Colon Cancer–Specific Cytochrome P450 2W1 Converts Duocarmycin Analogues into Potent Tumor Cytotoxins

Sandra Travica, Klaus Pors, Paul M. Loadman, Steven D. Shnyder, Inger Johansson, Mohammed N. Alandas, Helen M. Sheldrake, Souren Mkrtchian, Laurence H. Patterson, and Magnus Ingelman-Sundberg

Abstract

Purpose: Cytochrome P450 2W1 (CYP2W1) is a monooxygenase detected in 30% of colon cancers, whereas its expression in nontransformed adult tissues is absent, rendering it a tumor-specific drug target for development of novel colon cancer chemotherapy. Previously, we have identified duocarmycin synthetic derivatives as CYP2W1 substrates. In this study, we investigated whether two of these compounds, ICT2705 and ICT2706, could be activated by CYP2W1 into potent antitumor agents.

Experimental Design: The cytotoxic activity of ICT2705 and ICT2706 in vitro was tested in colon cancer cell lines expressing CYP2W1, and in vivo studies with ICT2706 were conducted on severe combined immunodeficient mice bearing CYP2W1-positive colon cancer xenografts.

Results: Cells expressing CYP2W1 suffer rapid loss of viability following treatment with ICT2705 and ICT2706, whereas the CYP2W1-positive human colon cancer xenografts display arrested growth in the mice treated with ICT2706. The specific cytotoxic metabolite generated by CYP2W1 metabolism of ICT2706 was identified in vitro. The cytotoxic events were accompanied by an accumulation of phosphorylated H2A.X histone, indicating DNA damage as a mechanism for cancer cell toxicity. This cytotoxic effect is most likely propagated by a bystander killing mechanism shown in colon cancer cells. Pharmacokinetic analysis of ICT2706 in mice identified higher concentration of the compound in tumor than in plasma, indicating preferential accumulation of drug in the target tissue.

Conclusion: Our findings suggest a novel approach for treatment of colon cancer that uses a locoregional activation of systemically inactive produgs by the tumor-specific activator enzyme CYP2W1.

Clin Cancer Res; 19(11); 2952–61. ©2013 AACR.

Introduction

Colorectal cancer is the third most frequently diagnosed malignancy in both men and women and a leading cause of cancer-related deaths in the Western world. While surgery remains the primary curative modality for the localized disease, advanced colon cancer (stage II, III, and IV) requires the introduction of chemotherapeutics to prevent disease relapse in a significant proportion of patients (1). In a neoadjuvant setting, chemotherapy can add benefit by shrinking the cancer before surgery, using the still intact tumor vasculature to deliver a therapeutic dose not only at the primary tumor site but sites of tumor spread (2). Advances in neoadjuvant/adjuvant chemotherapy (including 5-fluorouracil, irinotecan, and oxaliplatin) as well as radiotherapy have improved the clinical outcome; however, overall impact of these amended treatment regimens has been relatively modest, leaving metastasized or recurrent disease largely incurable by conventional approaches and requiring the development of better therapeutics (3).

Targeted chemotherapy aimed towards more effective advanced colon cancer treatment could be achieved by the use of agents that are systemically inactive but selectively converted to potent cytotoxins locoregionally. Several commonly used chemotherapeutics, notably the produgs oxazaphosphorines, dacarbazine, and tegafur, are directed against tumor tissue but in fact are activated by hepatic cytochrome P450 (CYP) enzymes (4) introducing side effects associated with the systemic exposure to toxic metabolites. CYPs are a superfamily of hemoproteins that play an essential role in oxidative metabolism of a wide range of...
Translational Relevance

Progress in the chemotherapy of advanced colon cancer has been confined to improvements in established classes of cytotoxics, notably the platins, camptothecins, and pyrimidine antimetabolites. It is therefore an attractive option to identify chemotherapeutics with novel mechanisms of action and with lack of conventional resistance that could complement existing therapies without adding to overall toxicity. Here, we present an opportunity for novel chemotherapy that uses the cytochrome P450 2W1 (CYP2W1) with a unique colon cancer–specific expression. This enzyme is shown to efficiently convert inert duocarmycins into potent tumor cytotoxins, without the systemic side effects common to chemotherapeutics.

