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A technology platform to assess multiple cancer agents simultaneously within a patient's tumor

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Abstract

A fundamental problem in cancer drug development is that antitumor efficacy in preclinical cancer models does not translate faithfully to patient outcomes. Much of early cancer drug discovery is performed under in vitro conditions in cell-based models that poorly represent actual malignancies. To address this inconsistency, we have developed a technology platform called CIVO, which enables simultaneous assessment of up to eight drugs or drug combinations within a single solid tumor in vivo. The platform is currently designed for use in animal models of cancer and patients with superficial tumors but can be modified for investigation of deeper-seated malignancies. In xenograft lymphoma models, CIVO microinjection of well-characterized anticancer agents (vincristine, doxorubicin, mafosfamide, and prednisolone) induced spatially defined cellular changes around sites of drug exposure, specific to the known mechanisms of action of each drug. The observed localized responses predicted responses to systemically delivered drugs in animals. In pair-matched lymphoma models, CIVO correctly demonstrated tumor resistance to doxorubicin and vincristine and an unexpected enhanced sensitivity to mafosfamide in multidrug-resistant lymphomas compared with chemotherapy-naïve lymphomas. A CIVO-enabled in vivo screen of 97 approved oncology agents revealed a novel mTOR (mammalian target of rapamycin) pathway inhibitor that exhibits significantly increased tumor-killing activity in the drug-resistant setting compared with chemotherapy-naïve tumors. Finally, feasibility studies to assess the use of CIVO in human and canine patients demonstrated that microinjection of drugs is toxicity-sparing while inducing robust, easily tracked, drug-specific responses in autochthonous tumors, setting the stage for further application of this technology in clinical trials.

INTRODUCTION

Attrition rates for new oncology drugs in clinical trials are higher than those of almost all other therapeutic areas, and survival rates for patients with advanced cancers are persistently low (1, 2). Currently, only 7% of agents that demonstrated anticancer activity in preclinical studies demonstrate sufficient efficacy in phase 3 testing to warrant U.S. Food and Drug Administration (FDA) approvals (3, 4). Meanwhile, costs associated with drug development continue to escalate, with the current estimate exceeding \$2.6 billion per approved drug (5). The factors that contribute to cancer therapy failures are numerous and intertwined. One key issue is a heavy reliance on cell-based models that do not closely represent clinical malignancies (6–9). Conditions such as hypoxia or acidity in the tumor microenvironment can perturb the efficacy of drugs as compared to well-nourished cancer cells in culture. Furthermore, the time-dependent duration and extent of tumor exposure to drug in the clinic are very different than the homogeneous and static exposure tested in cell-based systems. Although three-dimensional (3D) cultures overcome some of the limitations posed by the 2D in vitro setting, such models are simply approximations of the true tumor microenvironment. As a consequence, assessments of the impact of potential new drugs are often flawed, and seemingly promising agents that kill cancer cells under standard tissue culture conditions translate poorly into effective treatments in human cancer patients.

To enable earlier and more predictive analyses of anticancer agents in vivo and ultimately in cancer patients, we developed a platform called CIVO (10). CIVO consists of a device engineered to introduce multiple drugs transcutaneously into discrete and mapped locations within a growing tumor in a living subject. The device is complemented by an automated analysis package for quantification of multiple histological biomarkers of tumor response to drug. An array of needles at the distal end of the device enables simultaneous delivery of microliter volumes of candidate therapies directly into localized regions in tumors. By delivering drugs in a localized fashion to tumors in doses that would not induce systemic toxicity, we hypothesized that we would be able to observe spatially defined tumor responses, discern the mechanism of drug action, and ascertain tumor responsiveness to each drug in a way that has potential to predict therapeutic response to systemically delivered therapy.

The CIVO technology essentially allows for medium-throughput screening of drug activity in living animals, evaluating up to eight drugs per tumor, greatly increasing the amount of data generated from each tumor sample compared with the traditional one drug–one animal approach. We tested the CIVO application in human xenografted mouse models, including a model of chemoresistant lymphoma, and in canine patients. We also describe the first-in-human testing of CIVO in four patients, representing an important step toward future, personalized clinical application of this technology, to compare and prioritize drugs in the ultimate context of validation—the cancer patient.

RESULTS

The CIVO platform consists of an arrayed microinjection device coupled with automated image analysis

The CIVO platform consists of a device that simultaneously delivers microgram quantities of test drugs into defined positions within a living tumor, coupled with automated, quantitative image-based analysis of specific tumor responses (Fig. 1). Drugs are co-injected with a chemically inert, visible injection tracking dye (ITD) to denote injection position (Fig. 1, A to C). Injections are performed so that a constant microliter volume of drug is delivered per millimeter extruded via retraction of each needle through the tumor. This design leaves a uniform, column-like track of drug through the *z* axis of the tumor (Fig. 1D), allowing investigators to sample multiple tissue depths to assess consistency of tumor response to drug. Furthermore, accounting for tumor heterogeneity observed in solid tumors, the drug column provides the potential to interrogate how different tumor microenvironments affect drug efficacy; for instance, normoxia versus hypoxia where a GLUT1 signal accumulates with increasing distance from the nearest endothelial cell as indicated by CD31 staining, then drops at distances greater than 250 μm as a result of tumor necrosis, a consequence of poor vascularization.

