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Antitumor Activity of a Duocarmycin Analogue Rationalized to Be Metabolically Activated by Cytochrome P450 1A1 in Human Transitional Cell Carcinoma of the Bladder

Mark Sutherland¹, Jason H. Gill^{1,2}, Paul M. Loadman¹, Jonathan P. Laye¹, Helen M. Sheldrake¹, Nicola A. Illingworth¹, Mohammed N. Alandas¹, Patricia A. Cooper¹, Mark Searcey³, Klaus Pors¹, Steve D. Shnyder¹, and Laurence H. Patterson¹

Abstract

We identify cytochrome P450 1A1 (CYP1A1) as a target for tumor-selective drug development in bladder cancer and describe the characterization of ICT2700, designed to be metabolized from a prodrug to a potent cytotoxin selectively by CYP1A1. Elevated CYP1A1 expression was shown in human bladder cancer relative to normal human tissues. RT112 bladder cancer cells, endogenously expressing CYP1A1, were selectively chemosensitive to ICT2700, whereas EJ138 bladder cells that do not express CYP1A1 were significantly less responsive. Introduction of CYP1A1 into EJ138 cells resulted in 75-fold increased chemosensitivity to ICT2700 relative to wild-type EJ138. Negligible chemosensitivity was observed in ICT2700 in EJ138 cells expressing CYP1A2 or with exposure of EJ138 cells to CYP1B1- or CYP3A4-generated metabolites of ICT2700. Chemosensitivity to ICT2700 was also negated in EJ138-CYP1A1 cells by the CYP1 inhibitor α -naphthoflavone. Furthermore, ICT2700 did not induce expression of the AhR-regulated CYP1 family, indicating that constitutive CYP1A1 expression is sufficient for activation of ICT2700. Consistent with the selective activity by CYP1A1 was a time and concentration-dependent increase in γ -H2AX protein expression, indicative of DNA damage, associated with the activation of ICT2700 in RT112 but not EJ138 cells. In mice-bearing CYP1A1-positive and negative isogenic tumors, ICT2700 administration resulted in an antitumor response only in the CYP1A1-expressing tumor model. This antitumor response was associated with detection of the CYP1A1-activated metabolite in tumors but not in the liver. Our findings support the further development of ICT2700 as a tumor-selective treatment for human bladder cancers. *Mol Cancer Ther*; 12(1); 27–37. ©2012 AACR.

Introduction

Bladder cancer is a common malignancy with an estimated incidence of 356,600 new diagnoses annually (1). In the United States and United Kingdom, it ranks as the third and fourth most frequent form of cancer in men, respectively (1, 2). Transitional cell carcinoma (TCC)

accounts for approximately 90% of all bladder cancer cases and is classified as either superficial or muscle invasive. Muscle-invasive disease is particularly difficult to treat and is associated with a poor prognosis, being lethal in approximately 50% of patients. In contrast, superficial TCC is usually managed well with intravesical therapy, with a much better prognosis. However, approximately 70% of superficial tumors will recur, with about 20% of these presenting as aggressive invasive disease (1, 2). Therefore, there is a clear need for better treatment for TCC, especially high-grade or muscle-invasive disease.

The cytochrome P450 enzymes (CYP) are a family of constitutive and inducible oxidases with central roles in the phase I metabolism of xenobiotics (3). As such, the CYPs have been extensively studied with regards to their functions as mediators of the toxicology and pharmacokinetics of many compounds to which humans are exposed. The contribution of CYP1 family (CYP-1A1, 1A2, and 1B1) mediated oxidation of xenobiotics including polycyclic aromatic hydrocarbons and aromatic amines to mutagenic and carcinogenic metabolites is well established (4). This is pertinent to bladder cancer in which there is a strong epidemiologic association between development of this neoplasia and exposure to aromatic

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amines either from cigarette smoke or occupational exposure (5). This is supported by a study of human exfoliated urothelial cells, which has shown that CYP1A1 protein was expressed in a significantly greater proportion of cells from tobacco smokers than nonsmokers (6). Furthermore, a statistically significant association between genetic polymorphism of *CYP1A1* and development of bladder cancer has also been shown (7, 8).

It is now well accepted that, in addition to metabolism of carcinogens, CYPs can activate and deactivate chemotherapeutic agents and as such influence the response of tumors to anticancer drug therapy (9, 10). In addition, elevated expression of several CYP family members, notably CYP1A1, 1B1, 2S1, and 2W1, has been shown in tumors relative to normal human tissues (10–15), and therefore, these CYPs can be viewed as molecular targets for the development of new tumor-selective therapeutic strategies (10, 16). In this regard, a potential approach for bladder cancer is to use the oxidation functionality of CYP1A1 to restore potent toxicity to an inactivated chemotherapeutic selectively within the tumor, thereby facilitating reduced systemic toxicity and a consequent improved therapeutic index.

In our laboratory, we have previously identified the duocarmycin family of natural products (17) to possess a pharmacophore suitable for modification to create CYP-activated chemotherapeutic prodrugs (18). We have synthesized a truncated chloromethylpyrroloindoline (ICT2700), which we have shown to undergo CYP1A1-mediated oxidation, resulting in production of a potent and highly specific DNA minor groove alkylating agent (19). In this study, we identify CYP1A1 expression in human bladder cancer and address the potential for CYP1A1-activated therapy based on the ultrapotent duocarmycins by showing, for the first time, CYP1A1 selective efficacy of ICT2700.

Materials and Methods

Cell culture

The human bladder carcinoma cell lines, RT112 and EJ138, were obtained from the European Collection of Cell Cultures (Salisbury, UK) and were authenticated morphologically. Cell lines were grown as monolayers in RPMI 1640 supplemented with 10% (v/v) FBS, 1 mmol/L sodium pyruvate, and 2 mmol/L of L-glutamine at 37°C in 5% CO₂. All cell lines were used at low passage in our laboratory and were tested regularly to confirm the absence of *Mycoplasma* infection.