These results indicate for the first time the possibility for drug design against highly malignant colon cancers using CYP2W1 as a tumor resident target for locoregional activation of a class of ultrapotent chemotherapeutics.

endogenous and exogenous compounds including more than 70% of clinically used drugs. The polymorphic CYPs in families 1–3 have a primary role in xenobiotic metabolism and are capable of both activating and inactivating anticancer drugs (5). Cyclophosphamide (CPA) and ifosfamide (IFA) are oxidized by CYP isozymes CYP2B6 and CYP3A4 with subsequent release of highly reactive alkylating agents capable of inducing DNA strand breaks leading to cell death. This class of prodrugs has also been investigated in gene-directed enzyme prodrug therapy (GDEPT), a strategy that largely depends on the cancer cell–specific delivery and expression of a therapeutic gene (6). Although as a general concept, GDEPT promises to improve the therapeutic index by a dose enhancement effect at the tumor site (7), this technology has yet to progress into the clinic. More attractive targets for anticancer treatment are endogenous tumorspecific holoenzymes. Use of drug-metabolizing enzymes that are intrinsic to the tumor could mediate the local cytotoxicity generation obviating the need for complex delivery systems. The tumor expression of CYPs that are also commonly found in some normal tissues, notably CYP1A1 and CYP1B1, is reported and their potential for metabolizing inactive prodrugs into potent reactive species with anticancer properties is identified (4, 8, 9). Regardless of the approach used, the therapeutic index is achieved not only via the direct activation of the prodrug, but also by a strong bystander killing effect on adjacent nonactivating enzyme expressing cancer cells that have taken up the toxic metabolites.

One of the latest members of the CYP family, cytochrome P450 2W1 (CYP2W1), has emerged as a potential target for cancer drug activation. This orphan enzyme is endogenously expressed in fetal colon and then silenced by methylation after birth (10). Expression of CYP2W1 in adults is a tumor-specific event occurring in approximately 30% of colon cancers, in particular in the higher grade phenotypes, whereas the expression in healthy tissues remains absent or insignificant (10, 11). Clinical studies indicate that increased levels of CYP2W1 expression positively correlate with the degree of tumor malignancy, as well as with decreased 10-year survival in patients with colon cancer (12, 13). So far, several groups have reported on the metabolism of a few candidate substrates (i.e., benzopyrene, sterigmatocystin, aflatoxin B1, lysophospholipids, and fluorobenzothiazoles) for CYP2W1 expressed in E. coli or mammalian cells (14–19). However, the potential of CYP2W1 as a specific target in cancer chemotherapy remains hitherto unexplored.

Recently, we have reported a synthesis of a novel chloromethylindolino ICT2700 as the first example of a duocarmycin analogue whose cytotoxic activity is dependent on metabolic activation by CYP1A1 (20). The mechanism behind the cytotoxicity involves CYP-mediated oxidation of ICT2700 followed by spirocyclization and production of an N3-adene covalent adduct. Because the substrate recognition site of CYP2W1 shares the highest sequence identity with CYP1A1 (21), we hypothesized that certain chloromethylindolinos might also represent CYP2W1 substrates. Our initial screen has identified several derivatives of ICT2700 as first candidate CYP2W1 substrates (Sheldrake and colleagues, submitted, under review). In the present study, we have analyzed the interaction of 3 chloromethylindolines with CYP2W1 in a colon cancer cell milieu. We show that the chloromethylindoline ICT2726 is metabolized by CYP2W1 into innocuous metabolites, whereas ICT2705 and ICT2706 are specifically converted into cytotoxic products capable of inducing rapid tumor cell death. We show the enzyme-dependent cytotoxic effect of these prodrugs using CYP2W1-transfected cell lines and a human colon cancer xenograft murine model. This communication represents a first report on CYP2W1 targeting by novel chloromethylindolines both in vitro and in vivo and establishes a basis for a possible novel antitumor strategy in the treatment of colon cancer.

