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Probing cytochrome P450 (CYP) bioactivation with chloromethylindoline bioprecursors derived from the duocarmycin family of compounds

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ABSTRACT

The duocarmycins belong to a class of agent which has great potential for use in cancer therapy. Their exquisite potency means they are too toxic for systemic use, and targeted approaches are required to unlock their clinical potential. In this study, we have explored seco-OH-chloromethylindoline (CI) duocarmycin-based bioprecursors for their potential for cytochrome P450 (CYP)-mediated cancer cell kill. We report on synthetic and biological explorations of racemic seco-CI-MI, where MI is a 5-methoxy indole motif, and dehydroxylated analogues. We show up to a 10-fold bioactivation of de-OH CI-MI and a fluoro bioprecursor analogue in CYP1A1-transfected cells. Using CYP bactosomes, we also demonstrate that CYP1A2 but not CYP1B1 or CYP3A4 has propensity for potentiating these compounds, indicating preference for CYP1A bioactivation.

1. Introduction

The cytochrome P450 enzymes (CYP) are a family of constitutive and inducible oxidases that play central roles in the metabolism of xenobiotics and endogenous compounds. Members belonging to the CYP1 subfamily participate in the metabolism of carcinogens originating from chemical pollutants,[1] including polycyclic aromatic hydrocarbons (PAHs), nitroaromatics, and arylamines.[2] Exposure to such xenobiotics could have a long-term effect on human health, including increased risk of developing cancer. The CYP1A1 isoform often generates more reactive intermediates through its catalytic activity of PAHs that are capable of binding with DNA and causing genetic mutations.[1] However, due to frequent intratumoral expression and innate capacity to metabolise xenobiotics, some CYPs including CYP1A1 could be a target for locoregionally activated cancer therapeutics.[3–7]

The phenol-containing duocarmycins, e.g. duocarmycin SA (**1**, Fig. 1) are a family of natural products recognized as ultrapotent cytotoxins.[8,9] Their mechanism of action involves spirocyclization of the deep-embedded *seco*-OH-chloromethylindoline (CI) fragment to trigger production of an N3-adenine covalent adduct upon binding of the minor groove of DNA.[10] Four duocarmycins have entered clinical evaluation, however the lack of tumour selectivity and lack of therapeutic index have prevented further progression and regulatory ~~a~~approval.[11–13] In recent years, several efforts have been pursued that are focused on the development of prodrugs[9] and antibody–drug conjugates,[14–16] as strategies to selectively deliver these ultrapotent duocarmycin chemotoxins to tumour tissue. Many of these approaches have focussed on modification of the CI trigger unit via the pendant phenolic OH (or NH_2) to deactivate these agents and prevent the spirocyclization mechanism necessary for DNA alkylation and cell killing.[17]

Instead of synthetic manipulation of the phenolic group, our strategy has been focused on the inactivation of the duocarmycins by complete removal of the key OH group. This deactivated pharmacophore, has been suggested to be evolved from ancestral precursors[18] although a recent report[19] on the biosynthetic pathway of CC-1065 provides evidence that the installment of the key phenolic OH is generated from a tyrosine building block by 4-hydroxyphenylacetate 3-hydroxylase. Regardless of origin, the de-hydroxylated compound is highly lipophilic and lends itself towards being a substrate for phase 1 CYP metabolism. In previous reports, we successfully demonstrated that duocarmycins lacking the phenolic OH, such as the cyclopropapyrroloindole (CPI)-derived bioprecursors **2** (ICT2700) and **3** (ICT2706) (Fig. 1), are capable

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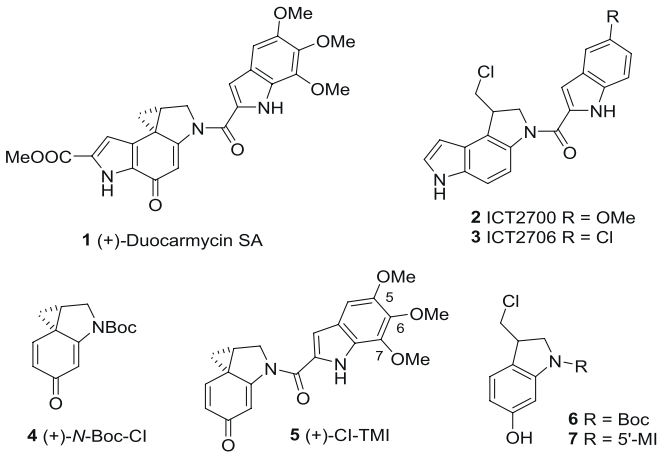


Figure 1. Duocarmycin SA, bioprecursors ICT2700 and ICT2706 and CI-based analogues; TMI = trimethoxyindole and 5′-MI = 5-methoxyindole.

of undergoing regioselective aryl oxidation by CYP1A1[20-22] and CYP2W1[21, 23] to generate cytotoxins via *seco*-duocarmycin OH metabolites in a tumour-selective manner (bioactivation outlined in Fig. 2).

Studies conducted on the CI pharmacophore, which is structurally the simplest member of the duocarmycins, indicated that while the Boc-protected analogue **4** and trimethoxyindole (TMI) **5** (Fig. 1), for example, retain potent DNA alkylation, only the latter retains low-nM antiproliferative activity against L2110 cells.[24,25] Since CI is the minimum potent pharmacophore of all of these alkylating natural products, and to complement previous data, we were interested in exploring whether dehydroxy analogues of **4** were also suitable for targeting tumour-expressed CYPs. Our previous studies[20, 21] have indicated that the 5-methoxy indole (MI) motif on the "right-hand" segment of the pharmacophore is best tolerated for CYP1A1 bioactivation, and here we have utilised the same motif linked to the deactivated CI pharmacophore to probe potential for CYP bioactivation. Herein, we report on the synthesis of *seco*-CI-MI **7** (synthesis reported in Scheme S1) and its dehydroxy analogue **16** and investigate their potential for bioactivation by CYP1A1, 1A2, 1B1 and 3A4, and also report on the metabolic profile. For