Overexpression of CYP1A1 in EJ138 bladder cell line

Full length CYP1A1 mRNA was amplified by PCR from CHO-CYP1A1 cells (a kind gift from the late Dr. T Friedburg, University of Dundee, Nethergate, Dundee, Scotland, UK) and full length CYP1A2 was amplified from RT112 cells, cloned into the mammalian expression vector pcDNA6-V5-His (Invitrogen), transfected into EJ138 cells using ExGen500 (Fermentas), and stable clones selected using blasticidin (Invitrogen). The transfected cell lines,

termed EJ138-CYP1A1 and EJ138-CYP1A2, were regularly tested by reverse transcription PCR (RT-PCR) to confirm expression of CYP1A1 and 1A2 and by monitoring of 7-ethoxyresorufin O-de-ethylation (EROD; ref. 20) to determine levels of enzymatically active CYP1 proteins.

Human bladder tissue samples

A total of 39 freshly resected and snap-frozen specimens of human bladder TCC and 18 histologically normal human bladder tissue specimens (excised distant to the tumor mass) were analyzed for genetic expression of CYP1 family members in this study. In addition, a tissue microarray (TMA) containing 25 formalin-fixed, paraffin-embedded specimens (in triplicate) of human bladder TCC, representing superficial (11 pTa) and invasive (9 pT1; 5 ≥ pT2) stages of TCC were analyzed for CYP1A1 protein expression. Specimens showing the presence of carcinoma *in situ* were excluded from both genetic and protein analyses. Informed consent was obtained from all patients before specimen collection and all patient details were anonymized to ensure confidentiality. All experiments were conducted 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.

Quantification of CYP expression by real-time RT-PCR

Gene expression analysis of *CYP1A1*, *CYP1A2*, and *CYP1B1* in cell lines and clinical tissues was conducted by either semiquantitative or real-time reverse transcriptase PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR), respectively. Details of the methodology and primers are shown in the Supplementary Methods. Expression of the CYP1 isoforms in clinical tissue determined by qRT-PCR was calculated relative to β-actin using the previously validated comparative C_T method (21).

Expression of CYPs in normal tissues (*n* = 20) was evaluated using the Human Total RNA Master Panel II (Clontech) by qRT-PCR. Expression was determined relative to β-actin using the comparative C_T method (21), and classified on the basis of the 2^{-ΔCT} value: undetected 0 to 10⁻⁴; low expression 10⁻⁴ to 10⁻³; moderate expression 10⁻³ to 0.01; high expression 0.01 to 0.1; and very high expression greater than 0.1.

Immunohistochemistry

CYP1A1 protein expression was assessed by immunohistochemistry in a TMA of human bladder TCC, as previously described (22). The specific protocol for CYP1A1 expression is shown in Supplementary Methods.

Chemical compound

(1-(Chloromethyl)-1,2-dihydropyrrolo[3,2-*e*]indol-3(6*H*)-yl)(5-methoxy-1*H*-indol-2-yl)methanone (ICT2700);

chloromethylpyrroloindoline; Fig. 2) was synthesized in-house and shown to be authentic and greater than 97% pure (19). For cellular analyses, the compound was prepared as a 10 mmol/L stock solution in dimethyl sulfoxide (DMSO).

Cellular chemosensitivity to ICT2700

In vitro chemosensitivity of RT112, EJ138, EJ138-1A1, and EJ138-1A2 cells to ICT2700 was determined using the MTT assay, described elsewhere (23). Cells (2,000 per well) were allowed to attach to the 96-well plate for 24 hours then exposed to ICT2700 (1 nmol/L–10 μ mol/L) or its solvent (DMSO). Solvent concentrations did not exceed 0.1% and were not cytotoxic. Cell survival was determined following 96 hours exposure to ICT2700. Survival curves were obtained and used to calculate the IC₅₀ values.

Assessment of CYP induction following exposure to ICT2700

The potential induction of *CYP1A1*, *CYP1A2*, or *CYP1B1* by ICT2700 was evaluated *in vitro*. RT112 cells (5×10^5 cells per flask) were exposed to ICT2700 (1 μ mol/L) or the solvent alone (DMSO). Following 24 hours exposure, RNA was extracted and the expression of the CYPs characterized by qRT-PCR, as detailed above.

Role of CYPs in chemosensitivity of ICT2700

Involvement of specific CYP isoforms in the activation of ICT2700 was determined by evaluating the chemosensitivity of CYP-generated metabolites of ICT2700. Metabolites were created via incubation of ICT2700 (50 μ mol/L) in the reaction mixture: [2 mmol/L NADPH, 1 mmol/L MgCl₂, 50 mmol/L Tris-HCl (pH 7.4), 20 pmol of either CYP1A1, 1A2, 1B1, or 3A4 batosomes (Cypex)]. Control reactions were carried out using CYP-null batosomes. Following 60 minutes incubation at 37°C, metabolites were extracted using acetonitrile and centrifugation at 10,000 *g* for 10 minutes. The resultant supernatant was removed, dried using vacuum evaporation (Genevac), and the resultant pellet resuspended in DMSO. The chemosensitivity of metabolites generated were assessed by the MTT assay following 96 hours exposure to EJ138 cells (CYP1A1 null), as described above.

To confirm an involvement of CYP1 activity in increased chemosensitivity to ICT2700, cells were exposed to ICT2700 in the presence of the CYP1 selective inhibitor, α -naphthoflavone (α -NF, 0.001–1,000 nmol/L). Following 24-hour exposure to ICT2700 and α -NF, drug was removed and fresh media was added to the cells. Cell survival was determined 96 hours posttreatment, as described above.

γ -H2AX induction as a measure of ICT2700-induced DNA damage

RT112 cells were treated with ICT2700 for 24 hours (0.01, 0.1, and 1 μ mol/L) and 4 hours (1 μ mol/L) or with solvent alone (DMSO). Expression of γ -H2AX was deter-

mined by Western blot analysis. Detailed methodology is shown in Supplementary Methods.

Involvement of CYP1 family in ICT2700-mediated induction of γ -H2AX

To confirm an involvement of CYP1 activity in ICT2700-mediated DNA strand breaks, RT112, EJ138, and EJ138-1A1 cells were exposed to ICT2700 in the presence of the CYP1 selective inhibitor, α -NF (1 μ mol/L) for 24 hours. Induction of γ -H2AX was measured as a marker of DNA strand breaks by immunoblotting, as outlined above.