Materials and Methods

Chemicals

The chloromethylindoline analogues of duocarmycin used in this study were (1-(chloromethyl)-1,2-dihydropryrolo[3,2-e]indol-3(6H)-yl)(3-fluoro-1H-indol-2-yl)methanone (ICT2705), (5-chloro-1H-indol-2-yl)(1-(chloromethyl)-1,2-dihydropryrolo[3,2-e]indol-3(6H)-yl)methanone (ICT2706), and (8-(chloromethyl)-7,8-dihydro-6H-furo[3,2-e]indol-6-yl)(5-fluoro-1H-indol-2-yl)methanone (ICT2726; Supplementary Fig. S1), synthesized in-house and shown to be authentic and more than 97% pure using Nuclear magnetic resonance, mass spectrometry, and high-performance liquid chromatography (Sheldrake and colleagues, submitted, under review).

cDNA constructs

The cDNA of CYP2W1 was cloned into the HindIII and EcoRV sites of both Flp-In System expression vector.
pcDNA5/FRT (Invitrogen) generating the pcDNA5/FRT/2W1 construct, and pcDNA3.1 vector generating pcDNA5/FRT/2W1 construct.

Cell lines and transfections

The Flp-In system (Invitrogen) was used for the generation of the human colon cancer SW480-2W1 cell line stably expressing CYP2W1. SW480 Flp-In host cell line was kindly provided by Céline Gongora (IRCM INSERM, France; ref. 22). Cotransfection of pcDNA5/FRT/2W1 vector with Flp Recombinase expression plasmid (pOG44) yielded SW480-2W1 cell line, whereas the cotransfection of an empty pcDNA5/FRT vector with pOG44 generated SW480-mock cells. After transfections, zeocin (Invitrogen) was omitted from the medium and stable clones were selected for hygromycin B (Invitrogen) resistance (50 μg/mL). Colo320 human colon cancer cell line (American Type Culture Collection) was maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin 100 U/mL, and streptomycin 100 μg/mL (Invitrogen). Colo320-2W1 cells transiently expressing CYP2W1 were generated with pcDNA3.1-2W1 plasmid using Lipofectamine 2000 reagent (Invitrogen). Corresponding Colo320-mock cells were generated by transient transfection with empty pcDNA3.1 vector.

Cell viability assay

Cells were seeded on 48-well plates and incubated with chloromethylindolines for 60 hours in triplicate. Compounds were dissolved in dimethyl sulfoxide DMSO (Sigma) whose final concentration in the cell medium did not exceed 0.25%. Viability of the cells following incubation was determined using EZ4U assay, according to manufacturer’s guidelines (Biomedica).

Bystander effect

SW480-2W1 cells were incubated with ICT2706, DMSO (vehicle), and NP40 (negative viability control) for 30 hours. Medium from all incubations was collected, centrifuged for 10 minutes at 13,000 rpm, and then transferred to SW480-mock cells whose intact medium was previously aspirated. SW480-mock cells were incubated with the conditioned media for 50 hours before their viability was determined using the EZ4U assay.

Western blot analysis

Colo320 and SW480 cell lines transfected with CYP2W1 or mock transfected, were lysed at 4°C for 15 minutes in 1% Triton X-100 in PBS with addition of a protease inhibitors cocktail (Roche), or in radioimmunoprecipitation assay buffer with subsequent centrifugation at 13,000 × g. Snap-frozen xenograft tumor samples were homogenized using the Bullet Blender Homogenizer (Next Advance Inc.) in a buffer containing 100 mmol/L Tris–HCl and 1 mmol/L EDTA, pH 7.5, with addition of protease inhibitors cocktail. Cell lysates and tumor homogenates were centrifuged at 13,000 × g for 10 minutes. The aliquots of supernatants were subjected to SDS-PAGE and subsequent immunoblotting using CYP2W1, γH2AX, or ERp29 antibodies (Supplementary Methods).

CYP2W1 functional activity assay

SW480-2W1 and corresponding mock-transfected cells were incubated with ICT2726 (4 μmol/L) for 24 hours, and ICT2706 (2 μmol/L) for 6 hours. Following incubations, the cell media were collected while the cells were harvested and centrifuged at 3,000 rpm for 5 minutes. Cell pellets were resuspended with an equal volume of ice-cold acetoni-trile, vortexed, and centrifuged at 13,300 rpm for 20 minutes. The supernatant was collected, mixed with collected medium, and centrifuged again at 13,300 rpm for 20 minutes. Combined cell extract and cell medium was analyzed by liquid chromatography/mass spectrometry (LC/MS; Supplementary Methods).