Responses to drugs were assessed after resection of the tumor at a predetermined time point (typically 24 to 72 hours). Multiple biomarkers, including those for mechanism-based drug effects, target or pathway engagement, and apoptotic response, were tracked by sampling 4- μm -thick histological sections at 2-mm intervals along the injection column (Fig. 1E). High-resolution whole-slide scanning was used to capture images of every cell from each tissue

section (Fig. 1F), and these images were then quantitatively processed by a custom image analysis platform called CIVO Analyzer, which comprises automated injection site detection, cross section registration, and biomarker-specific tissue and cellular segmentation to streamline analysis of induced tumor response (Fig. 1, G and H).

Spatially defined drug distribution enables multidrug analysis in tumors

CIVO platform performance and drug distribution were first assessed in xenografted human tumor models using a panel of radiolabeled compounds of varied physico-chemical properties, including molecular weight, lipophilicity, and protein binding. Drug distribution was assessed by ^{14}C or ^3H count distribution within 2 mm of the injection epicenter and at an adjacent site microinjected with saline. Three different xenograft models [Ramos lymphoma, H2122 non-small cell lung cancer (NSCLC), and H292 NSCLC] and three time points (15 min, 4 hours, and 24 hours) were evaluated. On average, more than 96% of each drug remained within the 2-mm radius around the targeted injection site and was not detected at adjacent sites (table S1).

Further characterization of drug distribution after CIVO microinjection was performed with the antimetabolic agent vincristine. Vincristine distribution was directly tracked by microinjection of ^3H -labeled drug into discrete positions of xenografted Ramos lymphoma tumors. Tumors were resected at 2, 8, 24, 48, and 72 hours after injection, and fixed cryosections cut perpendicular to each injection column were subjected to autoradiography. The resulting autoradiograms revealed spatially defined (with a maximum radial extent of about 1500 μm) and graded regions of drug distribution with respect to the injection site origin (Fig. 2A). Radioactivity, plotted as a function of distance from the injection, confirmed a monotonic drop in drug content with increasing radial distance. Within 400 μm of the injection epicenter, a rapid drop in drug content over time was observed (Fig. 2A). At radial distances between 500 and 1500 μm , the activity remained fairly stable over time, suggesting a steady-state drug concentration profile with diffusion into the zone balancing drug elimination, at least through 72 hours after injection. These data indicate that none of the drugs tested distributed beyond 2 mm of the injection epicenter.

The distribution pattern was compared with histological biomarkers of tumor response to drug in parallel Ramos tumors microinjected with the same amount of unlabeled vincristine. All injections of vincristine resulted in spatially constrained regions of tumor response, with a leading edge of phospho-histone H3 (pHH3)-positive cells arrested in mitosis (500 to 1500 μm) surrounding an inner core of apoptotic cleaved caspase-3 (CC3)-positive cells (0 to 900 μm) emanating from the site of drug delivery (Fig. 2B); these findings are consistent with the known mechanism of action of vincristine. In contrast, sites microinjected with vehicle were pHH3- and CC3-negative but exhibited variable evidence of modest tissue damage directly adjacent (< 200 μm) to the region where the needle passed through the tissue. The same distribution pattern of biomarker response to vincristine was observed upon microinjection into a patient-derived xenograft model of diffuse large B cell lymphoma (fig. S2A).

The graded drug distribution and corresponding response allowed each injection site to be evaluated as a range of drug concentrations, where the drug gradient is a function of distance

from the original site of localized drug delivery. This concept is similar to that described by Patel *et al.* (11), where drug distribution and biomarkers were plotted as a function of distance to the nearest blood vessel to assess concentration dependence of tumor responses. Our data demonstrate an increasing magnitude of tumor response upon increasing drug (vincristine) input concentration at each microinjection site, observed as a dose-dependent increase in CC3⁺ cells when plotted against radial distance (Fig. 2C). Dose-dependent increases were also observed for the local pHH3⁺ cell population. However, in contrast to CC3, pHH3 staining revealed a parabolic profile with cells nearest to injection sites (<500 μ m) no longer expressing the pHH3 antigen. Histological analysis suggests that pHH3 loss is due to extensive cell death in this core region, because loss of pHH3 directly correlates with increased CC3 and the appearance of morphologically damaged cells in a dose-dependent manner.

These experiments were repeated with mafosfamide (an active surrogate for the pro-drug cyclophosphamide), doxorubicin, and prednisolone (an active surrogate for the pro-drug prednisone), because these represent or simulate components of the CHOP [cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), prednisone] regimen, a first-line lymphoma therapy. All positions were co-injected with an ITD, except for doxorubicin, because the autofluorescence from doxorubicin interferes with detection of the dye (fig. S3). Similar to responses observed with vincristine, CIVO microinjection of either mafosfamide or doxorubicin resulted in dose-dependent, spatially defined regions of cellular response in Ramos lymphoma xenograft tumors (Fig. 3A). Tumor responses were specific to the established mechanism of action of each drug as shown in Fig. 3B and quantified in Fig. 3C. Of the four agents tested, only vincristine resulted in pHH3⁺ cells, corresponding with its antimitotic activity (Fig. 3, B and C). Consistent with their DNA-damaging activity, both doxorubicin and mafosfamide resulted in phospho-histone H2AX (γ H2AX) expression in cells adjacent to injection sites (Fig. 3, B and C). Mafosfamide, doxorubicin, and vincristine all induced a measurable apoptotic response (CC3⁺) above vehicle control by 72 hours.