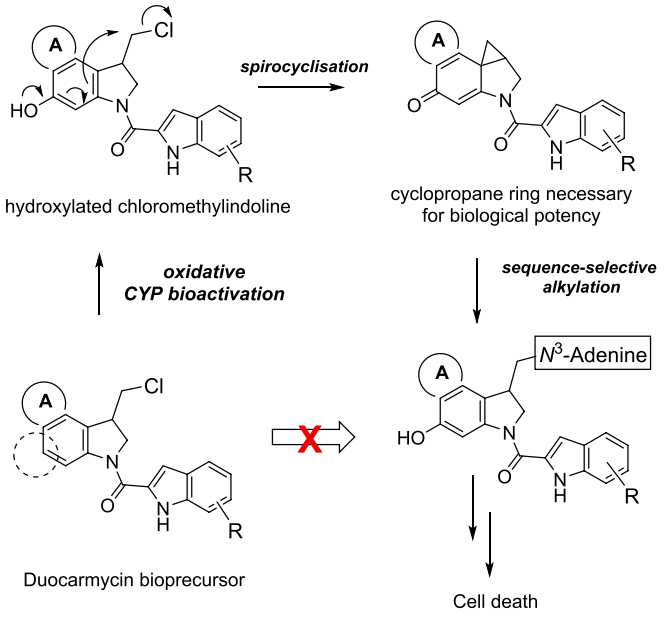


Figure 2. Oxidative bioactivation by CYP isoforms is affected by A ring configuration in the alkylating subunit and the presence of R fragments on the DNA minor groove binding motif.

mechanistic understanding and comparison, we also synthesised analogue **17** bearing a 5-positioned fluoro group and *ortho* to the point of hydroxylation to generate the classical CI pharmacophore.

2. Results and discussion

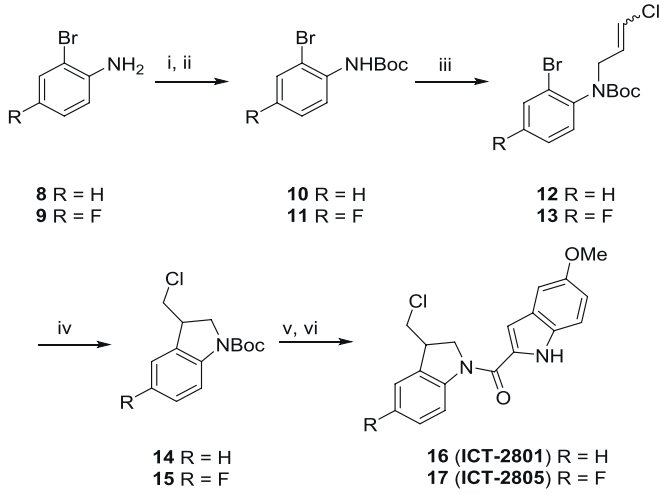
2.1. Synthesis

The synthesis of the dehydroxy alkylation subunit of ICT2700 (**2**) and ICT2706 (**3**)[20,21] utilised a 5-*exo-trig*-radical cyclization onto a vinyl chloride,[26] and this was the initial approach taken to the more simple CI analogues (Scheme 1). Starting from commercially available 2-bromoaniline or 2-bromo-4-fluoroaniline, di-Boc-protection of the amino group with subsequent removal of one protecting group was more efficient than attempts to mono-Boc protect directly. Alkylation with 1,3-dichloropropene and subsequent cyclization using tris(trimethylsilyl)silane (TTMSS) followed by Boc deprotection and conjugation with 5-methoxy-indole 2-carboxylic acid gave target compounds **16** and **17** in reasonable yield (Scheme 1, strategy A).

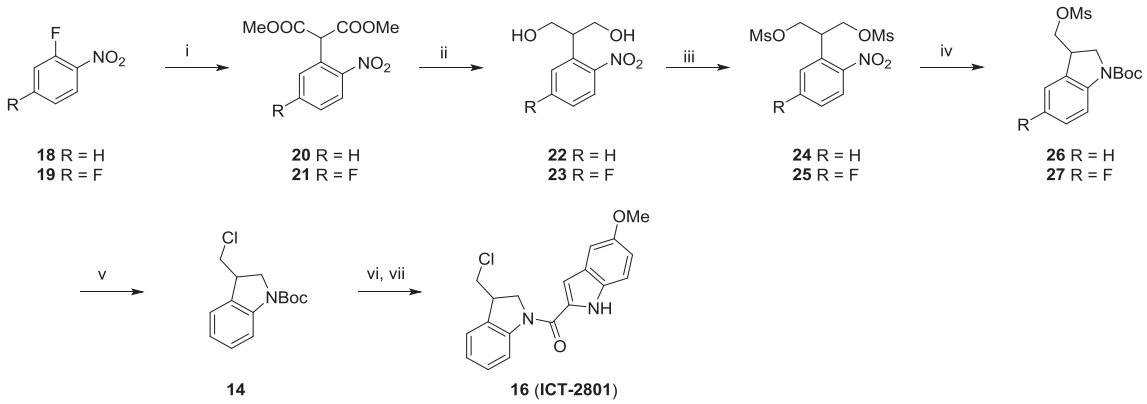
In order to investigate a potentially stereoselective synthesis, the lengthier synthetic strategy based on the method established by Warpehoski to prepare pyrrole chloroindoline duocarmycin analogues was also studied and was utilised for the synthesis of the active analogue *seco*-CI-MI (Scheme S1).[27] Both fluoro-2-nitrobenzene and 1,3-difluoro-5-nitro-benzene were treated under basic conditions with dimethyl malonate to generate compounds **20** and **21**. Reduction and conversion to the mesylates **24** and **25** was followed by a one-pot reduction/protection/intramolecular alkylation to generate target subunits **26** and **27**. While diol **22** was efficiently transformed into respective **24**, synthesis of fluoro derivative **25** was accomplished in poor yield (34%), and further attempts to improve the yield of this reaction were unsuccessful. Equally, the one-pot ring closure to afford fluoro derivative protected indoline **24** (yield = 34%) gave poor conversions. The presence of the fluoro substituent on the aromatic ring impacted negatively on the generation of target compounds and hence this approach was shown to be only suitable for the preparation of unsubstituted dehydroxy-*seco*-CI-MI **16** (Scheme 2, strategy B).