Human colon cancer xenograft model

All in vivo studies were approved by the local animal ethical committee in Stockholm, Sweden, and animal care was carried out in accordance with Institutional guidelines. For treatment with ICT2706 or vehicle, 20 severe combined immunodeficient (SCID) female mice (MTC, Karolinska Institutet, Sweden) were divided into test and control groups. The test group was inoculated subcutaneously in both flanks with SW480-2W1 cells (7 × 105) and the control group with SW480-mock cells (7 × 105). In both test and the control group, 20 tumors developed and were allowed to grow to average volume of 60 mm3 before the onset of treatment. ICT2706 (100 mg/kg) was dissolved in DMSO and further diluted in corn oil. In the single injection volume of 200 μL, DMSO solvent accounted for 10%. Vehicle formulation comprised of corn oil and 10% DMSO. Half of the animals from both test and control groups received intraperitoneal (i.p.) injections of 100 mg/kg ICT2706 once daily for 8 days, whereas the remaining animals received intraperitoneal injections of vehicle following the same dosing schedule. The tumor volumes were measured by caliper (width2 × length × 0.52 = mm3), with the final measurement conducted on the day 9, 18 hours after the last treatment dose. Extracted tumors were weighed and divided into 2 equal parts with one being snap-frozen and the other preserved in paraformaldehyde.

ICT2706 pharmacokinetics

12 SCID female mice were inoculated subcutaneously with SW480-2W1 cells (7 × 105) in both flanks, and the xenografts were allowed to grow to an average volume of 70 mm3 before the animals received a single intraperitoneal dose of 100 mg/kg ICT2706. Following treatment, animals were sacrificed at consecutive time points (t = 0 minutes, 5 minutes, 30 minutes, 1 hour, 4 hours, and 24 hours). 2 animals per time point, and plasma, tumor, and liver tissue were removed, snap-frozen in liquid nitrogen, and analyzed for the presence of ICT2706 (Supplementary Methods).

Immunohistochemistry

Formalin-fixed xenograft tissue sections were rehydrated, incubated in 3% hydrogen peroxide, and blocked using
2.5% horse serum for 20 minutes (Vector Laboratories, Inc.), followed by incubation with CYP2W1 antibody (11) in a dilution of 1:1,250 at 4°C overnight. After rinsing, the sections were incubated with ImmPRESS reagent for 30 minutes (Vector Laboratories, Inc.), followed by 3,3-diaminobenzidine enzymatic development (Invitrogen). Sections were mounted and visualized by light microscopy.

**Statistical analyses**

The GraphPad Prism 5 software package was used for all statistical analyses. Results were compared using a Student’s t-test, one-way or a two-way ANOVA (Dunnett or Bonferroni). All data were expressed as means ± SEM. Differences were considered statistically significant at a *P* < 0.05.

**Results**

**CYP2W1 sensitizes colon cancer cells to cytotoxic prodrugs in vitro**

To identify substrates metabolized by CYP2W1 to potent chemotherapeutics, we have synthesized and screened a library of chloromethylindolines (Sheldrake and colleagues, submitted, under review). In the present study, we investigate ICT2705, ICT2706, and ICT2726 as substrates for CYP2W1 to examine their potential as colon cancer-selective cytotoxins. For this purpose, we established 2 colon cancer cell lines, SW480-2W1 with a stable expression of CYP2W1, and Colo320-2W1 with transient CYP2W1 expression. Presence of CYP2W1 protein in both cell lines was confirmed by immunodetection (Fig. 1A and B, Figure 1. CYP2W1 mediates cytotoxicity of ICT2705 and ICT2706. Human colon cancer cell lines SW480 (A) and Colo320 (B), expressing wild-type CYP2W1 and corresponding mock-transfected cells, were seeded on 48-well plates and treated with ICT2705 (2705), ICT2706 (2706), and ICT2726 (2726). Cell viability was analyzed using EZ4U assay after 60-hour incubation. NP40-treated cells were used as a negative control for viability. Values represent the means of at least 3 independent experiments ± SEM, each conducted in triplicate. Statistically significant differences in viability between enzyme-transfected and mock-transfected cells are depicted by asterisks (*, *P* < 0.05; ***, *P* < 0.001). Inserts, immunodetection of CYP2W1 protein following transfection. C, chloromethylindoline-induced DNA damage. SW480-2W1 and SW480-mock cells were treated with 4 μmol/L ICT2705, ICT2706, ICT2726, and doxorubicin (DOX; positive control) for the indicated time periods. Cell lysates containing 80 μg of total protein were subjected to 15% SDS-PAGE with subsequent immunodetection of γH2AX.

