiently and accurately test early-stage investigational agents is needed to advance translational oncology and ultimately improve the successful development of novel cancer therapies.

Disclosure of Potential Conflicts of Interest

K.R. Gundle reports receiving commercial research grants from Presage Biosciences. S.M. Pollack is an employee/paid consultant for GlaxoSmithKline, Lilly, Seattle Genetics, Bayer, Tempus, Daiichi Sankyo, and Blueprint, and reports receiving commercial research grants from Merck, EMD Serono, Incyte, Presage, Janssen, Oncosec, and Juno. J.L. Davis is an employee/paid consultant for Bayer/LOXO/Lilly and reports receiving other remuneration from Galloway Farm. W. Kerwin holds ownership interest (including patents) in and is an advisory board member/unsalaried consultant for Presage Biosciences. J.A. Bertout, M.O. Greoley, J. Frazier, and R.A. Klinghoffer are employee/paid consultants for and hold ownership interest (including patents) in Presage Biosciences. K.H.W. Sottero, E. Beirne, and M. Ellison are employees/paid consultants for Presage Biosciences. R.G. Maki reports receiving commercial research grants from Presage Biosciences. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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Gundle et al.

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