2.2. Chemosensitivity

seco-CI-MI (**7**) and *seco*-CI-NHBoc (**6**, Scheme S1) and bioprecursors



Scheme 1. Strategy A: Synthesis of dehydroxy-*seco*-CI-MI derivatives. *Reagent and conditions*: i. Boc_2O, cat. DMAP, THF, reflux, 16 h, ii. K_2CO_3, MeOH, reflux, 3 h; **10**: 83%, **11**: 76%; iii. NaH, DMF, 0 °C, 30 min, then 1,3-dichloropropene, RT, 3 h; **12**: 87%, **13**: 80%; iv. TTMSS, AIBN, toluene, 3 h, 90 °C; **14**: 74%, **15**: 77%; v. HCl in EtOAc (2.7 M soln), RT, 3 h; vi. EDC, 5-methoxyindole-2-carboxylic acid, DMF, RT, 16 h; **16**: 62%, **17**: 64%.



Scheme 2. Strategy B: Synthesis of dehydroxy-*seco*-CI-MI derivatives. *Reagent and conditions*: i. NaH, $(CO_2CH_3)_2$|CH, THF, 0 °C, then reflux, 16 h.; **20**: 88%, **21**: 86%; ii. DIBAL-H, THF, 0 °C, 1 h.; **22**: 43%, **23**: 28%; iii. MsCl, Et_3N, CH_2Cl_2, 0 °C, 1 h; **24**: 71%, **25**: 35%; iv. H_2 , Pd/C, Et_3N, Boc_2O, THF, RT, 16 h; **26**: 70%, **27**: 34%; v. TBAC, DMF, 90 °C, 5-16 h, **14**: **86%**; vi & viii. See Scheme 1 for conditions.

14, **16** and **17** (Scheme 1) were evaluated for their ability to inhibit the growth of cell lines deficient in CYPs (EJ138 and CHOwt) and proficient in CYP1A1 (RT112 and CHO1A1). IC_{50} values for compound **6** were in the range of 6–11 μM and are akin to results reported previously,[25] while *seco*-CI-MI was significantly more potent with IC_{50} values in the range 30–55 nM (Table 1). Amongst the *seco*-CI analogues, it was expected that replacement of *tert*-butyloxy group CI-Boc (Scheme S1) with the planar 5-MI motif in CI-MI would favor association of this compound in the DNA minor groove leading to an increase in antiproliferative activity; the data here are in accordance with observations made between compound CI-Boc and the *seco*-CI-TMI analogue.[24] Bioprecursors **16** and **17** displayed a notable enhanced antiproliferative activity in the CYP1A1-expressing RT112[22] and CHO1A1[20, 22] cell lines, with **16** approximately 10-fold more potent in CHO1A1 compared with CHOwt cells. Fluorinated analogue **17** appeared to be slightly less potent than **16** suggesting introduction of a fluoro group is detrimental to bioactivation by CYP1A1. Although this differential is relatively small, the data suggest that antiproliferative activity of bioprecursors **16** and **17** may be potentiated in the presence of CYP1A1.

2.3. CYP metabolism of the CI pharmacophore

To further elucidate involvement of CYPs in the bioactivation of bioprecursors **16** and **17**, and to establish the CYP isoforms involved, compounds **16** and **17** were evaluated against a panel of recombinant CYP enzymes. This was accomplished by incubating **16** and **17** with several bactosomes (CYP null as control, 1A1, 1A2, 1B1, and 3A4) for 1 h at 37 °C, and any metabolites produced following incubation with specific CYP bactosomes were extracted and added to the EJ138 bladder cancer cells for antiproliferative activity evaluation using the previously reported methodology.[21]

The CYP1A1-extracted metabolite fractions were shown to produce a 7-fold potentiation for bioprecursor **16** and 5-fold for bioprecursor **17** (Table 2), which is largely in accordance with the CHO/CHO1A1 cell data for these compounds. Furthermore, CYP1A2, but not CYP1B1 or CYP3A4, was also shown to potentiate the antiproliferative activity of compounds **16** and **17**. CYP1A1 and 1A2 belong to the same CYP1 family member with over 70% homology, and the smaller size of the CI pharmacophore when compared with previously reported CBI and CPI pharmacophores[21] suggests that truncated CI duocarmycins can be accommodated by the active site of CYP1A2, perhaps explaining why bioprecursor **16** showed extensive metabolism when incubated with human CYP1A1 bactosomes, however we were not able to identify the authentic hydroxylated metabolite **7**. Similar extensive metabolism and lack of compound **7** identification was observed with CYP1A2 bactosomes (Fig. S1). Incubation of **16** in the presence of glutathione as a way to detect a putative spirocyclic active species by CYP1A2 also failed to identify a recognisable conjugate between the highly reactive glutathione with the electrophilic spirocyclopropane (Fig. S2).

In an attempt to identify the active metabolite(s), we decided to use human recombinant CYP1A1 and assay conditions previously described.[28] Bioprecursor **16** (50 μM) was incubated for 1 h with a reconstituted protein system (RPS; consisting of human NADPH cytochrome P450 reductase, freshly prepared 1,2-dilauroyl-*sn-glycero*-3-phosphocholine, 100 mM potassium phosphate, pH 7.4) and subsequent analysis revealed not only the presence of compound **7** but also higher amounts of metabolites M2-M6 (Fig. 3A). The results indicate the use of RPS provides a superior assay when attempting to identify very small amounts of metabolites; 61.4% of bioprecursor **16** was metabolised after 1 h of which 0.9% was identified as metabolite **7** (Fig. S5). Furthermore, incubation of authentic metabolite **7** with CYP1A1 led to the generation of a major metabolite M1 (M4 in Fig. 3B) with $m/z = 327.1$. This is a mass unit loss of 30, indicating the possibility of OCH_3 loss from the DNA minor groove binding motif and perhaps indicates preferred site of metabolism.

The antiproliferative activity of bioprecursor **17** indicated that bioactivation would also occur (Tables 1 and 2). Using RPS/CYP1A1 system we performed a time-dependent experiment and monitored metabolite generation over a period of 60 min (Fig. 4). Two mono-hydroxylated metabolites were generated with intact chloroethyl fragment, however as with compound **16** we were not able to identify spirocyclized product. The latter can be generated in position 6 (natural product configuration) or in position 4, which is theoretically a possible route for bioactivation.[29]

Next, we performed studies with liver endoplasmic reticulum samples to reflect the major site of drug metabolism to fully address the multiple CYP interactions with the most promising bioprecursor **16**. Accordingly, **16** was incubated in the presence of human and rat liver microsomes, both representing a rich source of drug-metabolising CYP enzymes. In both sets of microsomes, a time-dependent disappearance of compound **16** accompanied by concomitant formation of metabolites M16, M22 and M23 was observed, though not in similar proportion

Table 1
Growth inhibition (IC_{50} = μM) of chloromethylindolines against human bladder carcinoma cell lines, EJ138 and RT112, parental CHO cell line and the CYP1A1-transfected variant.

