**Showcasing research from the Institute of Cancer Therapeutics, University of Bradford**

Modification of the Duocarmycin Pharmacophore enables CYP1A1 Targeting for Biological Activity

CYP-mediated activation of agents based on the anticancer duocarmycins to address their intrinsic toxicity. The mechanism is exemplified by CYP1A1 mediated oxidative spirocyclisation of a chloromethylindoline pharmacophore resulting in DNA-alkylation and cell death. The DNA damage signalling cascade can be depicted by confocal laser scanning microscope green imaging of immunostained γH2AX, a marker for foci of DNA double strand breaks against a red background of total DNA staining using DRAQ5™.

As featured in:
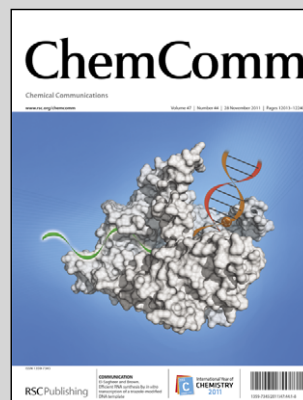
See Laurence H. Patterson *et al.*,
Chem. Commun., 2011, **47**, 12062.

RSCPublishing

www.rsc.org/chemcomm

COMMUNICATION

Modification of the duocarmycin pharmacophore enables CYP1A1 targeting for biological activity†

Klaus Pors,[a] Paul M. Loadman,[a] Steven D. Shnyder,[a] Mark Sutherland,[a] Helen M. Sheldrake,[a] Meritxell Guino,[b] Konstantinos Kiakos,[c] John A. Hartley,[c] Mark Searcey[b] and Laurence H. Patterson*[a]

The identification of an agent that is selectively activated by a cytochrome P450 (CYP) has the potential for tissue specific dose intensification as a means of significantly improving its therapeutic value. Towards this goal, we disclose evidence for the pathway of activation of a duocarmycin analogue, ICT2700, which targets CYP1A1 for biological activity.

The cytochromes P450 (CYPs) are a superfamily of mixed function oxidases of which CYP1-4 subfamily members are unique in their ability to oxidise xenobiotics including drugs. There is now growing evidence that CYP1A1, 1B1, 2J2, 2S1 and 2W1 are over-expressed in many human tumour types.[1] The presence of certain CYPs may reflect a resistance mechanism by diminishing the pharmacological activity of anticancer drugs whilst specific CYPs can also modulate cell proliferation by the formation or conversion of endogenous signalling molecules.[2] The potential for CYP-selective metabolism of xenobiotics coupled to their broad substrate specificity provides a unique opportunity to design drugs whose activity is dependent on a critical functional group that can be unmasked or restored by CYP oxidation selectively in tumour tissue. Several clinically used cancer chemotherapeutics, notably the DNA alkylating oxazaphosphorines and nitrosoureas are known to be metabolised by CYPs to reactive intermediates primarily in the liver.[3]

The phenol-containing *seco*-compounds of the natural products CC-1065 and the duocarmycins are recognized as ultrapotent cytotoxins.[4] Their mechanism of action involves spirocyclisation of the deep-embedded chloromethylindoline fragment to trigger production of an N^3-adenine covalent adduct upon binding of the minor groove of DNA. Nature's evolution of the duocarmycins may have been derived from ancestral precursors, which did not encompass the phenolic hydroxyl group in their structural architectures.[5] We hypothesise that de-hydroxylated (de-OH) chloromethylindolines possess a pharmacophore capable of undergoing oxidation by CYPs to generate the ultrapotent cytotoxin. Essentially, site specific oxidation restores the hydroxyl group as one route to the spirocyclisation mechanism necessary for DNA alkylation concomitant with potent cell killing (Fig. 1a).

We have synthesised a chloromethylindoline (ICT2700) and investigated the potential for this agent as a candidate for the development of ultrapotent cytotoxins that are inactive until CYP-activated in tumour tissue. This communication is the first demonstration of a pharmacophore based on the duocarmycin scaffold whose biological activity is dependent on metabolic activation by CYP1A1.

