The Tumor-Targeted Prodrug ICT2588 Demonstrates Therapeutic Activity Against Solid Tumors and Reduced Potential For Cardiovascular Toxicity.

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ABSTRACT

Development of therapeutic strategies for tumor-selective delivery of therapeutics, through exploitation of the proteolytic tumor phenotype, has significant scope for improvement of cancer treatment. ICT2588 is a peptide-conjugated prodrug of the vascular disrupting agent (VDA) azademethylcolchicine developed to be selectively hydrolyzed by matrix metalloproteinase-14 (MMP-14) within the tumor. In this report, we extend our previous proof-of-concept studies and demonstrate the therapeutic potential of this agent against models of human colorectal, lung, breast, and prostate cancer. In all tumor types, ICT2588 was superior to azademethylcolchicine, and was greater or comparable to standard clinically used agents for the respective tumor type. Prodrug activation in clinical human lung tumor homogenates relative to stability in human plasma and liver was observed, supporting clinical translation potential. A major limiting factor to the clinical value of VDAs is their inherent cardiovascular toxicity. No increase in plasma von Willebrand factor (vWF) levels, an indicator of systemic vascular dysfunction and acute cardiovascular toxicity, was detected with ICT2588, thereby supporting the tumor-selective activation and reduced potential of ICT2588 to cause cardiovascular toxicity. Our findings reinforce the improved therapeutic index and tumor-selective approach offered by ICT2588 and this nanotherapeutic approach.

Keywords: Cancer Therapy; Drug Delivery; Nanotherapeutic; Peptide-conjugate; Matrix Metalloproteinase; Vascular Disrupting Agent.

Abbreviations: MMP, matrix metalloproteinase; MTD, maximum tolerated dose; NSCLC, non-small cell lung cancer; VDA, Vascular disrupting agent; vWF, von Willebrand Factor.

INTRODUCTION

The standard approach for the treatment of advanced or disseminated cancer involves the systemic administration of antiproliferative cytotoxic agents. The major drawback to the non-selectivity of these agents against cancer is dose-limiting toxicity, with the consequent impact upon therapeutic efficacy. Over the past decade, in reflection of these limitations, a profound change in the direction for cancer drug development has evolved. Based upon the acquisition of addiction to specific oncogenic signaling pathways, approaches are now focused toward inhibition of specific dysregulated molecular pathways involved in cancer development and progression[1, 2]. In principle 'molecularly targeted' agents are highly selective agents against growth and survival of tumor cells, whilst sparing normal cells. However, despite improved treatment success and reduced toxicity relative to conventional cytotoxic chemotherapeutics, these approaches are not devoid of problems. It is becoming evident that efficacy of these such agents is hindered by genetic instability and heterogeneity within the cancer cell population, resulting in acquired drug resistance[3]. Furthermore, on- and off-target activities of many of these agents against particular 'normal' cell populations has led to induction of specific toxicities and detrimental physiological effects, and subsequent sub-therapeutic drug concentrations in the tumor[2-4]. Therefore the development of new therapeutics to circumvent these issues, with improved tumor selectivity, lower systemic toxicity, and limited potential for drug resistance is an important trajectory for improved cancer treatment.

One strategy is to develop agents targeting the tumor phenotype rather than intracellular signaling pathways, such as the tumor supporting vasculature or increased proteolytic activity of tumors[5-10]. The selective disruption of the established blood supply to tumors is now known to be an effective therapeutic strategy, with several vascular disrupting agents (VDAs) currently in late stage clinical trial[11-14]. The majority of these agents bind to the colchicine-binding domain of β-tubulin causing microtubule depolymerisation and consequent

morphological changes within endothelial cells[15]. The advantages of VDAs over other cancer therapeutics are that they are not required to extravasate from the blood vessel and penetrate across several cell layers for their activity, they avoid undesirable acquired drug resistance, and they target a fundamental process for survival of all solid tumors [11-13]. Furthermore, it is now well established that the disruption of a single tumor blood vessel results in death of the large number of tumor cells it supports [11, 13, 14]. Additionally, the destabilization of the tumor endothelium leads to a temporal enhancement of vascular permeability and consequent drug retention, with significant potential for therapeutic enhancement[14-16]. However, it is becoming increasingly apparent that the significant therapeutic value for these colchicine-related VDAs is compromised by their intrinsic toxicity, particularly cardiac toxicities[17, 18]. Therefore, strategies to reduce systemic exposure, increase tumor-selectivity and improve their therapeutic index are required.