In contrast to the other agents tested, prednisolone exposure did not result in overt tumor responses at the time points and the concentrations of drug examined (Fig. 3, B and C). Consistent with a role as a chemoenhancer in some lymphomas, co-injection of prednisolone with vincristine resulted in a modest but significantly enhanced drug-induced tumor cell apoptotic response across a range of drug concentrations as observed by plotting response as a function of radial distance from the injection site (fig. S4).

Localized tumor responses predict response to systemically delivered drugs

To assess the clinical predictive capabilities of the CIVO platform, we investigated whether localized tumor responses correlated with responses to systemic delivery of drug in mice. Because most novel drugs are first tested in patients who have failed first-line therapies, we extended our analysis to include a new drug-resistant variant of the Ramos lymphoma line, called Res-Ramos, which is refractory to 500 nM doxorubicin *in vitro*, likely due in part to increased activity of the P-glyco-protein (Pgp/MDR1/ABCB1) drug efflux pump (table S2) (12). Nude mice bearing either parental Ramos or Res-Ramos tumors were CIVO-injected

or treated systemically with one of the four CHOP agents. Short-term (24 to 72 hours) CIVO responses were compared to longer-term (up to 29 days) systemic responses to drug. Similar to previous experiments, CIVO microinjection of doxorubicin, vincristine, and mafosfamide all resulted in localized tumor responses in the parental Ramos tumors, with vincristine inducing the greatest apoptotic effect and prednisolone having no effect (Fig. 4A).

Tumor cell response after CIVO microinjection mirrored tumor growth inhibition after systemic treatment of Ramos tumors (Fig. 4B). CIVO was also able to predict response in a tumor context-specific manner (both resistance and increased sensitivity). In our model of drug resistance, Res-Ramos tumors exhibited the expected reduced response to microinjection of doxorubicin, as well as to vincristine—also a Pgp substrate (Fig. 4, A and B). Failure to induce a localized response was mirrored by lack of tumor growth inhibition after systemic exposure to vincristine, doxorubicin, or prednisone (Fig. 4B).

In contrast to the other drugs, CIVO microinjection of mafosfamide induced significantly greater cell death responses in the Res-Ramos tumor model (Fig. 4A), which was confirmed in vivo after systemic cyclophosphamide administration (Fig. 4B). Res-Ramos mice treated with cyclophosphamide also demonstrated significant improvement in overall survival compared with Ramos mice treated with the same drug (Fig. 4B). At the end of the study, 70% of the Res-Ramos mice treated with cyclophosphamide were tumor-free (average tumor volume, 60 mm^3) compared with only 15% of the Ramos mice, in agreement with the prediction drawn from CIVO microinjections in Fig. 4A.

Cell-based proliferation assays were also performed to assess the correlation between in vitro and in vivo responses to drug treatment. Drug sensitivity of the parental Ramos line in vitro approximated in vivo responses but did not register sensitivity to mafosfamide and falsely indicated sensitivity to prednisolone (Fig. 4C). Res-Ramos cells exhibited resistance to doxorubicin and vincristine in vitro (Fig. 4C), which was expected. However, in contrast, the increased sensitivity of Res-Ramos cells to mafosfamide, observed by both in vivo approaches (CIVO and systemic therapy), was not seen in the cell-based in vitro assay (Fig. 4C).

In vivo screening with CIVO indicates chemoresistant lymphoma sensitivity to mammalian target of rapamycin inhibitors

To expand on the CHOP results and to identify candidate agents to treat chemoresistant lymphoma, we performed a pilot CIVO-based screen in the Res-Ramos tumors using the 97-compound Developmental Therapeutics Program (DTP) version III set of approved oncology drugs from the National Cancer Institute (NCI). In vivo assessment of all 97 drugs was completed within 2 weeks from the initiation of the screen by microinjecting 8 compounds per tumor into replicate Res-Ramos xenografts and resecting and staining at 72 hours (fig. S5A). Of the 202 injection sites (replicate DTP set plus vehicle controls), 195 resulted in clear detection of localized injection site dye (96.5% success rate).

Of the 97 drugs tested, 5 resulted in a measurable increase in apoptotic cells ($>15\% \text{ CC3}^+$) around the site of injection (table S3). One of the five agents was the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibitor rapamycin, suggesting a dependence of

Res-Ramos tumors on active mTORC1 activity for survival (fig. S5B). To confirm that rapamycin was exerting its expected mechanism of action, down-regulation of the mTOR pathway, we micro-injected Res-Ramos tumors with rapamycin and resected them for examination of mTOR pathway status after 2 hours of localized (CIVO-mediated) drug exposure. Local exposure to rapamycin led to a marked decrease in phosphorylation of the mTORC1 substrate eIF4E-binding protein 1 (4EBP1) (p4EBP1) (fig. S5C). Pathway inhibition occurred before observable increase in apoptosis (CC3) or morphological changes indicative of cell death. This suggests that mTOR pathway inhibition precedes rapamycin-induced apoptosis. These results also suggest that use of CIVO is not limited to classic cytotoxic chemotherapy agents but can be used to evaluate inhibitors targeting specific pro-oncogenic pathways.