www.aacrjournals.org Clin Cancer Res; 19(11) June 1, 2013 2955
inserts). Incubation of ICT2705 and ICT2706 in either of the cell line resulted in a significant dose-dependent loss of viability, whereas the treatment with ICT2726 had no detrimental effect. The dependence on CYP2W1 expression for the observed cytotoxicity was confirmed by the absence of effect on mock-transfected cells (Fig. 1A and B).

Treatment with ICT2705 and ICT2706 induces DNA damage in CYP2W1-expressing SW480 cells

To show whether DNA damage is associated with the cellular effects of the 3 chloromethylindolines, we analyzed the appearance of phosphorylated H2AX histone (γH2AX), a positive indicator of DNA damage (23). SW480-2W1 and SW480-mock cells were treated with ICT2705, ICT2706, ICT2726, and doxorubicin (positive control), for 0, 8, 24, and 48 hours. Using immunodetection of γH2AX, we show a time-dependent increase in DNA damage following treatment with ICT2705 and ICT2706 in SW480-2W1 cells, but not in SW480-mock cells. Significant accumulation of γH2AX was not detectable after treatment with ICT2726 consistent with its lack of cytotoxicity (Fig. 1C).

Metabolism of ICT2706 and ICT2726 by CYP2W1

LC/MS analysis of the extracts of SW480-2W1 cells incubated with ICT2706 reveals a specific metabolic product (MW 363.2, m/z 364.2), consistent with a hydroxylated derivative of ICT2706 (Fig. 2A). As the cytotoxicity of the chloromethylindolines is known to be mediated by a regiospecific hydroxyl group (20), the cytotoxicity observed for ICT2706-treated SW480-2W1 cells is consistent with formation of a cytotoxic metabolite generated in the mono-oxygenase reaction typical for CYP enzymes.

As a part of the present investigation, we also characterized a metabolite of ICT2726 in SW480-2W1 cells. The metabolite formed (m/z 351) was consistent with the conversion of the chloromethyl moiety to the nontoxic hydroxymethyl derivative (Fig. 2B). Such a metabolite is rationalized not to undergo spirocyclization to the cyclopropane moiety, a process previously shown to be necessary for triggering the formation of the chloromethylindoline cytotoxic metabolite (20). No metabolites of ICT2706 and ICT2726 were detected in the mock-transfected cells (Fig. 2A and B) confirming the requirement for CYP2W1 in the cytotoxicity of these agents.

Figure 2. CYP2W1 catalyzed metabolism of chloromethylindolines. A, ICT2706 (2 μmol/L) was incubated with SW480-2W1 cells for 6 hours. Formation of the specific metabolites in the cell extracts was analyzed by LC/MS (Supplementary Methods). ICT2706 (m/z 384.2) was detected at RT 29.1 minutes. The active metabolite (m/z 364.2) was detected only in the SW480-2W1 incubation at RT 20.6 minutes. Insert, structure of the ICT2706 and its active metabolite. B, ICT2726 metabolite formation in SW480-2W1 cells was monitored as above. ICT2726 eluted at 22.6 minutes (m/z 368.9). The CYP2W1-specific metabolite (m/z 364.2) had an RT 14.9 minutes. Insert, structures of the ICT2726 and its metabolite. Some unidentified metabolites were present in both SW480-2W1 and SW480-mock cells.
ICT2706 inhibits SW480-2W1 tumor growth in vivo