The target compound ICT2700 was synthesised using de-OH starting materials and procedures similar to those previously described for the synthesis of *seco*-CPI-MI (ICT2740).[6] The route to preparation of ICT2700 is outlined in Supporting Information (Scheme S1†). In brief, the synthesis involved the common key steps: reaction of Boc-protected nitrogen with 1,3-dichloropropene afforded a vinylchloride, which upon treatment with Bu$_3$SnH (or TTMSS) and AIBN provided the chloromethylpyrroloindoline subunit *via* 5-*exo-trig* radical cyclisation. Removal of the Boc-protection under acidic conditions was followed by EDC-mediated coupling to 5-methoxy-indole-2-carboxylic acid to provide the target compound ICT2700.

Given the high level of genomic instability and mutations seen in tumours, resistance mechanisms are likely to be complex and multifactorial, allowing the cancer cell many escape routes to survival.[7] Accordingly, a duocarmycin prodrug must not only demonstrate conversion by CYP metabolism from an inactive form to an ultrapotent compound but once activated also evade drug resistant mechanisms associated with natural product efflux and DNA repair from alkylating agents. To explore this we investigated chloromethylpyrroloindolines ICT2700 and ICT2740 in both wild-type and resistant A2780 ovarian cancer cell lines. The results demonstrate that ICT2740, the *seco*-chloromethylpyrroloindolone, was cytotoxic to A2780 cells (IC$_{50}$ < 1 nM) whereas ICT2700 was at least 1000-fold less cytotoxic (Fig. 1c). Furthermore, it was demonstrated that

[a] *Institute of Cancer Therapeutics, University of Bradford, West Yorkshire BD7 1DP, UK.*
E-mail: L.H.Patterson@Bradford.ac.uk
[b] *Cancer Research UK Drug-DNA Interactions Research Group, UCL Cancer Institute, London, WC1E 6BT, UK*
[c] *School of Pharmacy, University of East Anglia, Norwich, Norfolk NR4 7TJ, UK*

† Electronic supplementary information (ESI) available: Synthetic methods and characterization data of ICT2700. Experimental procedures and figures MRM analysis of CYP1A1, CYP1A1 bactosome metabolism, thermal cleavage and EROD assays. See DOI: 10.1039/c1cc15638a
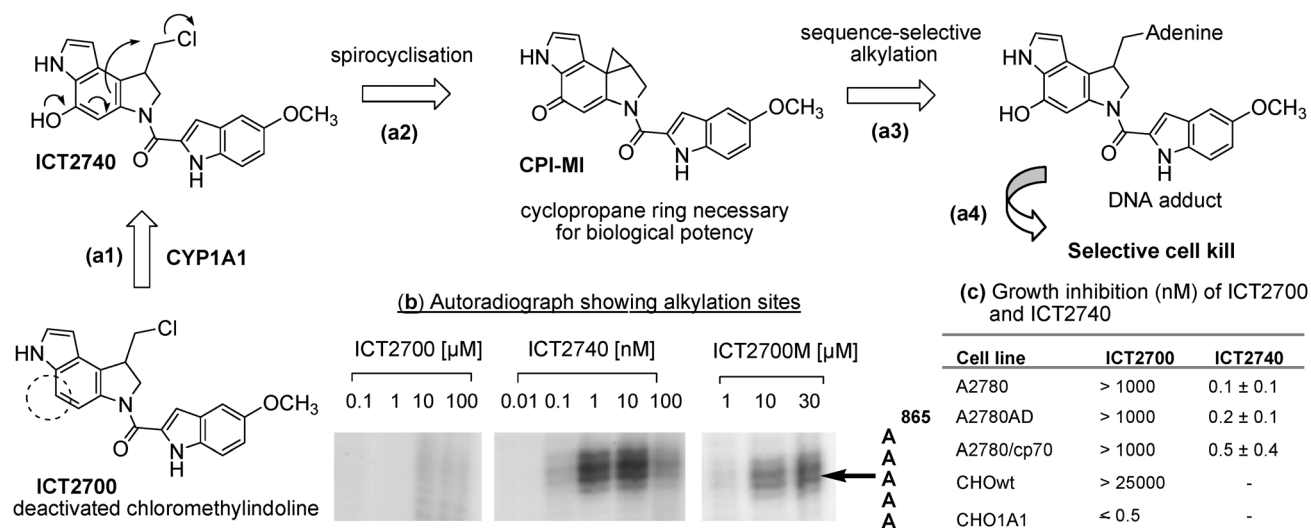
Fig. 1 Pathway of activation of ICT2700, DNA alkylation and chemosensitivity data. (a1) CYP1A1-mediated metabolism generates the *seco*-compound ICT2740. (a2) ICT2740 spirocyclises to produce an activated cyclopropane product.[5d] (a3) The cyclopropapyrroloindolone binds in a sequence-selective manner to cause DNA damage. (a4) The DNA adducts generated by CYP1A1-mediated activation causes selective cell kill. (b) The proposed pathway of CYP1A1 activation is supported by experimental evidence (inserted autoradiograph); Drug–DNA incubations were carried out at 37 °C for 2 h. ICT2700M is a metabolite extract from incubation of ICT2700 with CYP1A1/NADPH bactosomes for 30 min at 37 °C. (c) The cytotoxicity of compounds was measured against a panel of ovarian A2780 carcinoma as well as CHO cells lacking or expressing CYP1A1. Cells were treated for 96 h with ICT2700 or ICT2740 in a suitable concentration range. All data represent the mean of three independent experiments. For full experimental conditions, see Supplementary Information.