Another fundamental issue which impacts upon the success of several current small molecule cancer therapies is their limited ability to localize at the tumor site. Many cytotoxic drugs demonstrate poor biodistribution *in vivo*, with tumor doses for common agents such as doxorubicin and paclitaxel reported to be around 5-10% of the dose accumulating in healthy organs[19, 20]. In addition to reduced efficacy, this also has implications for intrinsic systemic toxicity. Therefore, there is a considerable requirement for the development of a new generation of anticancer agents, capable of selective localization at the tumor and with reduced potential for systemic toxicity. One approach which addresses this need is the development of nanotherapeutic agents as tumor-targeted prodrugs, recently termed “cancer smartbombs” or “molecular grenades”[6], whereby the potent therapeutic entity is masked until being activated by exploitation of unique phenotypic differences present in the tumor environment [6, 21, 22].

Harnessing enzymes with elevated or specific activity within the tumor environment to convert a non-toxic prodrug into a potent anticancer agent is one approach with significant therapeutic scope[6, 8, 21, 23, 24]. A class of enzyme heavily implicated in cancer expansion and dissemination, and with clear applicability for prodrug development, are the proteolytic enzymes of the tumor degradome[21]. This strategy was recently exemplified through use of the tumor-endothelial expressed carboxypeptidase prostate-specific membrane antigen (PSMA) for activation of the non-selective toxin thapsigargin, a successful approach which has now progressed to phase II clinical trial[6, 7]. Another family of enzymes within the tumor degradome with demonstrated potential for tumor-selective prodrug development are the matrix metalloproteinases (MMPs)[21], particularly the membrane-type MMP (MT-MMP) subfamily. Of these, the prototypic member MMP-14 (MT1-MMP) has the majority of required traits as a prodrug activating target; being pivotal for tumor invasive behavior and tumor vascularization[25-30], presenting as a direct target in the tumor vasculature for prodrug activation[25-27, 29], and having the ability to selectively cleave specific peptide sequences[5, 31]. Based on this, we previously designed a MMP-14-targeted nanotherapeutic prodrug, ICT2588, comprising the potent VDA, azademethylcolchicine, inactivated through conjugation to a cleavable peptide, which was endcapped to prevent non-specific exopeptidase degradation (Figure 1)[5, 32]. The peptide sequence integral to this nanotherapeutic approach had been rationally developed to be selectively cleaved by MMP-14[5, 32]. The therapeutic strategy for ICT2588 involves hydrolysis by MMP-14 followed by subsequent nonspecific exopeptidase activity to produce azademethylcolchicine selectively within the tumor environment[5, 33]. This mechanism was supported in both *ex vivo* and *in vivo* studies through demonstration of MMP-dependent hydrolysis of ICT2588 followed by rapid production of active azademethylcolchicine, with no intermediate peptide-conjugated metabolites of ICT2588 detected[5]. In this study we have further evaluated the clinical

potential for this MMP-14 activated VDA strategy using a range of clinically relevant human solid tumor models and primary surgical samples. A reduced potential for drug-induced cardiovascular toxicity with ICT2588 was assessed by development of an assay to measure systemic levels of murine von Willebrand Factor (vWF), a validated biomarker of systemic vascular dysfunction and acute cardiovascular events[34-37].

EXPERIMENTAL SECTION

Synthesis of ICT2588

ICT2588 (Figure 1) was synthesized and characterized in-house as a C- and N-terminal modified peptide conjugate of azademethylcolchicine (Figure 1), as previously described[32]. For *in vitro* analyses, ICT2588 was prepared as a stock solution in dimethyl sulfoxide (DMSO).

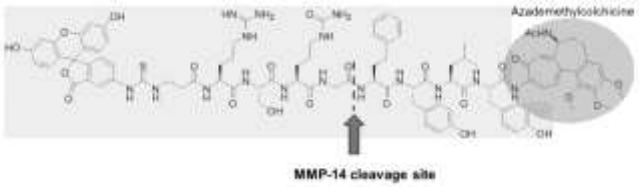

Figure 1: Structure of ICT2588 and azademethylcolchicine, indicating the MMP-14 selective scissile bond

Assessment of ICT2588 Activation in Human Clinical Samples ex vivo

To assess the clinical potential for tumor-selective activation of ICT2588, freshly resected and snap frozen specimens of non-small cell lung cancer (NSCLC) and their paired blood plasma, as well as human liver were used for this study. Informed patient consent was obtained from all patients prior to specimen collection and all patient details were

anonymized to ensure confidentiality. All experiments were performed after first obtaining consent from the local research and ethics committee according to Medical Research Council, UK Regulations. Human tissue samples were stored under the control of Ethical Tissue (Bradford, UK) who ensured concordance with regulations required by the UK Human Tissue Authority.