Two additional mTOR inhibitors—ridaforolimus (mTORC1 inhibitor) and CC-115 [inhibitor of mTORC1, mTORC2, and DNA-PK (DNA-dependent protein kinase); [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01353625): NCT01353625]—were microinjected into Ramos and Res-Ramos tumors, and p4EBP1 and CC3 staining was analyzed. Both ridaforolimus and CC-115 inhibited the mTOR pathway in the Ramos and Res-Ramos tumors (Fig. 5A). Like rapamycin, ridaforolimus induced apoptosis central to the region of mTOR pathway inhibition, and this activity was similar in both parental and Res-Ramos models. Whereas ridaforolimus exhibited no cell line preference, CC-115 activity was significantly increased in the Res-Ramos background within 24 hours (Fig. 5, A and B). Similar to the case of mafosfamide (Fig. 4), this apparent cell line-dependent activity was not detected in a standard 72-hour in vitro cell-based viability assay (Fig. 5C).

In vivo, CC-115-treated parental Ramos xenografts were overtly smaller than vehicle controls but continued to grow through the course of the experiment. In contrast, systemic delivery of CC-115 resulted in complete regression of Res-Ramos xenograft tumors (Fig. 5, D and E), consistent with localized apoptosis observed upon CIVO microinjection (Fig. 5, A and B). Regardless of the mechanism, these results emphasize the importance of efficacy testing in vivo, because the context-specific effect of CC-115 was not detected in a cell-based in vitro assay.

CIVO proves feasible and safe in cancer patients

As a first step to establish feasibility for use of CIVO in vivo in humans, we initiated a trial in lymphoma patients with a prototype device for arrayed microinjection. The primary goals of this trial were to assess the physician and patient experience during the procedure, to establish the choreography of using CIVO technology in the clinical setting, and to learn what aspects of the technology and methodology need to be improved. Four patients with enlarged lymph nodes were injected with microdoses of vincristine (1.5 µg) using the CIVO prototype (Fig. 6A). Although clear cell death was observed in cells surrounding the vincristine injection (Fig. 6B), the ITD indocyanine green (ICG) commonly used in human studies was difficult to detect (displayed as a separate image panel from the CC3 stain in Fig. 6C) owing to the loss of ICG signal during tissue processing. Technology modifications, including an improved method of tracking injection sites, were implemented on the basis of what was learned from pilot testing in humans (see Materials and Methods).

Early data regarding patient and physician experiences were encouraging. No grade 2, 3, or 4 adverse events were reported. All patients experienced transient grade 1 events, such as mild erythema and swelling, that resolved without intervention. An external review committee evaluated the interim results and raised no questions or concerns. On a pain scale of 0 to 10, with 0 being no pain and 10 being the worst possible pain, three patients reported 0.5, and one patient reported 3 (mild discomfort) (table S4).

Follow-up patient interviews revealed outstanding patient satisfaction, with one suggestion to improve the experience (table S5). In response to feedback from the various stakeholders involved in the clinical procedure (oncologist, pharmacist, patient, surgeon, and pathologist), the prototype device was reengineered as follows: (i) inclusion of ultrasound-based guide needle placement for optimized targeting of CIVO microinjections to cancerous tissue, (ii) improved materials and associated methods for retaining tumor orientation and injection site identification after tumor injection, and (iii) increased needle density to allow more drug samples to be investigated per patient. (Details on each modification, adopted for the canine study, are provided in Materials and Methods.)

Post-pilot CIVO modifications result in performance improvements in canine patients with lymphoma

CIVO technology modifications made after pilot testing in humans were applied in canine patients with spontaneous lymphoma in a clinical setting. Anesthetized dogs were subjected to CIVO microinjection of vincristine along with a fluorescent tracking dye (Fig. 7, A to D). Ultrasound-based image guidance, the use of a guide needle combined with modified needle positioning, and use of the improved ITD increased injection success in the clinic from 42% ($n = 20$ observed/48 total injection sites) before modifications, to >93% ($n = 15$ observed/16 total injection sites) in lymphoma tumors. The responses to vincristine in native tumors, as detected by both pHH3 and CC3 staining around easy-to-identify injection sites, were robust and reproducible in two canine patients, with a radial CC3 response similar in extent to that observed in xenograft tumors in mice (Fig. 7E). No toxicity was observed according to the Veterinary Cooperative Oncology Group standards (13). These clinical studies in both canine and human patients demonstrate that tumor response to drug can be tested in a toxicity-sparing, localized manner in vivo with CIVO technology.

DISCUSSION

Cancer drug developers face a fundamental challenge in the way new drug candidates are evaluated. Currently, novel cancer drugs are tested in preclinical, often in vitro cell-based models that poorly reflect human cancers. This discordance becomes evident when new agents enter human clinical trials: Nine of 10 drugs that exhibit promising antitumor efficacy in preclinical models fail to provide benefit to actual patients (3). This rate of failure comes at a tremendous cost to drug companies [10 to 15 years and >\$2.6 billion per approved drug (5)] and an even greater cost to cancer patients. Despite advances in 3D tissue culture technologies and nonhuman models of cancer, meaningful modeling of human cancer has proven challenging (2, 14, 15).