Metabolism of ICT2700 in mouse and human liver *ex vivo*

Microsomal preparations, fortified with NADPH were prepared from mouse and human liver as described previously (24) and incubated with ICT2700. Reaction aliquots were removed over a 120-minute period, proteins precipitated, and metabolites analyzed by liquid chromatography-mass spectrometry (LC-MS). Detailed methodology is shown in the Supplementary Methods. ICT2700 was quantified using absorbance measurement at 330 nm. Metabolic intermediates were detected as singularly charged ions and identified by mass spectrometry; ICT2700 = *m/z* 379.8, hydroxylated metabolite (ICT2740) = *m/z* 395.8. The loss of chlorine from metabolites was monitored by spectral analysis of chlorine isotopes.

Antitumor activity

Female BALB/cOlaHsd-Foxn immunodeficient nude mice implanted subcutaneously with 2 to 3 mm³ fragments of either wild-type or CYP1A1-expressing Chinese hamster ovary cell xenograft tumors (CHO-WT and CHO-1A1), and once tumor volumes reached approximately 32 mm³ mice were randomized into groups and received ICT2700 at its maximum soluble dose of 150 mg kg⁻¹ or solvent control (10% DMSO/arachis oil) via intraperitoneal (i.p.) administration. Additional groups of CHO-1A1 tumor-bearing mice received ICT2740, at its maximum-tolerated dose of 3 mg kg⁻¹. ICT2740 is the authentic metabolite of ICT2700. Tumor volume (measured by calipers) and animal body weight were recorded daily for up to 14 days, and normalized to the respective volume on day 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.

All procedures were carried out under a United Kingdom Home Office Project License, following UKCCCR guidelines (25).

Tumor and liver metabolism of ICT2700 *in vivo*

Mice-bearing CHO-WT or CHO-1A1 tumors were injected i.p. with either ICT2700 (150 mg kg⁻¹) or solvent (10% DMSO/arachis oil). Tumor and liver tissue were

collected 2 hours posttreatment. Samples were immediately frozen in liquid nitrogen before homogenization in ice-cold methanol. Following centrifugation, concentrations of ICT2700, its active-hydroxylated metabolite (ICT2740) and any further metabolites were determined in the resultant supernatants by LC-MS, as described above. Calibration samples were established using drug-free saline and tissues.

Results

The CYP1 family is differentially expressed in human bladder cancer and CYP1A1 is a potential target for chemosensitization of ICT2700

Initial studies were carried out to determine the relative genetic expression levels of the aryl hydrocarbon receptor (AhR)-regulated CYPs 1A1, 1A2, and 1B1 in clinical bladder samples, normal tissues, and the bladder cell lines RT112 and EJ138. In the clinical bladder samples (18 normal and 39 tumors) examined, CYP1A1 was found to be upregulated ($P < 0.05$; Fig. 1A). No difference in the levels of CYP1A2 ($P > 0.1$) and 1B1 ($P > 0.1$) expression was observed (Fig. 1B and C). In a panel of 20 normal tissues, CYP1B1 was negative in the majority of tissues with low levels of mRNA detectable in heart, prostate, skeletal muscle, testis, and trachea (Fig. 1D). CYP1A2 mRNA was found at high levels only in the liver (Fig. 1D). Although CYP1A1 mRNA was expressed at moderate levels in heart, liver and trachea, these levels were considerably lower than the very high expression detected in bladder cancer. A high level of CYP1A1 was observed in skeletal muscle, although this expression level was still considerably lower than normal bladder and bladder cancer (Fig. 1D).

To address whether elevated CYP1A1 mRNA is reflective of CYP1A1 protein, immunohistochemical analyses of human bladder TCC TMAs were conducted, an approach previously shown with TCC to be highly representative of whole histologic sections (22, 26). Expression of CYP1A1 was detected in all pathologic stages of TCC, with a correlation between increasing tumor stage and CYP1A1 expression (Supplementary Table S1.1). Furthermore, a significant difference in expression was observed between superficial nonmuscle invasive (pTa, pT1) and muscle invasive (\geq pT2) TCC (Supplementary Table S1.2; $P < 0.005$), CYP1A1 expression being higher in muscle invasive tumors.

Together these data indicate that clinical bladder cancer could be specifically chemosensitized to ICT2700, a synthetic derivative of the ultrapotent duocarmycins that we have shown to be metabolically activated by CYP1A1 (19).

To identify a preclinical model of bladder cancer for investigation of ICT2700, we determined the expression levels of the CYPs in the human bladder cancer cell lines, EJ138 and RT112, by real-time PCR. The EJ138 cell line expressed very low levels of CYP1A1, CYP1B1, and CYP1A2 (Fig. 1D), and was negative (below level of detection) or had very low levels of other extrahepatic

CYPs (data not shown); providing a model cell line for controlled expression of CYP1 family members. In contrast to EJ138, the RT112 and EJ138-CYP1A1 cell lines had very high levels of CYP1A1 (Fig. 1D and E). In both cases, the genetic sequence of CYP1A1 showed 100% homology to the published sequence for this gene, thus excluding the potential role of single nucleotide polymorphisms influencing the activity of the enzyme. Activity of CYP1A1, as determined by the EROD assay, was undetectable in microsomal incubates of the EJ138 cell line but was clearly measured in EJ138-CYP1A1 cells (Supplementary Fig. S1). CYP1-mediated 7-ethoxoresorufin metabolism was also detectable but lower in the RT112 cell line that may reflect the 30-fold differential in CYP1A1 mRNA levels between RT112 and EJ138-CYP1A1 (data not shown).