Clinical tumors and liver tissue were homogenized in MMP activity buffer (50 mmol/L Tris-HCl (pH 7.6), 1.5 mmol/L NaCl, 0.5 mmol/L $CaCl_2$, 1 μmol/L ZnCl, 0.01 % v/v Brij-35) and the resultant supernatants collected by centrifugation. The potential for activation of ICT2588 (final concentration 20μmol/L) was assessed in human plasma and tumor and liver supernatants (equivalent to 100 mg tissue per ml). Over a 90 minute period, reaction aliquots were removed, proteins precipitated using acetonitrile and ICT2588 metabolism analyzed by LC-MS, as previously described[5].

Determination of MT-MMP gene expression.

Assessment of MT-MMP gene expression as determined by quantitative real-time PCR (qRT-PCR) was performed as previously described[38]. All primers and fluorogenic probe nucleotide sequences were synthesized by Applied Biosystems (Warrington, UK) and had been shown previously to be MMP gene specific[38, 39]. The 18S ribosomal RNA gene was used as an endogenous control to normalize for any difference in the amount of total RNA in each sample, using previously validated procedures[38, 39]. The C_T (cycle at which amplification entered the exponential phase) was used as an indicator of the level of target RNA in each tissue, *i.e.* a lower C_T indicated a higher quantity of target RNA. For data interpretation, results were normalized to calibrated curve data for the 18S-rRNA and MMP genes, as previously described [38, 39].

Determination of MMP-14 protein expression.

Tumor homogenates were prepared as previously described[5]. Equal amounts of protein (equivalent to 0.1mg xenograft tissue) were resolved by 10% SDS-PAGE and blotted onto Hybond-P membrane (Amersham, UK). Non-specific antibody binding was blocked via incubation in 5% ECL blocking reagent (Amersham). The blot was probed with a monoclonal antibody to MT1-MMP (MAB3328; Chemicon International) overnight at 4°C followed by a rabbit anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Dako). Antibody reactivity was detected by chemiluminescence using ECL-Plus (Amersham).

Evaluation of Antitumor activity.

Female Balb/c immunodeficient nude mice (Harlan, Loughborough, UK), between the ages of 6 and 8 weeks, were used, Under brief general inhalation anesthesia, 2 to 3 mm^3 fragments of either A549 (non-small cell lung cancer); MCF7 (breast carcinoma); DLD1 (colorectal carcinoma); PC3 (prostate adenocarcinoma) or HT1080 (fibrosarcoma) human tumor xenograft tumors were implanted subcutaneously in the abdominal flanks. Once tumor volumes reached approximately 32 mm^3 (as measured by calipers) mice were randomized into groups (n=8) and received ICT2588 at 75 mg/kg or solvent control (10% DMSO/arachis oil) via intraperitoneal (i.p.) administration. For comparison to other chemotherapeutics, tumor-bearing mice received either azademethylcolchicine at 15 mg/kg (molar equivalent dose to 75mg/kg ICT2588), doxorubicin at 10 mg/kg intravenously (i.v.), 5-fluorouracil at 100 mg/kg i.p., Paclitaxel at 20 mg/kg i.p., or combretastatin-A4 phosphate (CA4P) at its maximum-effective dose of 150 mg/kg i.p. Tumor volume (measured by calipers) and animal body weight were recorded daily for up to 25 days. Tumor growth data is presented as the ratio of the mean tumor volume on individual days relative to the tumor volume on the initial day of treatment (day 0), with the mean relative tumor volume on day 0 being 1.0. Mann-

Whitney U tests were conducted to determine the statistical significance of any differences in growth rate (based on tumor volume doubling time) between control and treated groups.

Throughout the study, all mice were housed in air-conditioned rooms in facilities approved by the Home Office to meet all current regulations and standards of the United Kingdom. All procedures were carried out under a United Kingdom Home Office Project License, following UK National Cancer Research Institute Guidelines for the Welfare of Animals (UKCCCR) guidelines[40].

Measurement of systemic levels of von-Willebrand Factor (vWF)

Mice (Female Balb/c immunodeficient) were injected with either azademethylcolchicine (30 mg/kg i.p.), ICT2558 (150 mg/kg i.p.), doxorubicin (10mg/kg i.v.), or solvent control. At 4, 24 hours (all drugs) and 48 hours (ICT2588) post-treatment mice were sacrificed, blood collected into heparinized tubes, centrifuged at 2,000 x g for 10 minutes, and the resultant plasma aliquoted and stored at -80ºC until use. Normal and human tissues were subsequently removed and also stored at -80ºC. A purified vWF protein standard (from human plasma) was obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK), supplied at a concentration 11 IU/ml.