In response to these insufficient models of drug efficacy, we engineered a platform technology called CIVO that enables safe, ethical, and efficient (multiplexed) testing of cancer drugs directly in human tumors. The CIVO technology intentionally bypasses bioavailability, biodistribution, metabolism, and excretion issues associated with systemic dosing, making it possible to focus on whether a drug engages its target, how cancer cells respond to target engagement, and whether the ultimate fate of the exposed cells indicates potential for patient therapeutic response—all in the context of an actual tumor micro-environment.

Crossing the threshold into human studies required initial studies in the controlled environment of preclinical xeno-graft models of cancer. With an optimized device and protocol in place, we determined whether the short-term (24 to 72 hours) localized responses induced by CIVO predicted tumor response to systemic administration of the same drugs. The pair-matched set of Ramos and Res-Ramos (resistant) lymphoma tumors provided a simple model to test this correlation. Res-Ramos tumors were resistant to doxorubicin and had up-regulated Pgp drug efflux pumps; thus, the observed lack of responses to localized CIVO micro-injection of doxorubicin and vincristine was expected and served as fundamental demonstrations that our technology could detect resistance to cytotoxic drugs. We unexpectedly observed sensitivity of the Res-Ramos tumors to microinjection of mafosfamide, which was subsequently confirmed in vivo with systemically administered cyclophosphamide; standard in vitro analysis in cell culture, however, failed to reveal this sensitivity, indicating the importance of localized in vivo testing of drugs for more accurate systemic therapeutic predictions.

CC-115 was identified as a new anticancer agent for Res-Ramos tumors after an in vivo CIVO screen of 97 compounds. CC-115 indeed was able to shrink tumors in vivo in animals with the resistant tumors, further demonstrating the use of CIVO in identifying drugs that are effective in tumors already resistant to most therapies. Furthermore, in vitro cell-based proliferation assays did not detect the sensitivity to CC-115.

This observation is in line with previous studies demonstrating that in vitro tests have greater accuracy in predicting tumor resistance to drugs than sensitivity (16–20). The results presented here build on the body of literature emphasizing the limitations of anticancer agent assessment in vitro and provide additional evidence that cancer cells grown as a monolayer in tissue culture behave differently than those grown in the context of an intact tumor. Alternative in vitro and ex vivo technologies have been explored that are designed to predict clinical response to anticancer drugs, using platforms that conserve tumor heterogeneity and the native microenvironment, for example, using thin tumor sections cultured in wells coated with grade-matched tumor matrix in the presence of autologous serum (21). The authors established a correlation between the response of explanted samples and the response of corresponding xenografts, as well as the clinical responses to an approved cytotoxic combination of agents. Further investigation will be necessary to assess whether displacement of tumor tissue from the context of the host patient and, in turn, the immune system will affect the predictive accuracy of this approach.

In our study, CIVO was translated to the clinic for preliminary feasibility testing in four patients. None of the patients who received CIVO microinjections have exhibited adverse effects to date beyond mild grade 1 (transient erythema). From that pilot study, we further optimized the CIVO device and tested in canine patients. Similar to the responses observed in largely homogeneous lymphoma xenograft tumors in mice, we noted robust drug (vincristine)–specific cellular responses in heterogeneous, autochthonous lymphoma tumors. Although these results are preliminary, they support our expectation that CIVO will ultimately enable early drug discovery and treatment decisions in the clinic.

Several challenges remain to be addressed before widespread use of CIVO in the patients. CIVO devices are currently engineered to assess drug responses in lymphoma, melanoma, soft tissue sarcoma, and breast cancer, which, combined, affect about 500,000 U.S. patients and 4 million worldwide each year (22). Reengineering will be required to address tumors that cannot be accessed percutaneously (for example, colon). Another limitation of the current CIVO technology is the necessity to resect the tumor to use histological end points. This limits measurement of response to a single time point per tumor. Noninvasive imaging technologies and molecular or nanotechnology-based reporters may provide additional end point options in the future. Furthermore, clinical trials designed to demonstrate that CIVO-induced responses (or non-responses) correlate with treatment outcome in individual patients must still be performed. Ongoing assessment of CIVO in human patients is the next step toward defining the potential capabilities of this technology (www.clinicaltrials.gov/show/NCT01831505).

Ultimately, determination of whether a cancer drug can be commercialized depends on demonstrating activity in human patients. CIVO technology has the potential for testing experimental drugs directly in a patient with their own intact tumor microenvironment, their own immune system, and unique oncogenomic profile without the toxicities associated with typical clinical exposures. In combination with traditional preclinical toxicology studies, drug developers may then focus resources on the agents that demonstrate superior efficacy in the context of the true human disease before advancement to conventional clinical trials. The quantities of drug required to induce localized tumor responses, both for cytotoxic chemotherapy agents and for targeted inhibitors represented here by the mTOR inhibitors, all fall under established FDA phase 0 guidelines for microdosing studies (23, 24). CIVO technology will further complement genomic medicine. Unfortunately, current genomic approaches cannot reliably predict ultimate resistance to a particular therapy or which drug combinations would circumvent such resistance. This leaves an enormous information gap faced by cancer patients and oncologists between the biology and pharmacology of solid tumors growing in the body and clinical outcomes. We therefore envision a future where the power of genomics and an empirical approach enabled by CIVO are used to bridge this gap created by tumor heterogeneity, microenvironment influences, and drug resistance and to treat patients in a meaningful way.