Compd ID	EJ138	RT112	CHOwt	CHO1A1
6	8.1 ± 2.0	11.1 ± 2.8	7.3 ± 1.1	6.5 ± 0.8
7	0.038 ± 0.008	0.055 ± 0.004	0.030 ± 0.004	0.035 ± 0.004
14	>25.0	>25.0	>25.0	>25.0
16	15.0 ± 1.9	8.04 ± 0.9	11.4 ± 2.2	1.2 ± 0.5
17	18.6 ± 1.2	5.04 ± 0.9	14.8 ± 3.8	6.2 ± 1.3

[a]All IC_{50} values are in μM and the mean ± SD of at least three independent assays.

Table 2
Growth inhibition of bioprecursors **16** and **17** after incubation with CYP bactosomes against EJ138 bladder cancer cell lines.

	EJ138	EJ138 + C[a]	EJ138 + CYP1A1		EJ138 + CYP1A2		EJ138 + CYP1B1		EJ138 + CYP3A4	
Compd ID	IC_{50}[b]	IC_{50}	IC_{50}	PF[c]	IC_{50}	PF	IC_{50}	PF	IC_{50}	PF
16	15.0 ± 1.9	17.2 ± 4.1	2.5 ± 0.9	6.9	1.9 ± 0.9	9.1	13.5 ± 1.2	1.3	19.8 ± 6.8	0.9
17	18.6 ± 1.2	15.8 ± 3.2	3.1 ± 1.0	5.1	5.11 ± 0.5	3.1	11.9 ± 3.0	1.3	16.8 ± 5.1	0.9

[a]C = null bactosomes; [b]IC_{50} = μM; [c]Potentiation factor (IC_{50} in EJ-138 + control bactosomes/IC_{50} in EJ-138 + CYP isoform metabolites).

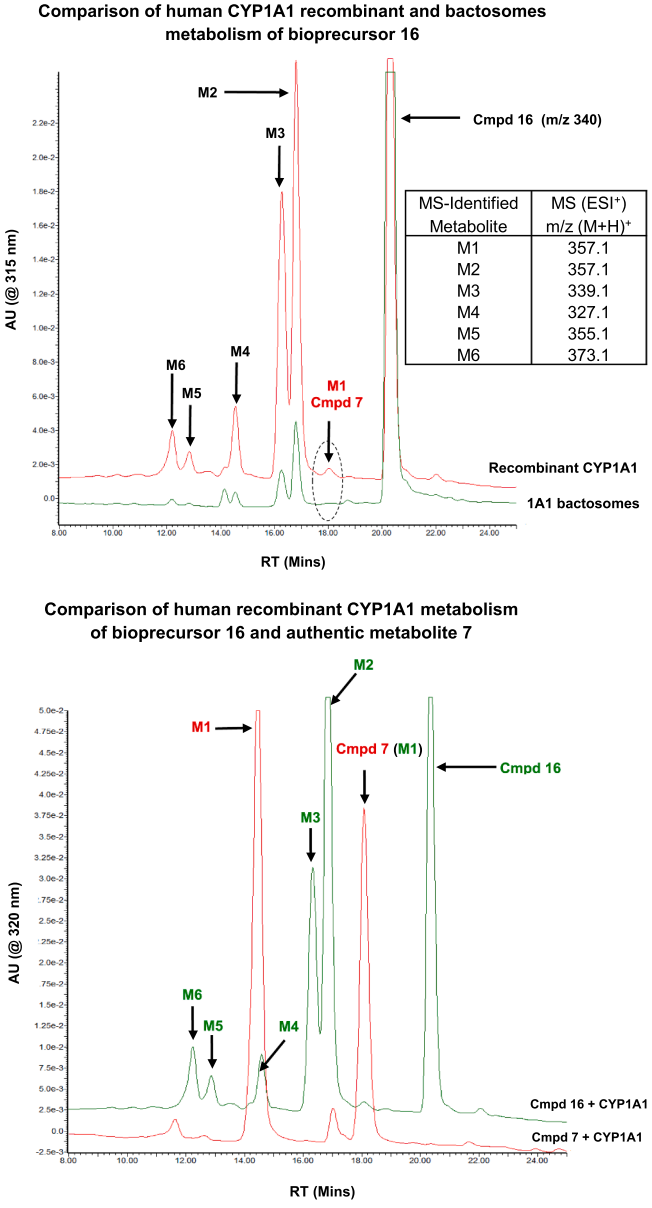


Figure 3. CYP1A1 metabolic studies of compounds **7** and **16**. *Top chromatogram*: comparison of metabolism profiles of **16** using purified human CYP1A1 recombinant enzyme and CYP1A1 bactosomes. *Bottom chromatogram*: comparison of metabolism profiles of compounds **7** and **16** using CYP1A1 recombinant enzyme. In both cases, incubation time was 1 h. Apart from M1, M = unidentified metabolites.

(Supporting Information). Metabolites M16 and M22 are the predominant metabolites produced by human liver microsomes (Fig. S4), while microsomes from rat liver produced mainly M23 (Fig. S3). Human CYP3A4 is one of the most abundant drug-metabolising CYP isoforms in human liver and, on average, accounts for half of the total CYP