the cytotoxicity of ICT2740 was not significantly affected by an elevated expression of the P-glycoprotein efflux pump or defect in DNA MMR repair present in doxorubicin (A2780AD) and cisplatin (A2780/cp70) resistant cancer cell lines, respectively. Distribution of ICT2700 and ICT2740 into A2780 cells was similar (Fig. 2), indicating that the high differential activity between ICT2700 and ICT2740 is not due to poor cellular uptake of ICT2700. Accordingly, the absence of a *para*-directing phenolic hydroxyl group in the pharmacophore of ICT2700 is consistent with an inability to undergo closure to the cyclopropapyrroloindolone necessary for DNA alkylation and attendant cell damage.[8]

Next we investigated the effect of ICT2700 on CHO cells stably transfected with functional CYP1A1 and showed this compound to be cytotoxic at pM concentrations. In comparison, ICT2700 was at least 5 orders of magnitude less potent in wild-type CHO cells in which CYP1A1 was not expressed (Fig. 1c). In the absence of a specific CYP1A1 antibody, we used MRM analysis to show CYP1A1 protein was expressed in the transfected cells but not wild-type cells (Fig. S1†). Oxidative O-deethylation of ethoxyresorufin as a test substrate for CYP1A1 catalytic activity confirmed that CYP1A1 was functionally active in the CHO CYP1A1 cells but not the CHO wild-type (Fig. S2†).

An experiment was performed to see if the observed cytotoxicity of ICT2700 in CYP1A1-transfected CHO cells could be correlated with CYP1A1-mediated metabolism and DNA damage. Incubation of ICT2700 with CYP1A1 bactosomes produced an extract (ICT2700M) that was analysed by LC/MS/MS to comprise unconverted ICT2700 (85.7%), ICT2740 (3.3%), spirocyclised cyclopropapyrroloindolone product CPI-MI (1.7%) and two mono-hydroxylated metabolites (9.1%), measured as percent total peak AUC's (Fig. 3). The latter two metabolites both possessed a molecular weight consistent with retention of the de-hydroxy chloromethylindoline moiety and hence not considered to contribute to DNA alkylation and cytotoxicity.

Next we probed purine-N^3 alkylation with the incubate ICT2700M. Using a thermal cleavage assay,[9] an autoradiograph of a known DNA sequence revealed a prominent ICT2700-induced thermal cleavage site around a cluster of adenines within the sequence ^{869}5'-AAAAA-3'^{865} (Fig. 1b) and minor alkylation sites at base pairs 764 and 772 (Fig. S3†). These adenine-rich sequences are reported as a consensus binding regions of both adozelesin[10] and (+)-CC-1065.[11] In line with our hypothesis, ICT2700M was also shown to alkylate these adenine sites albeit at a higher concentration than the intrinsically active *seco*-compound ICT2740. In contrast, ICT2700 was not observed to alkylate DNA under the conditions investigated, consistent with deactivation of the duocarmycin pharmacophore as a consequence of the removal of OH group in the embedded chloromethylindoline fragment.
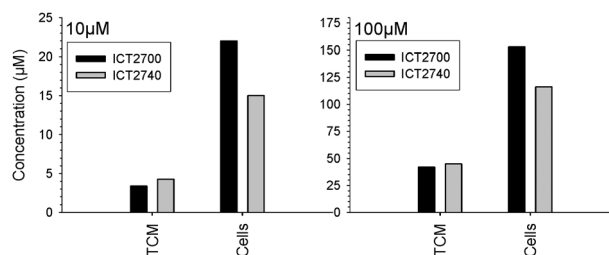