MATERIALS AND METHODS

Study design

This work in mice and dogs was reviewed and approved by the Institutional Animal Care and Use Committee and was done in accordance with all applicable laws, regulations, guidelines, and policies governing the use of animals in research.

Preclinical—Proof-of-concept studies were conducted using the human Ramos and Res-Ramos lymphoma cell lines xenografted into immunocompromised mice to demonstrate the capability of the CIVO platform for simultaneous assessment of numerous drugs within a single solid tumor. Multiple tissue sections representing distinct depths along the injection column were stained for biomarkers with replicates of at least three tumors per condition per time point analyzed, unless otherwise specified. Studies involving systemic drug administration in tumor-bearing mice were designed as follows. The study end point for Fig. 4 was the time until tumor volume reached 2000 mm³, at which point the mice were euthanized for ethical reasons. Mice were stratified by body weight and tumor size once tumors reached 250 (Fig. 4) or 500 (Fig. 5) mm³ and then randomized into the individual study arms (minimum $n = 6$ per treatment cohort) (25, 26). Power estimates were made using a two-sided, two-arm t test for mean differences (<http://stat-tools.crab.org>) with a $P < 0.05$ significance level. Studies were designed with >90% power to detect a 40 (Fig. 4) or 50% (Fig. 5) change in tumor volume in either of the treatment arms compared to the vehicle control. No outliers were excluded from the data presented.

Clinical, canine—Feasibility studies were conducted in pet dogs with naturally occurring cancer that had presented to local area veterinary clinics. All dogs recruited into this proof-of-principle study remained under the custody and care of their owners and the participating veterinary specialists. Informed consent was obtained from owners. The goal of the feasibility study in dogs was to demonstrate the capability of the CIVO platform for simultaneous assessment of numerous drugs within a single naturally occurring solid autochthonous tumor. Histological analysis was conducted on injected tumors. No outliers were excluded from data presented.

Clinical, human—An observational study in human cancer patients with newly diagnosed or recurrent lymphoma was conducted to determine how cancerous lymph nodes in situ respond to precisely injected standard-of-care therapeutics. All human studies using the CIVO device were done under the oversight of the Fred Hutchinson Cancer Research Center's (FHCRC's) Institutional Review Board (IRB). The feasibility study of the CIVO platform in lymphoma patients is currently open and underway ([ClinicalTrials.gov: NCT01831505](https://clinicaltrials.gov/ct2/show/study/NCT01831505)). All patients provided informed consent for treatment protocols approved by the FHCRC IRB. Patients 18 years or older were eligible if they had an enlarged lymph node highly suspicious of lymphoma or had persistent recurrent, or progressive, lymphoma. At least one enlarged lymph node had to be considered accessible for percutaneous injection by the investigator and of at least 2 cm in the longest dimension. Patients were excluded if the delay of surgery until the lymph node resection date or other factors associated with the study were not feasible. In addition, patients were excluded if they had central nervous

system disease, or any therapy that was potentially immunosuppressive or had anticancer activity in the 4 weeks before device microinjection. Patients with active fungal, viral, or bacterial infections and pregnant women were excluded.

Four patients underwent the microinjection procedure after signing informed consent. Volumes up to 8 μ l of FDA-approved chemotherapy agents were percutaneously injected in a columnar fashion into an enlarged lymph node. The day after microinjection, the injected lymph node was removed by a surgeon for assessment of local cancer cell response to the injected chemotherapy. The injected portion was fixed and processed for histological analysis. No outliers were excluded from the data presented. Observations made during the study included a patient's assessment of the pain he or she experienced, which was noted after the procedure using the Mosby pain rating scale, and adverse events related to the study procedures (microinjections or lymph node biopsy), which were captured up to day 28 after the microinjection.

Device design

The CIVO device is a handheld instrument designed to deliver multiple drugs into living tumors in an arrayed format. Devices contain between four and eight needles (25 to 26 gauge), which are connected to internal drug reservoirs, and range from 30 to 43 mm in length depending on the device model. The device was designed to avoid cross-contamination or drug mixing and to deliver drug with a constant flow rate. The device, once loaded with drug and trace amounts of ITD, is inserted percutaneously to the proper depth within a tumor using ultrasound guidance. Actuation of a lever allows all needles to retract from the tissue while an equal volume of drug is delivered simultaneously from each needle. Successful drug delivery is confirmed by visualization of the ITD.

Arrayed microinjections

Nude mice were inoculated subcutaneously with Ramos or Res-Ramos cancer cells; NSG [NOD (nonobese diabetic)–SCID (severe combined immunodeficient)–IL-2R $\gamma^{-/-}$] mice were inoculated with patient-derived tumors (LY0055F diffuse large B cell lymphoma), as described in Supplementary Materials and Methods. Tumor-bearing mice were anesthetized with vaporized isoflurane during all microinjection procedures. The arrayed microinjection device was inserted transcutaneously into flank tumors, and drugs were delivered from the needles (26 gauge) during their extrusion from the tumor. The typical column length was ~6 mm. ITD [previously inactivated VivoTag680-S, ICG, or a fluorescent tattoo dye] was added to each drug reservoir at a final concentration of 50 μ g/ml or as a 5% solution diluted in vehicle, for delivery along with drug. Microinjected mice were allowed to recover from anesthesia and return to normal activity for 24 to 72 hours before euthanasia and tumor resection.