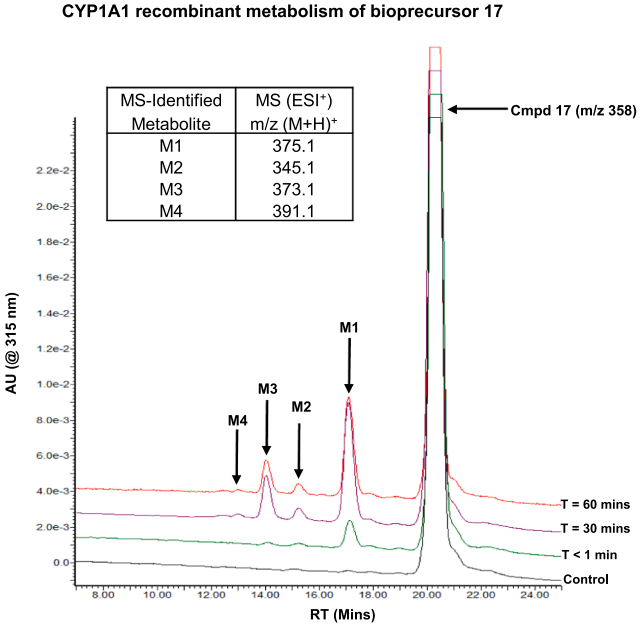


Figure 4. Time-dependent incubation of compound **17** with human CYP1A1 recombinant enzyme. M = unidentified metabolites.

expression in human liver. Growth inhibition data substantiate that this enzyme made no contribution to the bioactivation of **16** (Table 2), which is a desirable feature in the further development of these tumour-activated duocarmycins bioprecursors.

3. Conclusion

In this study, we have explored CI duocarmycin-based bioprecursors for their potential for CYP bioactivation. Collectively, the data indicate extensive metabolism of these compounds by CYPs; however, of those CYPs investigated, only CYP1A family members appear to be linked to potentiation of bioprecursors **16** and **17** in cancer cells. No metabolite from incubation of **16** with bactosomes, rat or human liver microsomes was shown to co-elute with the authentic CI-MI metabolite, however it was detected using human recombinant CYP1A1.

Although CYP1A2 was able to potentiate bioprecursors, it is possible that no significant toxicity will be seen in the liver as it is a detoxification organ and generally copes well with the metabolism of drugs such as cyclophosphamide, which is known to be activated in the liver to a DNA interstrand crosslinking agent.[30] Accordingly, targeting overexpressed CYP1A1 in tumours for therapeutic intervention remains a viable route given the liver is a robust organ capable of tolerating high concentration of chemicals without suffering severe damage in the short term. This work provides chemical exploration to target compound synthesis and enzymatic explorations of the simplest member of the duocarmycin family of compounds. Importantly, the data add further support to the concept of designing duocarmycin bioprecursors.

4. Materials and methods

4.1. Chemistry

All chemicals were reagent grade. All anhydrous solvents used were bought as such and presumed to conform to manufacturer's standards. NMR spectra were recorded on a Bruker Advance AM spectrometer at the frequencies of 400 MHz (^{1}H), 100 MHz (^{13}C). Chemical shifts (δ) are reported in parts per million (ppm). ^{1}H and ^{13}C chemical shifts were referenced to the residual solvent peak. Elemental analysis was carried out using a Carlo Erba CHN1108 Elemental Analyser. Melting points (mp) were recorded using a Bibby Stuart Scientific SMP3 Melting Point Apparatus. Infrared spectra were recorded as neat samples using a Nicolet Smart Golden Gate Spectrometer (Avatar 360 FT-IR E.S.P). Mass spectra (MS) were recorded using a ThermoQuest Navigator Mass Spectrometer operated under Electrospray Ionization in positive (ES+) or negative (ES−) modes. High-resolution mass spectra (HRMS) were recorded using a Micromass Q-TTOF Global Tandem Mass Spectrometer, and data were manipulated using the MassLab 3.2 software system. Chromatographic separations were performed on silica gel for flash chromatography (particle size 40–63 μm) Analytical-TLC was performed on Merck precoated silica gel 60 F254 TLC plates. The TLC plates were visualised using a variety of techniques: visualisation under UV light, phosphomolybdic acid (10% soln. in EtOH), ninhydrin (10% soln. in EtOH) followed by heating.

tert-Butyl N-(2-bromophenyl)carbamate (**10**[31]): 2-Bromoaniline (5 g, 29.1 mmol) was dissolved in anhyd·THF (50 mL) and treated with Boc_2O (14.3 g, 65.7 mmol) and DMAP (0.71 g, 5.81 mmol). The reaction mixture was then heated under reflux for 16 h. The solution was subsequently cooled and partitioned between HCl (0.5 M, 50 mL) and EtOAc (50 mL). The aqueous phase was further extracted with EtOAc (3 × 25 mL), and the combined organic extracts washed with sat. NaCl solution, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude oil was redissolved in CH_3OH (50 mL) and treated with K_2CO_3 (12 g, 87.3 mmol). This heterogenous reaction mixture was then heated under reflux for 3 h. The solution was subsequently cooled, filtered and partitioned between HCl (0.5 M, 50 mL) and EtOAc (50 mL). The aqueous phase was further extracted with EtOAc (3 × 25 mL), and the combined organic extracts washed with sat. NaCl solution. These were then dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography (100% hexane) gave **10** (6.55 g, 83%) as a very pale golden oil; R_f 0.57 (10% EtOAc in hexane); IR (neat) υ_{max} 3413, 2977, 2930, 1731, 1513, 1431, 1148, 745 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 (d, 1H, J = 8.2 Hz, ArH), 7.41 (dd, 1H, J = 8.0, 1.3 Hz, ArH), 7.19 (ddd, 1H, J = 8.2, 7.4, 1.2 Hz, ArH), 6.91 (br s, 1H, NH), 6.81 (ddd, 1H, J = 7.8, 7.6, 1.5 Hz, ArH), 1.45 (s, 9H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 152.4 (C═O), 136.3 (ArCNH), 132.2 (ArCH), 128.3 (ArCH), 123.8 (ArCH), 120.1 (ArCH), 112.4 (ArCBr), 81.1 ($C(CH_3)_3$), 28.3 (3C, $C(CH_3)_3$); MS (ES +) *m/z* calcd for $C_{11}H_{14}BrNO_2$ [*M*] 273.0, 271.0. Found $[M+1]^+$ 274.0/272.0; Anal calcd for $C_{11}H_{14}BrNO_2$: C, 48.55; H, 5.19; N, 5.15. Found: C, 48.30; H, 5.04; N, 5.20.