Differential activation of ICT2700 in human bladder cancer cell lines *in vitro*

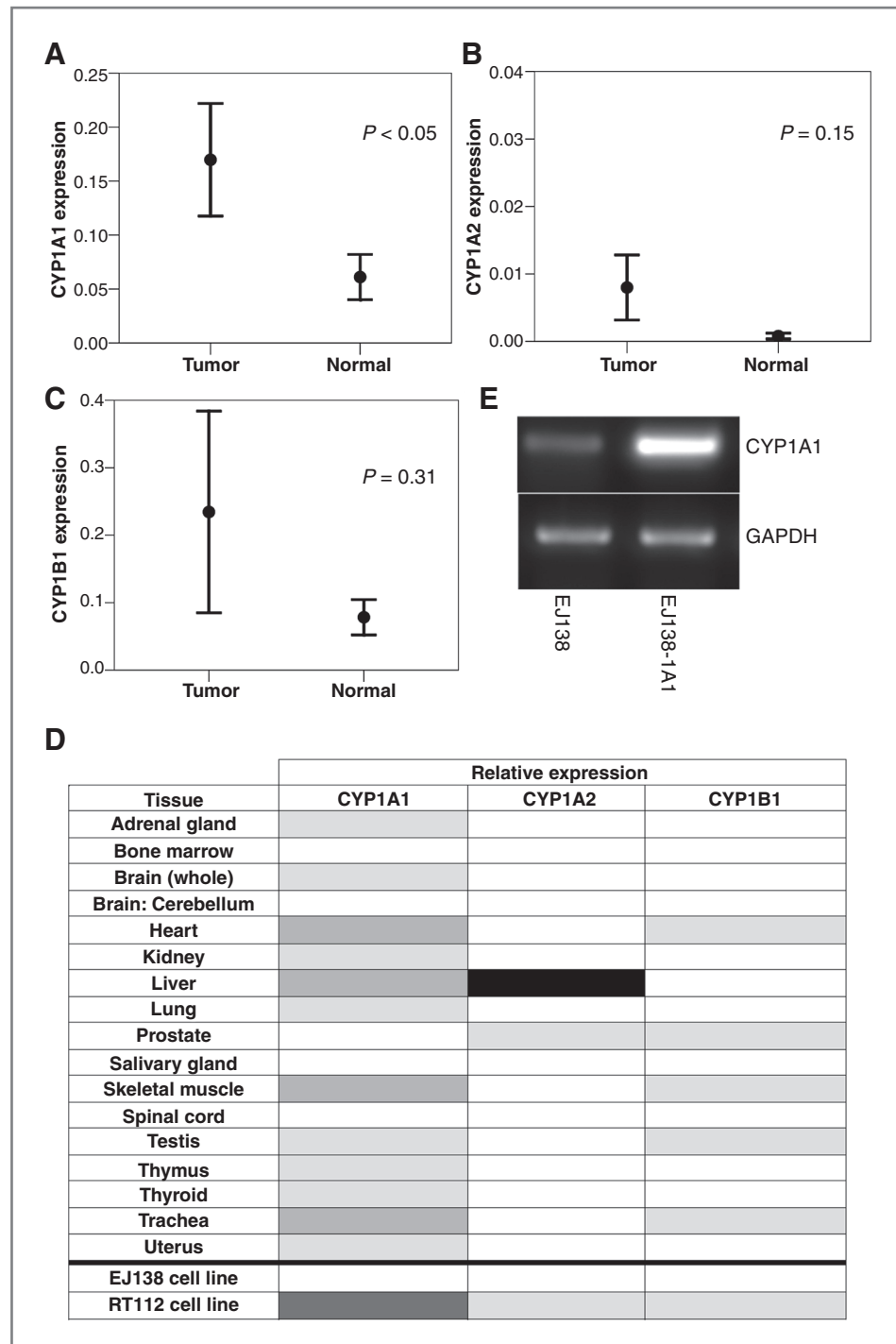
The mechanism of action of the naturally occurring duocarmycins is well documented and involves spirocyclization of the aryl-ring-fused subunit of the chloromethylpyrroloindoline moiety to trigger production of an N³-adenine covalent adduct upon binding to the minor groove of DNA (reviewed by ref. 17). We showed previously that CYP1A1 oxidation restores the hydroxyl group critical to the spirocyclization mechanism necessary for DNA alkylation (Fig. 2; ref. 19).

RT112 and EJ138 cells were used to assess whether the expression of CYP1A1 in bladder cancer cells influenced the activation and cytotoxicity of ICT2700. RT112 (CYP1A1 positive) were at least 20-fold more chemosensitive to ICT2700 than EJ138 cells (undetectable CYP1) *in vitro* (Table 1). This differential cytotoxicity was reflected pharmacodynamically by the induction of DNA damage, detected by the appearance of phosphorylated(Ser¹³⁹) H2AX (γ -H2AX; Fig. 3A). ICT2700 induced γ -H2AX protein levels in the RT112 cells, detectable at 4 hours post-treatment with a time-dependent (Fig. 3A) and dose-dependent (Fig. 3B) increase. In contrast, no increase in γ -H2AX expression was observed in the EJ138 cell line (data not shown).

Involvement of CYP1A1 in selective activation of ICT2700

To confirm a CYP1 selective role for CYP1A1 in the cytotoxic activity of ICT2700, cells overexpressing CYP1A1 and CYP1A2 were treated with ICT2700. The EJ138-1A1 cell line (Fig. 2E) was 75-fold more sensitive to ICT2700 than mock-transfected EJ138 cells (Table 1). In comparison, EJ138 cells engineered to express CYP1A2 did not show any significant difference in chemosensitivity relative to mock-transfected cells (Table 1). The results support the possibility of tumor CYP1A1-selective activation of ICT2700 in human bladder cancer to a cytotoxin with nanomolar activity while avoiding activation by CYP1A2, which is often constitutively expressed in normal tissue, notably the liver.

Figure 1. *CYP1A1*, *CYP1A2*, and *CYP1B1* are differentially expressed in clinical bladder samples, normal human tissues, and bladder cancer cell lines. A–C, constitutive expression of the AhR regulated *CYP1A1*, *CYP1A2*, and *CYP1B1*, respectively, in clinical bladder tumors and corresponding normal bladder tissue measured by real-time RT-PCR (qRT-PCR). D, expression of *CYP1A1*, *CYP1A2*, and *CYP1B1* in a panel of 20 different normal tissues and the bladder cell lines RT112 and EJ138 measured by qRT-PCR. E, elevated expression of *CYP1A1* in EJ138-CYP1A1 relative to mock-transfected EJ138 cells. □, Undetected; ◻, Low; ◼, Moderate; ◽, High; ◼◼, Very High.



The effect of CYP1B1 and CYP3A4 (the major hepatic xenobiotic metabolizing CYP) on ICT2700 chemosensitivity was then compared with CYP1A1, using CYP-expressing batosomes. Metabolites of ICT2700, produced following incubation with CYP null, 1A1, 1B1, or 3A4 batosomes, were extracted and exposed to EJ138 cells (Table 1). The metabolites produced by CYP1A1 batosomes were cytotoxic at subnanomolar concentrations, whereas no

significant increase in cytotoxicity was observed with metabolites produced by CYP1B1 and CYP3A4 batosomes compared with CYP null (control) batosomes (Table 1).