All microdoses were equivalent to or lower than what would be allowed under FDA guidelines for Exploratory IND (Investigational New Drug) studies and by solubility of drug into vehicle. For instance, on the basis of standard systemic dosing of vincristine in humans (0.4 to 1.4 mg/m²), 6 to 21 μ g of injected drug would be allowed for microdose studies for this compound under Exploratory IND guidelines (23, 24). Independent cohorts of mice

were administered drugs systemically for 4 weeks, as described in Supplementary Materials and Methods.

Whole-slide scanning and image analysis

Images of every cell from each tissue section stained were captured by digital, automated, high-resolution whole-tissue scanning, as described in Supplementary Materials and Methods.

Statistical analysis

Statistical analyses were performed using R version 3.0.2 (The R Project for Statistical Computing). Mean tumor growth inhibition was computed for each systemic drug and tumor line and compared to 0 using a Student's *t* test with Bonferroni correction for multiple comparisons. The Kaplan-Meier method was used to evaluate survival for mice treated with cyclophosphamide, and log-rank test was used to compare survival between the respective tumor lines. To account for different tumor growth rates in parental and resistant Ramos cell lines, the average tumor size for each line in the vehicle arms at day 8 was measured, and events were defined as the day on which a tumor exceeded the corresponding average. Comparison of histological results from CIVO Analyzer was assessed by a linear mixed-effects model, where each drug and tumor line (resistant versus parental) were modeled as an unknown fixed effect and each tumor was modeled as a random effect. Differences between drug effects and interactions between drug and tumor line were assessed by Wald test. All *P* values ≤ 0.05 , adjusted for multiple comparisons, were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The CIVO tumor microinjection platform

(A) The CIVO platform consists of a handheld array of up to eight needles capable of simultaneously penetrating subcutaneous tumors and delivering microdoses of candidate therapeutics. (B) For preclinical studies, tumors were grown as flank xenografts in immunocompromised mice and injected while mice were anesthetized. A chemically inert ITD was co-injected through each needle. (C) A representative example of the ITD signal from a tumor injected using a five-needle array visualized with a Xenogen In Vivo Imaging System (IVIS). (D) A longitudinal IVIS scan demonstrating the column-like distribution of the tracking dye signal from a single needle spanning the z axis of the tumor. (E) Tumor responses were assessed after resection of the tumor via histological staining of cross sections (4 μ m thick) sampled at 2-mm intervals perpendicular to the injection column. (F) High-resolution whole-slide scanning captured images of every cell from each 4- μ m-thick tissue section. (G) A representative tumor response to microinjected drug at a single injection site. Nuclei, DAPI (4',6-diamidino-2-phenylindole) (blue); ITD (green); a drug-specific biomarker (orange). (H) The resulting images were processed by a custom image analysis platform called CIVO Analyzer, which classifies the cells within each region of interest as biomarker-positive (green dots) or biomarker-negative (red dots).

Fig. 2. CIVO microinjections result in spatially defined, non-overlapping drug distribution and quantifiable tumor responses

(A) Intratumoral vincristine (VCR) distribution was directly tracked by microinjection of ^3H -labeled drug into discrete positions of xenografted Ramos lymphoma tumors. After injection, tumors were resected at 2, 8, 24, 48, and 72 hours, and sections were subjected to autoradiography and quantified as drug concentration as a function of distance. Data are averages $\pm$ SEM ($n = 4$ tumors per time point). (B) Parallel tumors were microinjected with the same amount of vincristine (1.5 μg) or a vehicle (Veh) control. Tumor responses at 24 hours at various distances from each injection site were visualized by staining for pHH3 or CC3. High-magnification hematoxylin and eosin (H&E) images are from the approximate regions designated by the red and blue boxes in the middle panels. The fraction of biomarker-positive cells was plotted as a function of radial distance from the injection site 72 hours after microinjection. Data are averages $\pm$ SEM ($n = 6$ tumors). $*P < 0.05$; $**P < 0.001$, Wald test. (C) Multiplexed microinjection of five different vincristine concentrations, each through a unique position within the array. Sections from resected tumors were stained and quantified. Data are averages $\pm$ SEM ($n = 5$ tumors at 24 hours, six tumors at 72 hours after microinjection).

Fig. 3. In vivo tumor responses are mechanism of action-specific and concentration-dependent (A and B) Ramos lymphoma tumors were injected with arrays containing a vehicle control, mafosfamide (MAF), doxorubicin (DOX), vincristine, and prednisolone (PRED), each delivered from a distinct needle within the array, and tumors were resected 24 hours later. Tissue sections from multiple depths along the injection column were stained with H&E or antibodies recognizing γ H2AX, pHH3, or CC3. (A) Representative whole-section images from resected tumors. (B) Individual injection sites from tumors. (C) Ramos lymphoma tumors were microinjected with varying concentrations of each drug, each concentration through a distinct needle within the array, and tumors resected 24 and 72 hours after microinjection. Tissue sections were stained with antibodies recognizing γ H2AX, pHH3, or CC3, and the fraction of biomarker-positive cells was plotted as a function of radial distance

from the injection site. Data are average responses across multiple tumors $\pm$ SEM (mafosfamide, $n = 5$; doxorubicin, $n = 15$; vincristine, $n = 8$; and prednisolone, $n = 4$).