Microplate wells (Nunc Maxisorp 96-well; Thermo Scientific, UK) were coated with 100µl of purified sheep anti-vWF capture antibody (AB11713; Abcam, UK) diluted 1:500 in phosphate buffered saline (PBS; 10mM, pH 7.2), covered and incubated overnight at 4ºC. Plates were washed three times in PBS / 0.05% Tween 20 using an automated platewasher (WellWash, Thermo Scientific), and non-specific binding blocked via incubation in PBS/1% BSA for 60 minutes at room temperature (RT). The vWF standard was serially diluted in PBS, creating a concentration range of 0.002-0.12 IU/ml, and murine plasma samples were

diluted 1:2 and 1:8 in PBS. After washing, 100 µl of each standard and sample were added to the plate, with PBS alone added as a blank control. Plates were incubated for 60 minutes at RT, with gentle agitation, and washed. Rabbit anti-vWF horseradish peroxidase-conjugated antibody (P0226; Dako) was added to each well (100µl) at a dilution of 1:400 in PBS, and the plate sealed and incubated for 60 minutes at RT, with gentle agitation. Wells were washed, 100µl TMB solution (Sigma-Aldrich) added to each well, and the reaction stopped after 5 minutes by addition of 100µl TMB stop solution (2 M H_2SO_4; Sigma-Aldrich). After a further 5 minutes of color development, absorbance of each well was measured at 450 nm, with correction at 550 nm, using a Multiskan EX platereader (Thermo Life Sciences). A calibration curve was generated using the vWF protein standards, and sample values were interpolated into this curve to obtain concentration values. Data were analyzed using SPSS, and mean differences considered statistically significant at the $P < 0.05$ level.

RESULTS

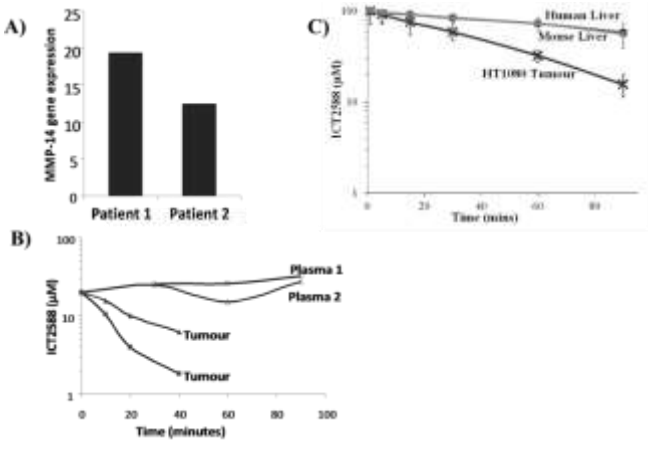

Figure 2: Selective activation of ICT2588 in human non-small cell lung cancer (NSCLC). (A) Expression of MMP-14 mRNA in two clinical NSCLC tumor biopsies as measured by quantitative RT-PCR, calculated relative to a calibration sample[38]. (B) Metabolism of

ICT2588 by clinical NSCLC biopsies relative to plasma from the same patients, and (C) metabolism of ICT2588 in homogenates of human liver relative to murine liver and the MMP-14-expressing HT1080 tumor model. For the metabolism studies, ICT2588 was quantified using liquid chromatography-mass spectrometry (LC-MS) and the degree of proteolytic metabolism indicated by the concentration of ICT2588 remaining over time, as previously described[5].

Metabolites detected by LCMS and expressed as concentration of ICT2588 remaining.

Selective metabolism of ICT2588 in human clinical NSCLC relative to normal tissue

The elevated expression of MT-MMPs in non-small cell lung cancer (NSCLC) relative to associated normal lung tissue, and a strong correlation between MMP-14 gene expression and proteolytic activity is previously reported by our group[38]. In order for ICT2588 to be a viable clinical therapeutic option, it must show activation in tumor tissue, limited activation in non-diseased tissue and the potential for systemic pharmacological stability. Metabolism of ICT2588 was studied *ex vivo* using homogenates of two clinical samples of NSCLC and their respective blood plasma. In both tumor samples MMP-14 expression was elevated (Figure 2A) and ICT2588 was metabolized rapidly ($t_{½}$ = 11.4 minutes and 23.2 minutes, respectively (Figure 2). In contrast, ICT2588 metabolism was minimal in plasma samples from these patients ($t_{½}$ > 90 minutes; Figure 2B) and human and mouse liver homogenates (Figure 2C).