The involvement of CYP1A1 in the therapeutic mechanism of ICT2700 was further assessed by determining cytotoxicity of ICT2700 in the presence of the CYP1 inhibitor α -NF. Incubation of cells with ICT2700 in the

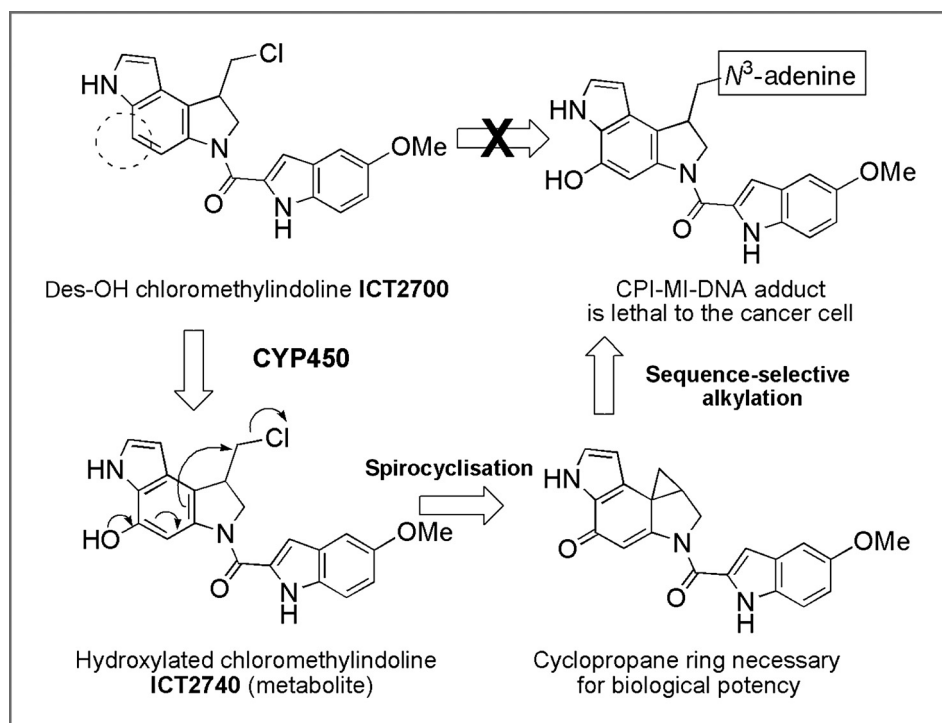


Figure 2. Structure and proposed mechanism of metabolism of ICT2700 to the active hydroxylated form. The authentic metabolite ICT2740 is identified in the scheme.

presence of α -NF resulted in a decrease in ICT2700 cytotoxicity in both RT112 and EJ138-CYP1A1 cell lines (Fig. 4A). In the presence of α -NF, a significant increase in cell survival was observed in the EJ138-1A1 cells exposed to a cytotoxic concentration of ICT2700 (Fig. 4A). α -NF alone did not induce cytotoxicity at any inhibitor concentrations evaluated (data not shown). A reduction in ICT2700-induced γ -H2AX protein

expression in RT112 cells was also evident in the presence of α -NF (Fig. 4B) consistent with inhibition of CYP1-mediated cytotoxicity by ICT2700.

Lack of induction of CYP1 enzyme expression by ICT2700

The possible contribution of AhR activation and consequent CYP1 induction by ICT2700 was investigated using qRT-PCR following treatment of RT112 cells with ICT2700. Following 24-hour drug exposure, the expression levels of all 3 CYPs were similar to those of the untreated controls (Supplementary Table S2). A previous study has shown that 12 to 24 hours is the optimal time for dose-dependent CYP1A1 induction in HepG2 cells by 3-methylcholanthrene (27). This indicates that ICT2700, at a therapeutically relevant concentration, does not induce expression of the AhR-regulated CYP1 family or adversely affect the CYP1A1 levels required for its oxidative conversion to a potent cytotoxin.

Antitumor activity of ICT2700

Mice-bearing CHO-WT or CHO-1A1 tumors were treated with the CYP1A1-activated agent, ICT2700 (150 mg kg⁻¹), with the CHO-1A1 tumors also being treated with its authentic cytotoxic metabolite, ICT2740 (3 mg kg⁻¹). Tumor growth following treatment was compared with vehicle-treated controls. ICT2700 resulted in a significant antitumor response in mice-bearing CYP1A1-expressing tumors ($P < 0.01$; Fig. 5A) but not in mice-bearing wild-type (CYP1 negative) tumors (Fig. 5B). Furthermore, no significant loss in mouse body weight was observed following administration of ICT2700 (<3% weight

Table 1. CYP1A1 selective chemosensitivity of ICT2700 against human bladder cell lines *in vitro*

Cell line	CYP bactosome	IC ₅₀ (μ mol/L)
RT112	—	0.18 \pm 0.11
EJ138	—	3.77 \pm 1.90
EJ138-CYP1A1	—	0.05 \pm 0.08
EJ138-CYP1A2	—	4.82 \pm 0.08
EJ138	Control	1.36 \pm 0.48
EJ138	1A1	<0.0005
EJ138	1B1	1.15 \pm 0.64
EJ138	3A4	1.00 \pm 0.21

NOTE: Bladder cancer cell lines were exposed to ICT2700 for 96 hours (upper table) or ICT2700 was incubated with bacto-somes expressing specific CYP isoforms and the resultant metabolites exposed to EJ138 cells for 96 hours (lower table). Values are reported as IC₅₀ and represent the mean of at least 3 independent experiments \pm SD.