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A In vivo: CIVO **B In vivo: Systemic** **C In vitro**

Fig. 4. Localized responses to CIVO microinjection detect context-dependent drug-specific resistance and sensitivity, which correlate with long-term systemic outcomes

(A) Ramos and Res-Ramos tumors were microinjected with arrays containing vehicle control, doxorubicin, vincristine, mafosfamide, and prednisolone, and tumor responses were visualized by staining 4- μ m sections from tumors resected 24 hours after microinjection (except for doxorubicin, shown at 72 hours). Tissue sections from multiple depths along the injection column were stained with antibodies recognizing γ H2AX, pHH3, or CC3. The fraction of CC3⁺ cells was quantified using CIVO Analyzer, and the difference in response between the Ramos and Res-Ramos tumors was plotted as a function of radial distance from the injection site for each drug. Data are average differences in response across a minimum of three tumors 24 hours after microinjection (except for doxorubicin curves, which are from 72 hours) $\pm$ SEM. (B) Ramos and Res-Ramos tumor-bearing mice ($n = 10$ per cohort) were

treated systemically with saline (control), doxorubicin (3.3 mg/kg), vincristine (0.5 mg/kg), cyclophosphamide (20 mg/kg), or prednisone (0.2 mg/kg). Doxorubicin, vincristine, and cyclophosphamide were each administered intravenously once per week (q1w) for 4 weeks. Prednisone was administered orally (PO) 5 days per week for 4 weeks. Efficacy was assessed via tumor volume measurements, and data are means $\pm$ SEM. Kaplan-Meier survival curves were determined for the cyclophosphamide treatment groups ($n = 10$ per cohort); P value was determined by a log-rank test using vehicle control for the comparison. Tumor growth inhibition (TGI) was calculated (see Materials and Methods) at day 8 for each treatment condition compared to vehicle. P values were determined by a two-sided Student's t test. (C) Cultures of drug-naïve and doxorubicin-resistant Ramos (Res-Ramos) cell lines were seeded in drug-free medium or medium with various doxorubicin, vincristine, mafosfamide, or prednisolone concentrations. Cell viability was measured 72 hours after drug exposure. Data are representative from three separate experiments.

Fig. 5. CIVO predicts enhanced experimental mTOR inhibitor CC-115 efficacy in doxorubicin-resistant tumors

(A) Ramos ($n = 4$) and Res-Ramos ($n = 3$ at 2 hours; $n = 5$ at 24 hours) tumors were microinjected with arrays containing vehicle control, ridaforolimus, or CC-115. Tumor responses were visualized by staining for p4EBP1 and CC3. (B) The fraction of CC3⁺ cells was quantified using CIVO Analyzer and plotted as a function of radial distance from the injection site. Data are average responses 24 hours after micro-injection $\pm$ SEM. * $P < 0.05$, Wald test. (C) Cultures of drug-naïve (Ramos) and doxorubicin-resistant (Res-Ramos) cell lines were seeded in drug-free medium or medium with various concentrations of CC-115. Cell viability was tested using the PrestoBlue assay after 72 hours. IC_{50} , median inhibitory concentration. (D and E) Ramos and Res-Ramos tumor-bearing mice ($n = 6$ per cohort) were treated systemically with saline (control) or CC-115 (5 mg/kg) administered PO daily

for 25 days. (D) Efficacy was assessed via tumor volume measurements. Data are means $\pm$ SEM. TGI was calculated at day 25 for each treatment condition. $*P < 0.05$ compared to saline control, two-sided Student's t test. (E) Representative images of tumors from each treatment arm on day 25.

Fig. 6. Pilot clinical use of CIVO demonstrates feasibility of inducing localized responses in human lymphoma tumors

(A) Microinjection procedure in a patient with a palpable cancerous lymph node using an early CIVO clinical prototype. The patient's tumor was injected with microdoses of vincristine (1.5 μg) and ICG. Lymph nodes were resected 24 hours later, and tumor responses were visualized by staining 4-mm sections for CC3. (B) The fraction of CC3⁺ cells was quantified from one patient's tumor using CIVO Analyzer and plotted as a function of radial distance from the injection site. Data are average responses across three sections from multiple depths along the injection column ± SEM. (C) Tissue sections were stained for CC3, and the localized cell death response to vincristine was verified by evaluation of parallel H&E-stained sections.

Fig. 7. CIVO performance in canine lymphoma tumors demonstrates spatially restricted responses linked to drug mechanism

(A to C) CIVO microinjection procedure adapted for a clinic-like, veterinary setting (A and B) by incorporating the placement of a “guide needle” into the optimal injection site using ultrasound guidance, (C) which aligned the microinjection device to target all needles of the handheld device into the optimal location in the tumor, increasing injection success. (D) ITD signal seen beneath the skin with a blue flashlight and yellow filter glasses, from a canine tumor injected using an eight-needle CIVO array. (E) Cancerous lymph nodes in two dogs were microinjected with the same amount of vincristine (1.5 μg) or a vehicle control. Tumors were resected 24 hours after microinjection. The ITD (green) and tumor responses (CC3, red; pHH3, yellow) were visualized in 4-μm sections from multiple depths along the injection column. Individual injection sites are shown for each dog. The fraction of biomarker-positive cells for each dog was plotted as a function of radial distance from the

injection site. Data are average responses across three sections at about 2-mm intervals along the injection column $\pm$ SEM.

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