tert-Butyl N-(2-bromo-4-fluorophenyl)carbamate (**11**[32]): Compound **11** was synthesized and purified as described above for **10**, starting from 2-bromo-4-fluoroaniline (5 g, 26.5 mmol). Yield: 76% (white solid). R_f 0.60 (10% EtOAc in hexane); mp = 35.5–37.7 °C; IR (neat) υ_{max} 3343, 3077, 2992, 1694, 1514, 1479, 1365, 1275, 1238, 1155, 1057, 1022, 850, 811, 775, 731 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.02–7.90 (dd, 1H, J = 9.1, 5.6 Hz, ArH), 7.18–7.16 (ddd, 1H, J = 9.1, 7.8, 2.9 Hz, ArH), 6.96–6.91 (dd, 1H, J = 7.8, 2.9 Hz, ArH), 6.77 (br s, 1H, NH), 1.45 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 157.8 (d, JCF = 246.1 Hz, ArCF), 152.5 (C═O), 132.8 (d, J_{CF} = 3.0 Hz, ArCN), 121.3 (d, J_{CF} = 7.8 Hz, ArCH), 119.2 (d, J_{CF} = 25.6 Hz, ArCH), 115.1 (d, J_{CF} = 21.6 Hz, ArCH), 112.5 (d, J_{CF} = 9.9 Hz, ArCBr), 81.2 ($C(CH_3)_3$), 28.3 (3C, $C(CH_3)_3$); MS (ES +) *m/z* calcd for $C_{11}H_{13}BrFNO_2$ [*M*] 291.0, 289.0. Found [*M* + 1] 292.0, 290.1; Anal calcd for $C_{11}H_{13}BrFNO_2$: C, 45.54; H, 4.52; N, 4.83. Found: C, 45.92; H, 4.54; N, 4.71.

tert-Butyl N-(2-bromo-phenyl)-N-(3-chloro-allyl)carbamate (**12**): Compound **10** (6.5 g, 24 mmol) was dissolved in DMF (60 mL), cooled to 0 °C and treated with NaH (60%, 2.87 g, 71.7 mmol) portionwise over 15 min. The resulting solution was stirred at 0 °C for a further 15 min. *E/Z*-1,3-Dichloropropene (7.4 mL, 71.7 mmol) was then added and the reaction mixture warmed to RT. After 3 h, the reaction was quenched with sat. NaCl and the aqueous phase extracted with EtOAc (3 × 25 mL). The combined organic extracts were then dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography (2% EtOAc in hexane) gave **12** (7.36 g, 87%) as a pale golden oil; R_f 0.40 (10% EtOAc in hexane); IR (neat) υ_{max} 3020, 2976, 2929, 1697, 1365, 1159, 754, 724 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.61 (d, 1H, J = 7.3 Hz, ArH), 7.30 (d, 1H, J = 6.8 Hz, ArH), 7.17 (m, 2H, ArH), 6.04 (m, 2H, =CH), 4.51 (dd, 1H, J = 15.7, 5.9 Hz, CH_2), 4.41 (dd, 1H, J = 12.4, 5.2 Hz, CH_2), 4.30 (dd, 1H, J = 15.8, 6.3 Hz, CH_2), 3.86 (dd, 1H, J = 12.0, 6.0 Hz, CH_2), 1.54 (s, 2H, $(CH_3)_3$), 1.35 (s, 7H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.1 (*E/Z* C═O), 153.9 (*E/Z* C═O), 140.9 (*E/Z* ArCN), 140.5 (*E/Z* ArCN), 133.2 (*E/Z* ArCH), 133.0 (*E/Z* ArCH), 130.5 (*E/Z* ArCH), 130.0 (*E/Z* ArCH), 128.8 (*E/Z* ArCH), 128.7 (*E/Z* ArCH), 128.4 (*E/Z* = CH), 128.0 (*E/Z* = CH), 127.5 (ArCH), 121.5 (*E/Z* = CHCl), 120.5 (*E/Z* = CHCl), 119.9 (ArCBr), 80.6 ($C(CH_3)_3$), 48.9 (*E* CH_2), 45.9 (*Z* CH_2), 28.4 (minor rotamer $C(CH_3)_3$), 28.1 (major rotamer $C(CH_3)_3$); MS (ES +) *m/z* calcd for $C_{14}H_{17}BrClNO_2$ [*M*] 347.0, 345.0. Found [*M* + 1] 348.0, 346.0; Analysis calcd for $C_{14}H_{17}BrClNO_2$: C, 48.51; H, 4.94; N, 4.04. Found: C, 48.66; H, 5.06; N, 4.07.

tert-Butyl N-(2-bromo-4-fluorophenyl)-N-(3-chloroallyl)carba-mate (**13**): Compound **13** was synthesized and purified as described above for **12**, starting from compound **11** (3.76 g, 14.2 mmol). Yield: 80% (oil); R_f 0.40 (10% EtOAc in hexane); IR (neat) υ_{max} 2977, 2928, 1699, 1488, 1379, 1366, 1295, 1254, 1193, 1159, 880, 859, 763, 672 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34 (m, 1H, ArH), 7.14 (m, 1H, ArH), 7.02 (m, 1H, ArH), 6.04 (m, 2H, =CH), 4.48 (dd, 1H, J = 15.4, 6.1 Hz, CH_2), 4.39 (dd, 1H, J = 14.7, 4.0 Hz, CH_2), 4.24 (dd, 1H, J = 15.8, 6.9 Hz, CH_2), 3.81 (dd, 1H, J = 14.9, 4.6 Hz, CH_2), 1.52 (s, 2.3H, $(CH_3)_3$), 1.35 (s, 6.7H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.1 (d, J_{CF} = 251.0 Hz, *E/Z* ArCF), 161.0 (d, J_{CF} = 251.2 Hz, *E/Z* ArCF), 154.0 (*E/Z* C═O), 153.8 (*E/Z* C═O), 137.2 (*E/Z* ArCN), 136.8 (*E/Z* ArCN), 131.3 (d, J_{CF} = 8.6 Hz, *E/Z* ArCH), 130.9 (d, J = 9.0 Hz, *E/Z* ArCH), 128.5 (*E/Z* = CH), 127.2 (*E/Z* = CH), 124.3 (*E/Z* ArCBr), 124.2 (*E/Z* ArCBr), 121.8 (*E/Z* = CHCl), 120.9 (*E/Z* = CHCl), 120.4 (d, J = 25.3 Hz, *E/Z* ArCH), 120.2 (d, J = 25.4 Hz, *E/Z* ArCH), 115.2 (*E/Z* ArCH), 115.0 (*E/Z* ArCH), 80.8 (C $(CH_3)_3$), 48.9 (*E* CH_2), 45.8 (*Z* CH_2), 28.3 (minor rotamer, $C(CH_3)_3$), 28.1 (major rotamer, $C(CH_3)_3$); MS (ES +) *m/z* calcd for $C_{14}H_{16}BrClFNO_2$ [*M*] 365.0, 363.0. Found [*M* + 1] 366.0, 364.0; Analysis calcd for $C_{14}H_{16}BrClFNO_2$: C, 46.11; H, 4.42; N, 3.82. Found: C, 46.11; H, 4.60; N, 3.65.