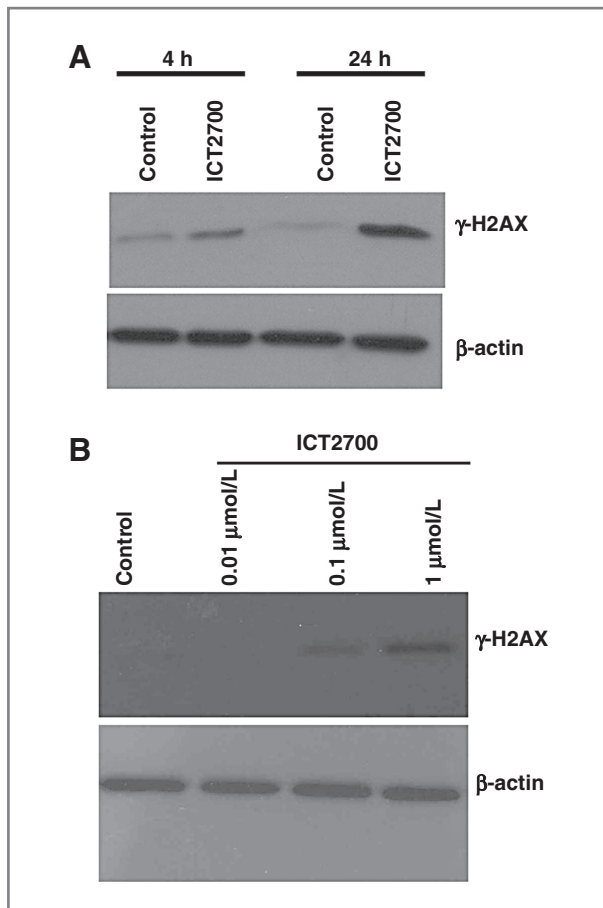


Figure 3. ICT2700 induces DNA strand breaks in CYP1A1-expressing bladder cells. **A**, RT112 cells were treated for either 4 or 24 hours with 1 $\mu\text{mol/L}$ ICT2700. The induction of DNA strand breaks was determined via the detection of γ -H2AX protein. **B**, the induction of γ -H2AX protein by ICT2700 occurs in a dose-dependent manner. Cells were treated with 0.01, 0.1, and 1 $\mu\text{mol/L}$ ICT2700 for 24 hours. In all cases, β -actin was detected and used as the loading control. Blots are representative of 3 separate experiments.

loss; Fig. 5C). In contrast, the authentic metabolite ICT2740 produced a significant antitumor response ($P < 0.01$; Fig. 5A), but this was associated with greater toxicity as evidenced by a significant loss in mouse body weight over the course of the study (15% maximum weight loss; Fig. 5C)

Formation of active metabolite (ICT2740) in tumor but not liver *in vivo*

In order for ICT2700 to be a viable therapeutic agent, it must show activation in tumor tissue and limited activation in nondiseased tissue. The fate of ICT2700 in tumor and liver was measured at 2 hours following its administration. Both tissues showed ICT2700 to predominate with a number of metabolites evident (Supplementary Fig. S2). The authentic-activated metabolite, ICT2740, was observed in tumor but was not detectable in liver (Fig. 5D). The absence of measurable ICT2740 in mouse liver following ICT2700 administration led us to evaluate metab-

olism of ICT2700 in human liver microsomes *ex vivo*. A number of ICT2700 metabolites were detected in human liver microsomes, but none corresponding to ICT2740 were detected (Supplementary Fig. S3).

Discussion

Therapy of bladder cancer is dependent upon whether the disease is superficial disease, whereby treatment is intravesical chemotherapy, or invasive disease, whereby treatment involves cystectomy and systemic chemotherapy. Despite therapy for superficial disease being largely successful, approximately 70% of these tumors will recur and of these about 20% will have progressed to aggressive disease. In the case of muscle-invasive disease, this is lethal in approximately 50% of patients and the success of systemic chemotherapy is limited by significant toxicity. Therefore, development of dose intensification therapeutic strategies by improved tumor-selectivity and efficacy with a concordant reduction in systemic toxicity are of the upmost importance for the treatment of bladder cancer. Toward this aim, we have identified CYP1A1 as a potential therapeutic target for tumor-selective prodrug activation in bladder cancer. Furthermore, we have shown that CYP1A1 expressing human bladder cancer cells are potentially sensitive to the chloromethylpyrroloindoline ICT2700. The importance of this study lies in the potential to use CYP-mediated activation of ICT2700, an agent based on the ultrapotent cytotoxic duocarmycins, to address their intrinsic toxicity with the promise of overcoming the lack of therapeutic index observed in clinical studies.

Although all members of the *CYP1* family were detectable at the mRNA level in clinical human bladder tissue, only *CYP1A1* has shown statistically significant elevated mRNA levels in bladder cancer relative to nonmalignant tissue. This observation was supported by the association between increasing CYP1A1 protein expression and tumor invasiveness. Previous studies have shown higher CYP1A1 protein in bladder urothelium from smokers, an epidemiologic association between bladder cancer and exposure to CYP1-metabolized aromatic amine and polycyclic aromatic hydrocarbons carcinogens, and a significant association between *CYP1A1* polymorphisms and bladder cancer development (6–8). Analysis of *CYP1A1* mRNA expression in a panel of normal tissues revealed low to moderate levels in several tissues, with higher levels detectable in skeletal muscle. The significantly greater levels of *CYP1A1* in bladder cancer relative to normal bladder together with an association between tumor invasion and CYP1A1 protein expression, and the strong association between CYP1A1 and bladder tumorigenesis provides a compelling case for exploring CYP1A1 as a potential-activating enzyme for prodrugs to treat bladder cancer.

Several approaches for targeting chemotherapy directly to malignant tissue have emerged, including antigen targeting, antibody- or gene-directed enzyme prodrug therapy, and tumor enzyme-activated chemotherapy