Fig. 2 Concentrations of ICT2700 or ICT2740 in A2780 cells or tissue culture medium (TCM) following 15 min incubation of 10 μM or 100 μM of ICT2700 or ICT2740 with 3×10^6 cells in 500 μL cell culture medium.
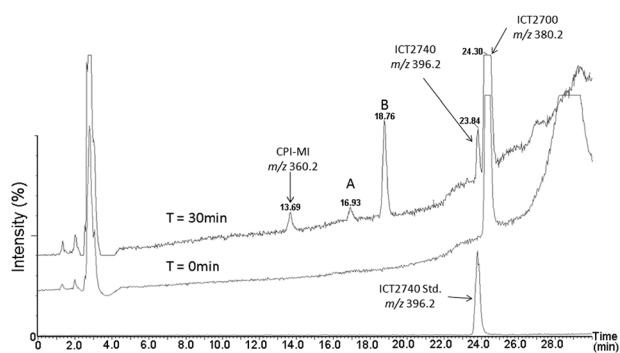
Fig. 3 Metabolite identification from CYP1A1 bactosomes incubation. A typical chromatograph demonstrating the appearance of hydroxylated metabolite ICT2740 (*m/z* 396.2) and its spirocyclised toxic metabolite (*m/z* 360.1) following a 30 min incubation of ICT2700 (*m/z* 380.1) with CYP1A1/NADPH bactosomes. Products A and B are mono-hydroxylated products, which *m/z* indicate intact chloromethyl fragment with no loss to spirocyclisation.

In summary, synthetic modifications of *seco*-duocarmycins have been the focus of addressing the unmet clinical potential of the (+)-CC-1065 and duocarmycin family of agents, which have failed to show an acceptable therapeutic index in clinical trials.[4] Previous studies have focused on inactivating the alkylating pharmacophore of the *seco*-duocarmycins with electron-withdrawing groups including carbamate derivatisation at the phenolic hydroxyl group or by substitution with functional groups with potential for bioreduction.[4,11,12] Although good differential activity and improved pharmacokinetics in pre-clinical models have been observed, the clinical trials with carzelesin and KW-2189 have not corroborated the potential benefit of carbamate prodrug design.[4]

Our strategy is to harness the unique capability of selected CYPs to affect regioselective aryl oxidation in order to generate an ultrapotent and non-reversible active (hydroxyl) metabolite. Towards this aim we have identified a de-activated pharmacophore based on the duocarmycins with potential to undergo tissue specific oxidative activation in which CYP1A1 is expressed. The importance of this study lies in the potential to use CYP-mediated activation of agents 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.