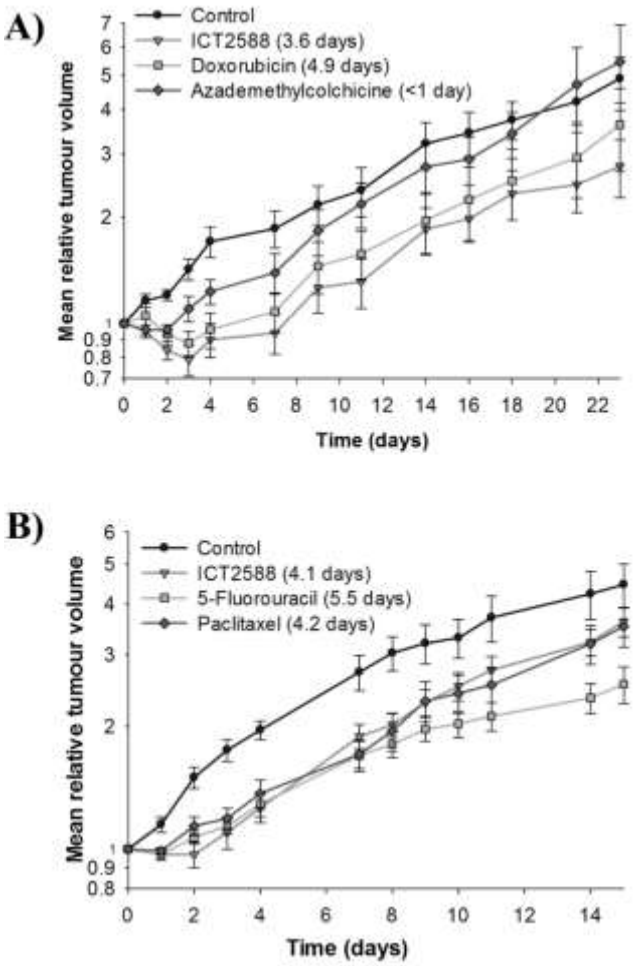

Figure 3: Antitumor activity of ICT2588 against human (A) NSCLC and (B) Colorectal tumor models. ICT2588 inhibits tumor growth to a greater extent than an equimolar concentration of azademethylcolchicine (active metabolite of ICT2588) and comparable to standard agents; doxorubicin for NSCLC (A) and Paclitaxel and 5-Fluorouracil for colorectal tumors (B). Mice were treated with a single intraperitoneal dose of ICT2588 (75 mg/kg; maximum effective dose, equivalent to 50% maximum deliverable dose). Mice bearing A549 NSCLC tumors were administered an equimolar dose of azademethylcolchicine (15 mg/kg, *i.p*) or doxorubicin (10mg/kg, *i.v.*), and those bearing DLD1 colorectal tumors were administered a single dose of paclitaxel (20 mg/kg, *i.p*) or 5-Fluorouracil (10mg/kg, *i.p.*). The tumor growth delay is defined as time taken for doubling of tumor volume relative to control, denoted in brackets in the legend of each figure.

Treatment with ICT2588 was comparable to, or an improvement upon current clinically used therapeutics. Against the A549 NSCLC tumor model, ICT2588 produced a significantly greater tumor response than azademethylcolchicine (active metabolite of ICT2588), and a similar response to that of doxorubicin (Figure 3A). In the colorectal DLD-1 tumor model, therapeutic response to ICT2588 was comparable to that of Paclitaxel and 5-Fluorouracil (Figure 3B).

Against the PC3 prostate xenograft model, VDAs resulted in a significant growth delay compared with vehicle-treated control ($P < 0.01$; Figure 4). The antitumor effect of a single dose administration was significantly greater for ICT2588, with a tumor growth delay of 9.3 days relative to 3.1 ($P < 0.05$) and 1.2 days ($P < 0.01$) for its' active metabolite (azademethylcolchicine) and Combretastatin-A4 Phosphate (CA4P), respectively (Figure 4). In all tumor models, administration of the various treatments did not cause any significant loss in mouse body weight (≤15%; data not shown).

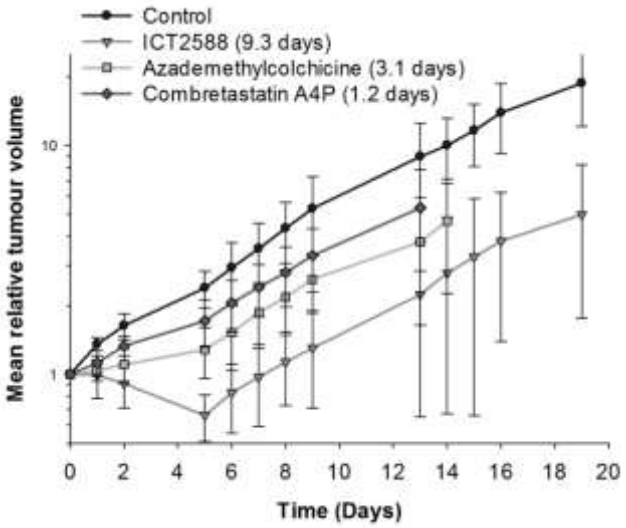

Figure 4: Antitumor activity of Vascular Disrupting Agents against human PC-3 prostate tumor model. Administration of a single intraperitoneal dose of ICT2588 results in a significantly greater inhibition of PC-3 tumor growth compared to the active metabolite (azademethylcolchicine) at equimolar concentrations, or Combretastatin-A4-Phosphate at 150mg/kg. The tumor growth delay is defined as time taken for doubling of tumor volume relative to control, denoted in brackets in the legend of each figure.