tert-Butyl 3-chloromethyl-2,3-dihydroindole-1-carboxylate (**14**): A solution of **12** (116.0 mg, 0.33 mmol) in anhyd. toluene (10 mL) was degassed with N_2 for 15 min and then treated with AIBN (12.6 mg, 0.077 mmol) and TTMSS (114 μl, 0.37 mmol) before heating at 90 °C for 3 h. The reaction was then cooled and concentrated *in vacuo*. Column chromatography (5% EtOAc in hexane) gave **14** (66.3 mg, 74%) as a clear colourless oil; R_f 0.83 (15% EtOAc in hexane); IR (neat) υ_{max} 2975, 1697, 1484, 1388, 1163, 1140, 1014, 856, 748, 708 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.88–7.49 (br d, 1H, ArH), 7.23 (m, 2H, ArH), 6.97 (ddd, 1H, J = 7.5, 7.5, 0.9 Hz, ArH), 4.11 (dd, 1H, J = 11.0, 9.7 Hz, CH_2N), 3.94 (m, 1H, CH_2N), 3.77 (dd, 1H, J = 10.6, 4.4 Hz, CH_2Cl), 3.69 (m, 1H, CH), 3.55 (dd, 1H, J = 10.4, 4.4 Hz, CH_2Cl), 1.59 (s, 9H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 151.3 (C═O), 142.1 (ArC), 129.4 (ArC), 127.9 (ArCH), 123.3 (ArCH), 121.2 (ArCH), 114.0 (ArCH), 80.0 (C $(CH_3)_3$), 51.0 (CH_2Cl), 46.2 (CH), 41.3 (CH_2N), 27.3 (3C, $C(CH_3)_3$); HRMS (ES +) calcd for $C_{14}H_{18}ClNO_2$ [*M* + 1] 268.1104. Found [*M* + 1] 268.1112.

Alternatively, a solution of **26** (0.47 g, 1.45 mmol) in DMF (4.7 mL) was treated with TBAC (1.01 g, 3.6 mmol) and heated at 90 °C for 5 h. The reaction mixture was concentrated *in vacuo*. Column

chromatography (5% EtOAc in hexane) gave **14** (335 mg 86%) as a clear colourless oil that matched the compound obtained by the alternative route.

tert-Butyl 3-chloromethyl-5-fluoro-2, 3-dihydroindole-1-carbox-ylate (**15**): Compound **15** was synthesized and purified as described above for **14**, starting from compound **13** (113.8 mg, 0.31 mmol). Yield: 77% (pale golden oil); R_f 0.32 (10% EtOAc in hexane); IR (neat) υ_{max} 2975, 2871, 1699, 1488, 1390, 1366, 1254, 1161, 1143, 880, 859, 763 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.82–7.37 (br d, 1H, ArCH), 6.93 (m, 2H, ArCH), 4.14 (m, 1H, CH_2), 3.94 (m, 1H, CH_2), 3.73 (dd, 1H, J = 10.4, 4.7 Hz, CH_2), 3.67 (m, 1H, CH_2), 3.57 (dd, 1H, J = 10.2, 8.2 Hz, CH_2), 1.57 (br s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.3 (C═O), 152.8 (ArCF), 142.4 (ArC), 138.5 (ArC), 116.3 (ArCH), 115.9 (ArCH), 115.7 (ArCH), 77.8 ($C(CH_3)_3$), 52.9 (CH_2Cl), 47.4 (CH_2N), 29.0 (3C, $C(CH_3)_3$), 14.7 (CH); HRMS (ES +) calcd for $C_{14}H_{17}ClFNNaO_2$ [M + Na]$^+$ 308.0829. Found [M + Na]$^+$ 308.0815.

(3-Chloromethyl-2,3-dihydroindol-1-yl)-(5-methoxy-1H-indol-2-yl) methanone (**16**): Compound **14** (0.15 g, 0.56 mmol) was added to a solution of HCl in EtOAc (1.5 mL, 2.7 M) and left stiirred at RT for 3 h. After this time, the reaction mixture was concentrated *in vacuo* and subsequently redissolved in DMF (1.5 mL). This solution was then treated with EDC (0.32 g, 1.68 mmol) and 5-methoxyindole-2-carboxylic acid (118.4 mg, 0.62 mmol), and stirred at RT for 16 h. After this time the reaction mixture was concentrated *in vacuo*. Column chromatography (15% EtOAc in hexane) gave **16** (118.5 mg, 62%) as an off-white solid. mp = 197.6–198.2 °C; IR (neat) υ_{max} 3266, 3067, 2932, 1623, 1521, 1482, 1404, 1199, 1167, 752, 730 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.53 (br s, 1H, NH), 8.34 (d, 1H, J = 8.1 Hz, ArH), 7.38–7.30 (m, 3H, ArH), 7.14 (m, 2H, ArH), 7.02 (m, 2H, ArH), 4.69 (dd, 1H, J = 10.6, 9.5 Hz, CH_2Cl), 4.51 (dd, 1H, J = 10.7, 4.5 Hz, CH_2Cl), 3.92–3.85 (m, 5H, OCH_3, CH, CH_2N), 3.63 (dd, 1H, J = 10.8, 9.2 Hz, CH_2N); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.4 (C═O), 154.7 (ArCOCH$_3$), 143.7 (ArC), 131.3 (ArC), 131.2 (ArC), 130.6 (ArC), 129.1 (ArCH), 128.3 (ArC), 124.5 (ArCH), 124.2 (ArCH), 118.2 (ArCH), 116.8 (ArCH), 112.7 (ArCH), 105.9 (ArCH), 102.5 (ArCH), 55.7 (OCH_3), 54.2 (CH_2Cl), 46.9 (CH_2N), 43.8 (CH); MS (ES −) m/z calcd for $C_{19}H_{17}ClN_2O_2$ [M] 340.1. Found [M − 1]$^-$ 339.2; Analysis calcd for $C_{19}H_{17}ClN_2O_2$: C, 66.96; H, 5.03; N, 8.22. Found: C, 66.93; H, 5.25; N, 8.29.