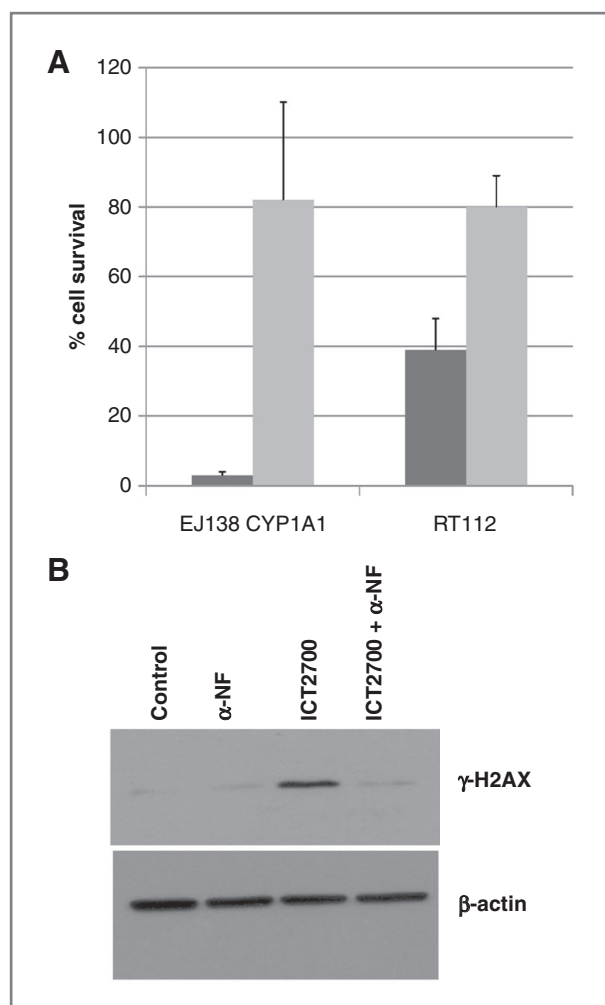


Figure 4. ICT2700 cytotoxicity is dependent upon CYP1 activity. **A**, RT112 and EJ138-CYP1A1 cells were exposed to ICT2700 for 96 hours in the presence or absence of the CYP1 inhibitor α -NF (1 μ mol/L). Cell viability was determined using the MTT assay. Dark gray bars depict cell survival following treatment with ICT2700, whereas the light gray bars denote cells survival in the presence of the CYP1 inhibitor α -NF. Values represent the mean of 3 independent experiments \pm SD. **B**, the presence of γ -H2AX protein, indicative of DNA damage, induced by ICT2700 was determined in the presence or absence of the CYP1 selective inhibitor α -NF (1 μ mol/L).

(28, 29). The concept of exploiting enzymes selectively in the tumor microenvironment to convert an inactive drug into its active chemotherapeutic metabolite has shown significant promise, exemplified by capecitabine (Xeloda; ref. 29), EO9 (Apaziquone, currently in Phase III clinical trials; ref. 30), and AQ4N (Banoxantrone; ref. 31), the latter 2 of which show promise against bladder cancer (30, 31). In this study, we evaluated ICT2700, a chloromethylpyrroloindoline, which is rationalized to undergo CYP1A1-mediated oxidation resulting in production of an ultrapotent DNA alkylating cytotoxin. ICT2700 is a synthetic modification of the *seco*-duocarmycins as an approach to address the unmet clinical potential of this family of agents, which have

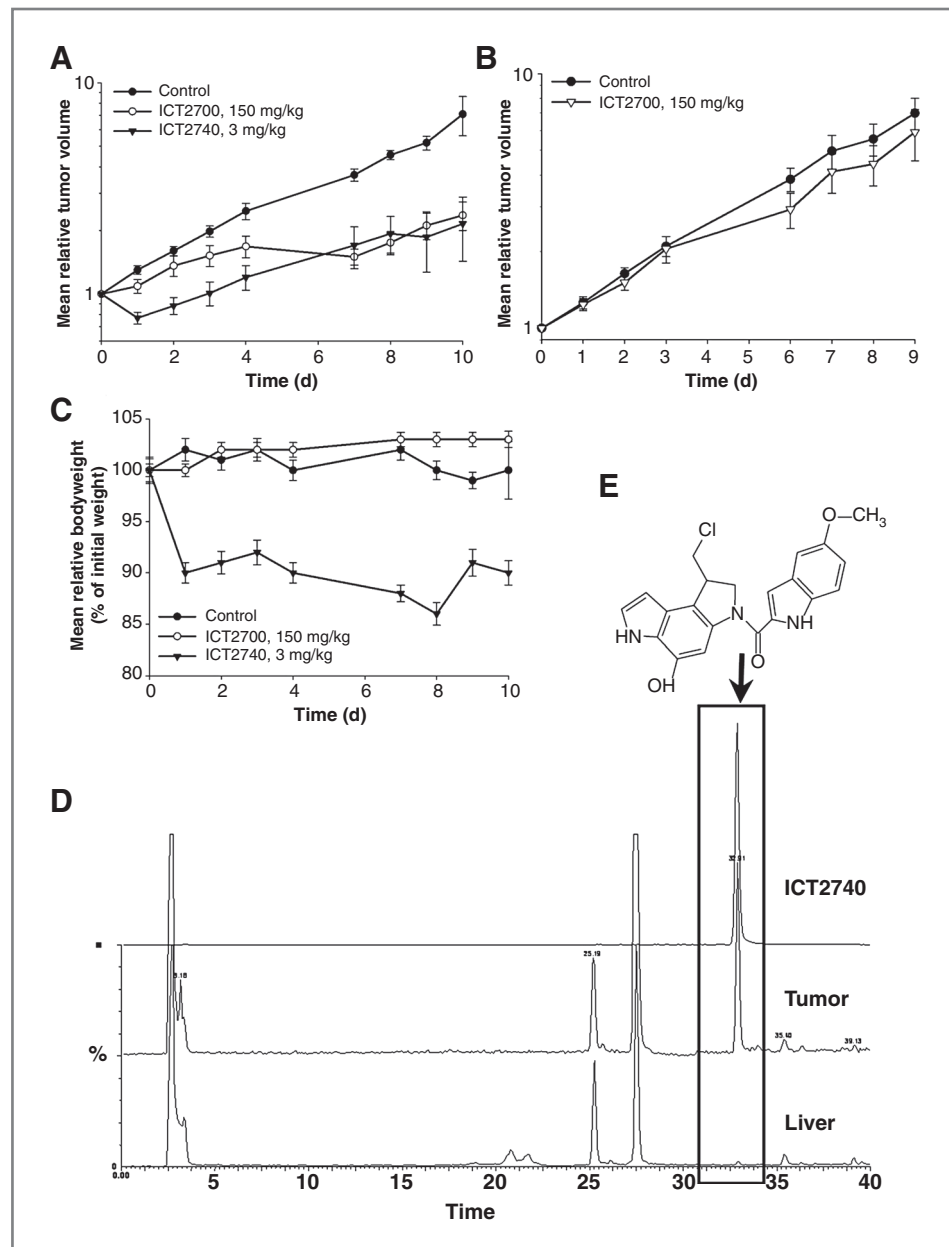
failed to show an acceptable therapeutic index in clinical trials (17).