Activity of ICT2588 against MCF-7 human breast carcinoma *in vivo*

A lack of activity of ICT2588 against an MMP-14 negative cell line (MCF7) *in vitro* had been demonstrated in our previous study, consistent with a requirement for MMP-14 involvement in ICT2588 activation[5]. However, in contrast to MCF7 cells grown *in vitro*, the presence of MMP-14 was detectable in *ex vivo* MCF7 tumor homogenates[5] (Figure 5). Whereas human MMP-14 mRNA was undetectable in the MCF7 xenograft model[5], moderate expression of murine MMP-14 was detected (Figure 5).

Administration of ICT2588 to mice bearing MCF7 tumors produced a significant tumor response compared with vehicle-treated controls, with a growth delay of 7.3 days. This is much greater than an equimolar dose (15 mg/kg) of the active metabolite (azademethylcolchicine) which produced a considerably diminished growth delay of 2.0 days (Figure 5B).

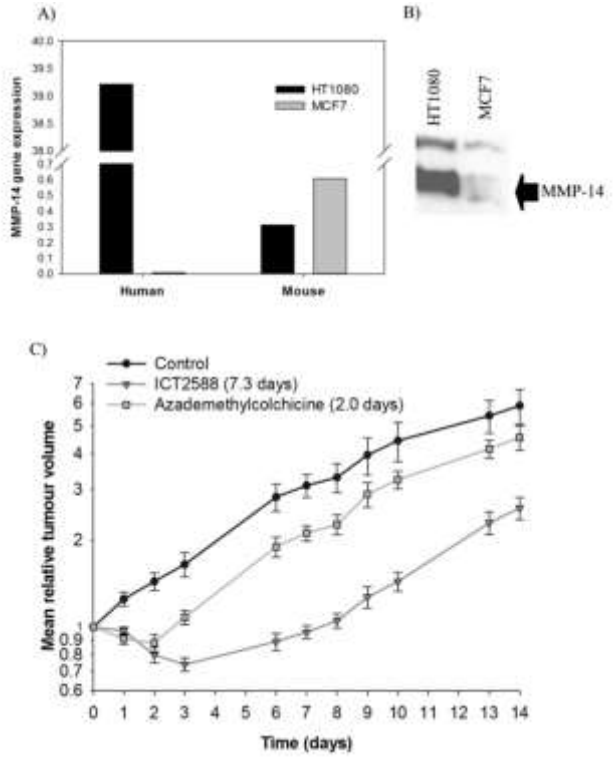

Figure 5: The role of the tumor microenvironment in activity of ICT2588. Although MCF-7 xenografts demonstrate negligible expression of human MMP-14 mRNA, the levels of murine MMP-14 are similar to those of the HT1080 xenograft (A). The MMP-14 protein expressed by the murine stromal and endothelial cells (B) is sufficient to activate ICT2588 and result in significant inhibition of MCF7 tumor growth compared to azademethylcolchicine at equimolar concentrations. Mice were treated with a single intraperitoneal dose of ICT2588 or azademethylcolchicine and tumor size determined daily. The tumor growth delay is defined as time taken for doubling of tumor volume relative to control, denoted in brackets in the legend of each figure.

Post-treatment changes in plasma vWF levels, as an indicator of cardiovascular effects.

Systemic levels of vWF were determined as an indicator of drug-induced detrimental effects upon the cardiovascular system, as previously reported[34-37]. Using vWF standards the ELISA assay was linear between 2.0×10^{-3} and 0.03 IU/ml ($R^2 = 0.975$), with an inter-assay

variability of <5% (Data not shown). The dilution-corrected concentration of plasma vWF in vehicle-treated control mice was between 0.07 and 0.10 IU/ml monitored at 4, 24 and 48 hrs (Table 1). This was comparable to that detected in non-treated mice plasma (Table 1). Administration of ICT2588 did not result in a significant increase in circulating levels of vWF at 24 hours over that detected in vehicle-treated controls (P = 0.6; Table 1 and Figure 6). In contrast, azademethylcolchicine at an equimolar dose caused a significant elevation in plasma vWF levels after 24 hours (P < 0.05; Table 1 and Figure 6). No increase in plasma vWF levels were detected following exposure to the chemotherapeutic agent doxorubicin (Figure 6). Additionally, no delayed rise in vWF levels was observed with the prodrug ICT2588, as at 48 hours the plasma vWF concentration was not significantly different to vehicle treated control mice (P = 0.50; Table 1).