Next, we examined the effect of the chloromethylindolines on growth of human SW480-2W1 colon cancer xenografts established as solid tumors in SCID mice. ICT2706 was selected as a lead compound because in SW480 cells it possessed the highest potency (Fig. 1A). Consecutive daily doses of ICT2706 (100 mg/kg i.p.) produced a persistent inhibition of tumor growth throughout the 8-day dosing period (Fig. 3A). SW480-mock xenografts, subjected to the same treatment regimen, showed no growth retardation in comparison with vehicle-treated tumors (Fig. 3B). The ICT2706 multiple dosing treatment used in this study was well tolerated in all animals as judged by appearance, behavior, and body weight changes of less than 10% (results not shown). After termination of the in vivo experiment on day 9, tumors were excised and weighed. CYP2W1-expressing tumors treated with ICT2706 showed marked depletion of CYP2W1-positive cells, as compared with the vehicle-treated SW480-2W1 tumors (Fig. 4A).

Growth inhibition in SW480-2W1 xenografts is associated with ICT2706-induced DNA damage

Using immunodetection of γH2A.X, we examined the levels of treatment-induced DNA damage in tumor samples resected from mice. The data show that ICT2706 treatment of SW480-2W1 tumors results in a strong γH2A.X signal. In comparison, vehicle-treated SW480-2W1 tumors produced little or no effect on γH2A.X formation, and no difference was found between the levels of γH2A.X in SW480-mock tumors subjected to either ICT2706 or vehicle treatment (Fig. 4B). Overall, a 50% increase in γH2A.X was measured in ICT2706-treated versus vehicle-treated SW480-2W1 tumors using quantitative densitometric analysis (data not shown).

Tissue distribution of ICT2706

To show tumor uptake of ICT2706 and determine the fate of this agent in vivo, we compared the distribution of ICT2706 in SW480-2W1 tumors, plasma, and liver. The distribution of ICT2706 in these tissues was monitored for up to 24 hours following its administration as a single intraperitoneal dose of 100 mg/kg. A summary of the...
pharmacokinetic parameters for ICT2706 is given in Supplementary Table S1, and the data are shown graphically in Fig. 5. The absorption from the peritoneum was delayed with a $T_{\text{max}}$ of 4 hours in plasma and tissue, however, ICT2706 was well distributed throughout the tissues. Tumor concentrations peaked at 7.7 $\mu$g/mL, which is 4-fold higher than the concentrations detected in plasma (1.78 $\mu$g/mL), though peak concentrations were considerably higher in liver (324 $\mu$g/g). All AUCs are approximated because of the limited number of sampled points. The results show also that the ICT2706 concentration–time curves followed a similar clearance profile. A direct comparison of AUCs calculated from this data showed that the AUC for ICT2706 was higher in tumor compared with plasma, whereas the highest AUC associated with the liver showed hepatic clearance to be an important route of elimination.
cells were incubated with 1 and 4 mock cells were seeded on 24-well plates without hygromycin. SW480—whether bystander cytotoxicity can occur, i.e., whether residual tumor tissue (Fig. 4B). We therefore investigated the active metabolite of ICT2706 is likely to transfer from the supernatants of centrifuged media from the differentially treated SW480-2W1 cells. The viability of the SW480-mock cells incubated with the conditioned media was determined using EZ4U assay after 50 hours of incubation. Values represent the means of 3 independent experiments ± SEM. Statistically significant differences in viability between differentially treated SW480-mock cells are depicted by asterisks (*, P < 0.05; ***, P < 0.001).

**Bystander effect of ICT2706 in vitro**

The formation of a cytotoxic metabolite of ICT2706 in a tumor cell is predicted to kill the cell in which it is produced. However, even though the majority of CYP2W1-positive cells have been eliminated from SW480-2W1 xenografts during ICT2706 treatment (Fig. 4A), significant DNA damage is still observed in the residual tumor tissue (Fig. 4B). We therefore investigated whether bystander cytotoxicity can occur, i.e., whether the active metabolite of ICT2706 is likely to transfer from one tumor cell to others in close proximity. SW480-mock cells incubated with the medium from ICT2706-treated SW480-2W1 cells show significant loss of viability, whereas no cytotoxicity occurs in the SW480-mock cells incubated with medium from the cells treated with vehicle (Fig. 6).