Notes and references

1 (*a*) G. I. Murray, W. T. Melvin, W. F. Greenlee and M. D. Burke, *Annu. Rev. Pharmacol.*, 2001, **41**, 297–316; (*b*) H. Chang, J. M. Su, C. C. Huang, L. C. Liu, C. H. Tsai, M. C. Chou and P. Lin, *Cancer Detect. Prev.*, 2005, **29**, 562–569; (*c*) P. Gibson, J. H. Gill, P. A. Khan, J. M. Seargent, S. W. Martin, P. A. Batman, J. Griffith, C. Bradley, J. A. Double, M. C. Bibby and P. M. Loadman, *Mol. Cancer Ther.*, 2003, **2**, 527–534; (*d*) P. Lin, H. Chang, W. L. Ho, M. H. Wu and J. M. Su, *Lung Cancer*, 2003, **42**, 255–261; (*e*) M. C. McFadyen, M. E. Cruickshank, I. D. Miller, H. L. McLeod, W. T. Melvin, N. E. Haites, D. Parkin and G. I. Murray, *Br. J. Cancer*, 2001, **85**, 242–246; (*f*) M. C. McFadyen, W. T. Melvin and G. I. Murray, *Br. J. Cancer*, 2004, **91**, 966–971; (*g*) S. Haas, C. Pierl, V. Harth, B. Pesch, S. Rabstein, T. Bruning, Y. Ko, U. Hamann, C. Justenhoven, H. Brauch and H. P. Fischer, *Int. J. Cancer*, 2006, **119**, 1785–1791; (*h*) J. G. Jiang, C. L. Chen, J. W. Card, S. Yang, J. X. Chen, X. N. Fu, Y. G. Ning, X. Xiao, D. C. Zeldin and D. W. Wang, *Cancer Res.*, 2005, **65**, 4707–4715; (*i*) M. Kumarakulasingham, P. H. Rooney, S. R. Dun-das, C. Telfer, W. T. Melvin, S. Curran and G. I. Murray, *Clin. Cancer Res.*, 2005, **11**, 3758–3765; (*j*) S. T. Saarikoski, H. A. Wikman, G. Smith, C. H. Wolff and K. Husgafvel-Pursiainen, *J. Histochem. Cytochem.*, 2005, **53**, 549–556; (*k*) M. Karlgren, A. Gomez, K. Stark, J. Svard, C. Rodriguez-Antona, E. Oliw, M. L. Bernal, S. Ramon y Cajal, I. Johansson and M. Ingelman-Sundberg, *Biochem. Biophys. Res. Commun.*, 2006, **341**, 451–458; (*l*) D. Downie, M. C. McFadyen, P. H. Rooney, M. E. Cruickshank, D. E. Parkin, I. D. Miller, C. Telfer, W. T. Melvin and G. I. Murray, *Clin. Cancer Res.*, 2005, **11**, 7369–7375; (*m*) M. A. Rieger, R. Ebner, D. R. Bell, A. Kiessling, J. Rohayem, M. Schmitz, A. Temme, E. P. Rieber and B. Weigle, *Cancer Res.*, 2004, **64**, 2357–2364.
2 (*a*) M. C. McFadyen, W. T. Melvin and G. I. Murray, *Mol. Cancer Ther.*, 2004, **3**, 363–371; (*b*) C. Rodriguez-Antona and M. Ingelman-Sundberg, *Oncogene*, 2006, **25**, 1679–91.
3 (*a*) L. Yu and D. J. Waxman, *Drug Metab. Dispos.*, 1996, **24**, 1254–1262; (*b*) L. H. Patterson and G. I. Murray, *Curr. Pharm. Des.*, 2002, **8**, 1335–1347.
4 (*a*) D. L. Boger and D. S. Johnson, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1438–1474; (*b*) S. E. Wolkenburg and D. L. Boger, *Chem. Rev.*, 2002, **102**, 2477; (*c*) N. Ghosh, H. M. Sheldrake, M. Searcey and K. Pors, *Curr. Top. Med. Chem.*, 2009, **9**, 1494–1524.
5 K. S. MacMillan and D. L. Boger, *J. Am. Chem. Soc.*, 2008, **130**, 16521–16523.
6 (*a*) D. L. Boger, R. J. Wysocki and T. Ishizaki, *J. Am. Chem. Soc.*, 1990, **112**, 5230–5240; (*b*) D. L. Boger, W. Y. Yun and B. R. Teegarden, *J. Org. Chem.*, 1992, **57**, 2873–2876; (*c*) D. L. Boger, C. W. Boyce, R. M. Garbaccio and M. Searcey, *Tetrahedron Lett.*, 1998, **39**, 2227–2230; (*d*) D. L. Boger, A. Santillan, M. Searcey, S. R. Brunette, S. E. Wolkenberg, M. P. Hedrick and Q. Jin, *J. Org. Chem.*, 2000, **65**, 4101–4111; (*e*) D. L. Boger, C. W. Boyce, R. M. Garbaccio and J. A. Goldberg, *Chem. Rev.*, 1997, **97**, 787–828.
7 R. D. Baird and S. B. Kaye, *Eur. J. Cancer*, 2003, **39**, 2450–61.
8 M. Ichimura, T. Ogawa, K. Takahashi, A. Mihara, I. Takhashi and H. Nakano, *Oncol. Res.*, 1993, **5**, 165–171.
9 K. Kiakos, A. Sato, T. Asao, P. J. McHugh, M. Lee and J. A. Hartley, *Mol. Cancer Ther.*, 2007, **6**, 2708–2718.
10 L. H. Hurley, V. L. Reynolds, D. H. Swenson, G. L. Petzold and T. A. Scahill, *Science*, 1984, **226**, 843–844.
11 J. P. Lajiness, W. M. Robertson, I. Dunwiddie, M. A. Broward, G. A. Vielhauer, S. J. Weir and D. L. Boger, *J. Med. Chem.*, 2010, **53**, 7731–7738.
12 M. Tercel, G. J. Atwell, S. Yang, A. Ashoorzadeh, R. J. Stevenson, K. J. Botting, Y. Gu, S. Y. Mehta, W. A. Denny, W. J. Wilson and F. B. Prujin, *Angew. Chem., Int. Ed.*, 2011, **50**, 2606–2609.