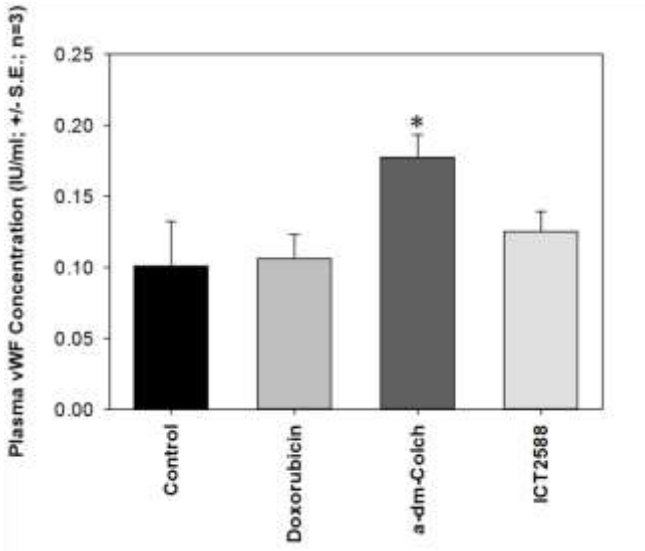

Figure 6: Levels of von-Willebrand (vWF) factor in mouse plasma following drug treatment. Mice were treated with ICT2588, azademethylcolchicine (a-dm-Colch), or doxorubicin and plasma vWF levels measured 24 h post-treatment. Graph represents mean values from 3 mice ± standard error. * P<0.05 relative to control.

Table 1. Plasma levels of von-Willebrand factor (vWF) following drug administration[2].

	Plasma concentration of vWF (IU/ml)		
Drug Treatment	**4 hrs**	**24 hrs**	**48hrs**
Control	0.09 ± 0.023	0.10 ± 0.031	0.07 ± 0.021
Azademethylcolchicine	0.12 ± 0.016	0.18 ± 0.016	*n.d.*
ICT2588	0.12 ± 0.017	0.12 ± 0.015	0.09 ± 0.020
Doxorubicin	0.10 ± 0.022	0.11 ± 0.016	*n.d.*

DISCUSSION

Drug-induced disruption of established tumor vasculature is a promising therapeutic strategy, with several colchicine based VDAs currently in late-stage development[11-13]. However, the off-target toxicities of these agents, primarily against the cardiovascular system, has limited their clinical progression[17, 18]. We previously reported a therapeutic strategy to overcome the limitations outlined above, which exploits the therapeutic potential of VDAs and exploits the tumor phenotype. The therapeutic 'smartbomb', ICT2588, was designed to selectively deliver the VDA, azademethylcolchicine, to the tumor through activation by membrane-type MMPs (MT-MMPs) endopeptidases specifically located within the tumor microenvironment[5].

ICT2588 illustrates how harnessing of the tumor degradome to convert an inactive 'smartbomb' into its active chemotherapeutic metabolite, has shown significant promise[5]. Elevated expression of membrane-type MMPs (MT-MMPs) has been demonstrated in many tumor types, both preclinically and clinically[5, 25, 27, 30, 38], with a direct relationship between genetic expression of MMP-14 (MT1-MMP) and its enzyme activity[38], strongly supporting these endopeptidases as tumor-selective activators of therapeutics[5, 21, 38]. Our previous study demonstrated the viability for MMP-14 activated tumor 'smartbombs' and illustrated proof-of-concept, using a human fibrosarcoma xenograft model known to express very high levels

[2] Values are dilution-corrected and represent the mean of 3 independent experiments ± standard error

of MMP-14[5, 38]. Together with the pivotal role MMP-14 plays in tumor expansion and dissemination[26, 29, 30], the implication is that this technology would be applicable to the treatment of all solid vascularized tumors. This is demonstrated in this study by the significant antitumor responses and the superior activity of ICT2588 relative to equimolar doses of its active metabolite (azademethylcolchicine), against NSCLC, colorectal, breast and prostate tumor models. The importance of the tumor stroma and vasculature in the therapeutic mechanism is exemplified with the MCF7 breast cancer model. These cells are negative for human MMP-14 *in vitro* and *in vivo*, but have detectable murine MMP-14 levels *in vivo*, representing expression within tumor stroma and neovasculature, as previously indicated[25, 27, 41, 42]. The significant growth delay observed following administration of ICT2588 indicates that in addition to expression of MMP-14 within the tumor mass, the presence of MMP-14 within the tumor microenvironment provides a further dimension to the therapeutic response of this approach. This indication of an integral role for the tumor microenvironment in activation of ICT2588 is also beneficial in terms of cancer therapy, as it effectively permits circumvention of downregulation of MMP-14 within the tumor mass as a mechanism of acquired drug resistance and any consequent reduction in therapeutic response.