**Discussion**

The conventional treatment of a variety of tumor types with CYP-activated agents (e.g., CPA, IFA, and nitrosoareas) is associated with substantial host toxicity due to the metabolism of the prodrug by several CYPs and the presence of these enzymes in the liver and other tissues (24). Ongoing efforts to improve the therapeutic index for drugs like CPA are based on viral CYP2B6 gene delivery to tumor tissue, pioneered by Waxman and colleagues (25–27). This GDEPT approach does significantly sensitize the cancer cells to administered prodrug, however, the toxicity problem remains. In contrast to such issues, confinement of CYP2W1 to cancer cells circumvents the need for gene delivery as well as the unwanted effects resulting from hepatic prodrug activation.

In the present study, we evaluate the potential of CYP2W1 targeting in a preclinical setting. Novel chloromethylindolylone prodrug design has yielded ICT2705 and ICT2706 (Supplementary Fig. S1) as substrates for the CYP2W1 enzyme that can be metabolically activated into potent cytotoxic agents. The hydroxylation of these compounds most likely triggers their alkylation of DNA, as chloromethylindolylones have previously been shown to mediate this event (20). We show that upon treatment with ICT2705 and ICT2706, cancer cells expressing CYP2W1 enzyme suffer substantial DNA damage with concomitant loss of viability.

In mice, xenograft growth is inhibited from the onset of ICT2706 dosing by continuous eradication of CYP2W1-expressing cells (Fig. 3A). The requirement of CYP2W1 enzyme was absolute in this study, as an identical treatment of mock-transfected xenografts produced no anti-tumor effect (Fig. 3B). After the cessation of ICT2706 dosing, CYP2W1-positive cells were undetectable in extracted SW480-2W1 tumors (Fig. 4A). This tumor tissue most probably represents a remnant of the cell population that has initially circumvented the stable expression of CYP2W1 and subsequently proliferated in the absence of antibiotic pressure. Nonetheless, significant levels of ICT2706-induced DNA damage can be detected in this residual tumor tissue through immunodetection of γH2AX (Fig. 4B). The comprehensive antitumor effect and the accumulation of DNA damage observed despite the destruction of CYP2W1-expressing cells, speak in favor of potential bystander cytotoxicity. For instance, the cytosine deaminase/5-fluorocytosine suicide-prodrug system triggers colon cancer xenograft regression in vivo with only 4% of successfully transfected cells (28). Our in vitro analyses suggest that a similar bystander mechanism might augment CYP2W1 targeting since SW480-mock cells suffer significant loss of viability after incubation with SW480-2W1-conditioned medium (Fig. 6). As in the case of CPA, whose CYP-generated 4-hydroxy metabolite alkylates the DNA of surrounding cells (27), the antitumor effect of ICT2706 is likely to be facilitated by the dissemination of the toxic metabolite, affecting even tumor tissue scarcely populated with CYP2W1-expressing cells.

In vivo, ICT2706 is distributed to the xenograft tissue at a concentration that is sufficient to produce a sustained antitumor effect. Analysis of ICT2706 from dosed mice identified higher concentration of the compound in tumor than in plasma, indicating preferential accumulation of drug in the target tissue. Furthermore, ICT2706 is cleared within 24 hours, inferring that on each day of treatment the tumor was exposed to an equal concentration of the prodrug. Predominantly higher concentration of ICT2706 in liver is consistent with hepatic elimination of highly lipophilic compounds (Fig. 5).