We showed a significant increase in *in vitro* cytotoxicity of ICT2700 in RT112 (CYP1A1 positive) but not in EJ138 (undetectable CYP1A1) human bladder tumor cell lines. Dependence of this activity upon the CYP1 family was shown by the inhibition of ICT2700 cytotoxicity in the presence of α -NF, a CYP1 family inhibitor. The specific involvement of CYP1A1, as opposed to the other CYP1 family enzymes, in the activation of ICT2700 was shown by cytotoxicity in EJ138 cells genetically engineered to express CYP1A1 but not EJ138 cells expressing CYP1A2 or EJ138 cells exposed to CYP1B1- or CYP3A4-mediated metabolites. Furthermore, DNA damage following exposure to ICT2700 was observed in RT112 and EJ138-1A1 but not wild-type EJ138 cells *in vitro*, as shown by the appearance of γ -H2AX that is a biomarker of DNA damage. The expression of γ -H2AX was inhibited by α -NF further supporting the involvement of CYP1A1 in cytotoxicity associated with ICT2700-mediated DNA damage.

Involvement of CYP1A1 in cancer prodrug activation has been shown for aminoflavone AFP464 (32) and the benzothiazole Phortress (33) with binding to the AhR and a resultant induction of CYP1A1 expression being a critical step in their mechanism of action (32, 34). CYP1A1-mediated DNA damage in tumors is identified as important to the antitumor activity of Phortress but also increases normal tissue toxicity, notably in the lung (35). Induction of CYP1A1 is also implicated in the mechanism by which ellipticine-derived DNA adducts are generated in tumor tissue but also healthy organs (36, 37). In contrast, we have shown a biologic response by ICT2700 in RT112 bladder cancer cells despite an absence of induction of CYP1 family members. This indicates that constitutive expression of CYP1A1 in bladder cancer cells is sufficient to activate ICT2700. This may also facilitate a therapeutic advantage as the results indicate that ICT2700 may not induce CYP1 family members and associated toxicity in normal tissue.

The demonstration of *in vitro* proof of principle for CYP1A1-selective oxidative activation and consequent cytotoxicity of the chloromethylpyrroloindoline ICT2700, led us to investigate whether this prodrug strategy was a viable approach *in vivo*. ICT2700 was shown to be sufficiently systemically stable *in vivo* and was identified as the major product detected in both tumors expressing CYP1A1 and the liver following administration. Efficacy against CYP1A1-expressing tumors, but not CYP1A1-null tumors provides compelling evidence for the importance of CYP1A1 in formation of a cytotoxic metabolite *in vivo*. The antitumor efficacy of the authentic metabolite ICT2740 has been shown previously to involve DNA-alkylation (19). Importantly, although both ICT2700 and its authentic metabolite ICT2740 have shown potent antitumor activity *in vivo*, only ICT2740 administration was associated with body weight loss, an indicator of normal tissue toxicity. This clearly indicates the benefit of a CYP1A1-targeted approach to improving the

Figure 5. ICT2700 is activated selectively in CYP1A1 expressing tumors and inhibits tumor growth. Mice-bearing CYP1A1-expressing (A) or CYP1A1-null (B) tumors were treated with a single 150 mg/kg dose (i.p.) of ICT2700. Mice-bearing CYP1A1-expressing tumors were also treated with its authentic metabolite ICT2740 (3 mg/kg, i.p.; A) and tumor size determined daily. C, lack of significant body weight loss, indicative of systemic toxicity, in CYP1A1 tumor-bearing mice following administration (i.p.) of ICT2700 (150 mg/kg) in contrast to body weight loss induced by administration of the authentic metabolite, ICT2740 (3 mg/kg). Values are presented as mean percentage of pretreatment mouse weight. D, detection by LC-MS of ICT2700 authentic metabolite, ICT2740 (E), only in CYP1A1-expressing tumor tissue, 2 hours after i.p. administration of ICT2700. Confirmation of the metabolite identified in tumor at 32.9 minutes being the C5 hydroxy-activated metabolite (ICT2740) was shown by coelution with the authentic standard (top trace) when extracted using specific SIR channels at m/z 395.8 and by production of an identical uv/vis spectrum (see Supplementary Fig. S1).



therapeutic index of agents based on the duocarmycins. The absence of a metabolite corresponding to ICT2740 amongst the several metabolites detected in liver following administration of ICT2700, or in solutions of ICT2700 incubated with mouse and human liver microsomes *ex vivo* further supports the tumor selectivity and subsequent therapeutic potential of this CYP-selective prodrug approach.

In bladder cancer, as with many other tumor types, there is considerable interest in the development of personalized medicine; the aim being to prescribe treatment based on the precise molecular abnormalities and molecular pathology of the individual patient and their malignancy (38). Patient selection for ICT2700 therapy would be dependent, in part, on identification of tumor CYP1A1

expression and hence addresses the goal of personalized medicine. Noninvasive detection of CYP1A1 in exfoliated urothelial cells shown previously to differentiate smokers from nonsmokers (6) would be a possible means to identify ICT2700 responders.

Although ICT2700 does not induce *CYP1A1* in studies to date, patient selection would necessitate consideration of other factors, such as smoking and diet, which may influence CYP expression in nontumor tissues. However, Phortress and aminoflavone (NSC686288, AFP464) are 2 cancer drugs currently in clinical development with a mechanism of action associated with their ability to act as powerful inducers of *CYP1A1*. Despite this, they have progressed to clinical trial based on suitable patient exclusion criteria, including exclusion of smokers.

In conclusion, the therapeutic efficacy, mechanism of action, tumor-selective metabolism to a potent cytotoxin, and potential for circumventing systemic toxicity associated with systemically active potent cytotoxins strongly support targeting tumor-expressed CYP1A1 and development of ICT2700 or analogues as a potential therapeutic strategy for the treatment of bladder cancer in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Sutherland, J.H. Gill, P.M. Loadman, J.P. Laye, M.N. Alandas, K. Pors, S.D. Shnyder
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Other: synthesis of ICT2700: K. Pors

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