An important indication for any developmental therapeutic is its clinical potential. The rapid metabolism of ICT2588 in homogenates of clinical specimens of NSCLC *ex vivo*, whilst remaining relatively stable in corresponding plasma samples and homogenates of human liver gives a strong indication for clinical translation of our therapeutic approach.

Treatment with ICT2588 is comparable to or an improvement on standard clinically applicable chemotherapeutics. The concentration of ICT2588 administered was previously shown to result in its maximal pharmacodynamics effect of vasculature shutdown, but to be significantly lower than its maximum tolerated dose (MTD; >150 mg/kg)[5]. Such a beneficial effect is a common attribute of colchicine-like VDAs, consistent with their effect upon the

tumor vasculature[5, 12, 13, 15]. One significant advantage of targeting a VDA, rather than an entity requiring direct interaction with tumor cells, is the proximity and accessibility of the cellular target. Whereas other smartbombs delivering tumor-cell focused agents must penetrate from the vasculature to instigate their effect, ICT2588 after being activated does not require such diffusion or transport for maximal activity as it acts upon the genetically stable tumor endothelium. Consequently, any activity against tumor cells is as an additional secondary or 'bystander' effect, rather than as a primary mode of action. A limitation to the use of VDAs is the persistence of a 'viable rim' of tumor cells at the periphery of the tumor mass, a factor resulting from these particular cells being supported by the normal established vasculature rather than the VDA-sensitive neovasculature[5, 11-13, 15]. Consequently, clinical VDA therapeutic regimens are rationalized to require co-treatment with agents to target the tumor cells in this rim[11, 13]. Our ability to effect tumor cures with ICT2588 and doxorubicin combination treatment is supportive of this[4e]. The superior response observed in several studies with co-treatment of a VDA and antiproliferative agent, as opposed to either agent alone, is likely a result of a VDA-induced temporal enhanced permeability and retention (EPR) effect within the tumor as a response to destabilization of the tumor endothelium[11, 14, 16], causing an enhancement of drug targeting and retention at the tumor site. Existence of this beneficial activity of ICT2588 is supported by the development of an MMP-14 activated theranostic agent, indicated by prolonged retention of the imaging nanoparticle within the tumor mass[33].

In the present study we evaluated ICT2588 against azademethylcolchicine and combretastatin-A4-phosphate (CA4P), two systemically active VDA's. ICT2588 was three-fold and seven-fold more active in terms of growth delay relative to azademethylcolchicine and CA4P respectively, indicating this tumor-activated molecule offers a benefit consistent with dose-exposure intensification previously reported for ICT2588 [5].

The reduced systemic exposure offered by ICT2588 through tumor-selective activation effectively reduces the likelihood of cardiovascular toxicities commonly associated with VDA therapy[13, 17, 18]. Such an assumption is based on previous studies wherein levels of azademethylcolchicine were below the level of detection in the circulation or cardiac tissue following ICT2588 administration[5]. However, a lack of detectable active agent does not account for effects upon endothelial and cardiovascular function. Increased plasma levels of von Willebrand factor (vWF) is a validated biomarker indicative of systemic vascular dysfunction and predictive of acute cardiovascular events[34-36]. Importantly the absence of raised vWF following ICT2588 administration to mice suggests that our targeted VDA is not overtly cardiotoxic, a problem commonly associated with VDA therapy[13, 17, 18]. Post ICT2588 treatment, levels of vWF were no greater than observed with the antiproliferative agent doxorubicin. Although doxorubicin is identified with clinical cardiotoxicity, this is a consequence of cumulative chronic drug exposure[43]. Acute exposure to doxorubicin is not associated with cardiovascular events, as previously reported in a preclinical study of breast cancer therapy[37]. In summary these results support our previous study which shows that tumor-selective activation of ICT2588 is producing an increased therapeutic index, not only due to dose enhancement at the tumor site, but also because of reduced systemic toxicity.

In conclusion, the activity of ICT2588 against colorectal, lung, breast and prostate tumors supports its widespread therapeutic potential and activation in clinical tumors associated with MMP-14 expression. This, in combination with lower and evidential reduction in potential for inducing cardiovascular events provides strong justification for progression of ICT2588 as a tumor-selective VDA.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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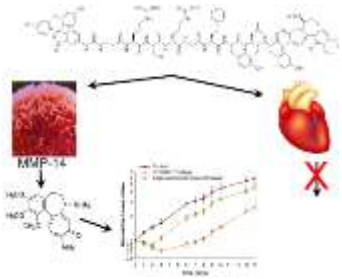
Graphical Abstract:



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