**Figure 6. In vitro analysis of bystander effect.** SW480-2W1 and SW480-mock cells were seeded on 24-well plates without hygromycin. SW480-2W1 cells were incubated with 1 and 4 μM ICT2706, DMSO (solvent; final concentration 0.25%), and NP40 (negative viability control; final concentration 1%), for 30 hours. Following the incubation, SW480-mock cell culture medium was replaced by the supernatants of centrifuged media from the differentially treated SW480-2W1 cells. The viability of the SW480-mock cells incubated with the conditioned media was determined using EZ4U assay after 50 hours of incubation. Values represent the means of 3 independent experiments ± SEM. Statistically significant differences in viability between differentially treated SW480-mock cells are depicted by asterisks (*, P < 0.05; ***, P < 0.001).
CYP2W1 enzyme is shown to be expressed at high levels, albeit in 30% of malignant colorectal cancers (11). Therefore, there is a need to identify the patients who would benefit from CYP2W1-targeted therapy. In this regard, ICT2726 is a novel chloromethylindoline that has potential as a biomarker for functional CYP2W1. We show that CYP2W1 oxidizes ICT2726 to an identified nontoxic metabolite in colon cancer cells (Fig. 2B). Unlike the chloromethylindolines that are converted to DNA-binding cytotoxins, ICT2726 is attractive for assessing the activity of CYP2W1 without the complication of induction of toxicity in the test system and with ease of extraction for quantitative detection.

The active site homology between CYP2W1 and CYP1A1 enzymes (21) implies a potential overlap in their substrate specificities. Expression of CYP1A1 is however confined to tumor tissues and generally dependent on xenobiotic induction, allowing for targeting by anticancer prodrugs such as Phortress (8). In the occurrence of in vivo targeting of CYP2W1 by ICT2706, no signs of unwanted host toxicity were apparent indicating that the activation of this agent by systemic CYPs is not contributing to the cytotoxicity observed.

The importance of CYP2W1 as a promising drug target is supported by in vitro studies of the metabolism of 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (GW-610, NSC721648) to a reactive intermediate shown to have a cytotoxic effect in selected breast and colon cancer cell lines (15, 16). However, the relevance of this to its CYP2W1 mediated antitumor activity remains to be verified by in vivo studies.

In summary, our study describes the first example of CYP2W1 targeting, using newly generated chloromethylindoline prodrugs, both in vitro and in vivo. We identify ICT2706 as a potential therapy, which could be trialed in an adjuvant setting of colon cancer to further prevent tumor growth or metastasis or in a neoadjuvant setting to assist tumor shrinkage or ablation before surgery.

cancer-confined CYP2W1 expression in combination with ICT2706 prodrug may present a novel candidate system for selective colon tumor treatment, minimizing the risk of systemic toxicity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Travica, K. Pors, P.M. Loadman, M.N. Alandas, L.H. Patterson, M. Ingelman-Sundberg
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Travica, K. Pors, P.M. Loadman, S. Mkrtchian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Travica, K. Pors, P.M. Loadman, M.N. Alandas, L.H. Patterson, M. Ingelman-Sundberg
Writing, review, and/or revision of the manuscript: S. Travica, K. Pors, P.M. Loadman, S.D. Shnyder, H.M. Sheldrake, M. Ingelman-Sundberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Travica, P.M. Loadman
Study supervision: P.M. Loadman, I. Johansson, S. Mkrtchian, M. Ingelman-Sundberg

Acknowledgments
The authors thank Dr. Mikhail Burmakin, MBB, Karolinska Institutet, for valuable advice regarding animal experiments; Tristan Reuillon, Institute of Cancer Therapeutics, for a valuable contribution towards ICT2706 synthesis; and Professor Hans Wigzell, Karolinska Institutet, for continuous interest.

Grant Support
This study was supported by The Swedish Cancer Foundation and The Swedish Research Council (to M. Ingelman-Sundberg, S. Mkrtchian, I. Johansson, and S. Travica); program grant and project grant (B207) from Yorkshire Cancer Research, United Kingdom (to I.H. Patterson, K. Pors, S.D. Shnyder, P.M. Loadman); and Research Councils-UK Academic Fellowship (to H.M. Sheldrake).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 30, 2013; revised March 11, 2013; accepted March 18, 2013; published OnlineFirst April 15, 2013.

References
Colon Cancer–Specific Cytochrome P450 2W1 Converts Duocarmycin Analogues into Potent Tumor Cytotoxins

Sandra Travica, Klaus Pors, Paul M. Loadman, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0238

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/04/17/1078-0432.CCR-13-0238.DC1

Cited articles
This article cites 28 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/11/2952.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/19/11/2952.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.