(3-Chloromethyl-5-fluoro-2,3-dihydroindol-1-yl)-(5-methoxy-1H-indol-2yl)methanone (**17**): Compound **17** was synthesized and purified as described above for **16**, starting from compound **15** (384.5 mg, 1.35 mmol). Yield: 64% (white powder); R_f 0.42 (30% EtOAc in hexane); mp = 186.4–187.9 °C; IR (neat) υ_{max} 3323, 3008, 2954, 2479, 1619, 1581, 1480, 1402, 1261, 1232, 1200, 1163, 1030, 810, 726, 708 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.49 (br s, 1H, NH), 8.32 (dd, 1H, J = 8.6, 4.8 Hz, ArH), 7.36 (d, 1H, J = 8.9 Hz, ArH), 7.13 (d, 1H, J = 1.7 Hz, ArH), 7.02 (m, 4H, ArH), 4.71 (dd, 1H, J = 10.3, 10.3 Hz, CH_2), 4.52 (dd, 1H, J = 10.7, 4.7 Hz, CH_2), 3.87 (m, 5H, OCH_3, CH, CH_2), 3.63 (dd, 1H, J = 10.6, 8.7 Hz, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.1 (C═O), 160.5/158.7 (J = 180 Hz, ArCF), 155.1 (ArCOCH$_3$), 140.1 (ArC), 133.4, 133.3 (*E/Z* ArC), 131.6, 131.5 (*E/Z* ArC), 130.6, 130.5 (*E/Z* ArC), 128.6 (ArC), 119.5, 119.4 (*E/Z* ArCH), 117.2 (ArCH), 116.0, 115.8 (*E/Z* ArCH), 113.0 (ArCH), 112.0, 111.8 (*E/Z* ArCH), 106.3 (ArCH), 102.8 (ArCH), 56.1 (OCH_3), 54.7 (CH_2Cl), 46.8 (CH_2), 43.8 (CH); MS (ES +) m/z calcd for $C_{19}H_{16}ClFN_2O_2$ [M] 358.1. Found [M + 1]$^+$ 359.0; Analysis calcd for $C_{19}H_{16}ClFN_2O_2$: C, 63.60; H, 4.49; N, 7.81. Found: C, 63.12; H, 4.11; N, 7.50.

Dimethyl 2-(2-nitrophenyl)malonate (**20**[33]): A solution of dimethyl malonate (24.8 mL, 0.21 mol) in anhyd·THF (150 mL) was cooled to 0 °C and treated with NaH (8.51 g, 0.21 mol). The resulting suspension was then stirred at 0 °C for 15 min and subsequently treated with a solution of **18** (7.55 mL, 0.07 mol) in anhyd·THF (50 mL). The reaction mixture was then heated to reflux for 16 h. Upon completion, the crude reaction mixture was poured into acetic acid (100 mL, 10% aq. soln. *v/v*) then extracted with EtOAc (3 × 25 mL). The organic phase was subsequently washed with sat. $NaHCO_3$ solution (2 × 25 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography (7% EtOAc in hexane) gave **20** (15.86 g, 88%) as a pale yellow solid; R_f 0.77 (50% EtOAc in hexane); mp = 59.7–63.9 °C; IR (neat) υ_{max} 3012, 2958, 2866, 1751, 1613, 1578, 1518, 1431, 1341, 1270, 1199, 1031, 794, 718 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.07, (dd, 1H, J = 8.2, 0.9 Hz, ArH), 7.66 (ddd, 1H, J = 7.6, 7.6, 0.9 Hz, ArH), 7.56–7.51 (m, 2H, ArH), 5.34 (s, 1H, CH), 3.81 (s, 6H, OCH_3); ^{13}C NMR ($CDCl^3$, 100 MHz) δ 167.6 (2C, C═O), 148.8 ($ArCNO_2$), 133.6 (ArCH), 131.4 (ArCH), 129.3 (ArCH), 127.9 (ArC), 125.3 (ArCH), 54.1 (CH), 53.1 (2C, OCH_3); MS (ES +) m/z calcd for $C_{11}H_{11}NO_6$ [M] 253.1. Found [M + Na]$^+$ 276.0; Anal. calcd for $C_{11}H_{11}NO_6$: C, 52.18; H, 4.38; N, 5.53. Found: C, 52.03; H, 4.46; N, 5.58.

Dimethyl 2-(5-fluoro-2-nitrophenyl)malonate (**21**[33]): Compound **21** was synthesized and purified as described above for **20**, starting from compound **19** (10.4 mL, 0.09 mmol). Yield: 86%; R_f 0.18 (10% EtOAc in hexane); mp = 78.8–79.7 °C; IR (neat) υ_{max} 3089, 2965, 2355, 1751, 1720, 1588, 1522, 1435, 1353, 1255, 1157, 1006, 834, 742 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.19 (dd, 1H, J = 9.0, 5.2 Hz, ArH), 7.26 (m, 2H, ArH), 5.43 (s, 1H, CH), 3.86 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.1 (2C, C═O), 164.75 (d, J_{CF} = 258 Hz, ArCF), 144.8 ($ArCNO_2$), 131.3 (d, J_{CF} = 9 Hz, ArC), 128.2/128.1 (J = 10 Hz, ArCH), 118.6 (d, J_{CF} = 25 Hz, ArCH), 116.4 (d, J_{CF} = 24 Hz, ArCH), 54.0 (CH), 53.3 (2C, OCH_3); MS (ES −) m/z calcd for $C_{11}H_{10}FNO_6$ [M] 271.1. Found [M − 1]$^-$ 270.2; Analysis calcd for $C_{11}H_{10}FNO_6$: C, 48.72; H, 3.72; N, 5.16. Found: C, 48.84; H, 3.77; N, 5.18.

2-(2-Nitrophenyl)propane-1,3-diol (**22**[34]): A solution of **20** (5.14 g, 20.3 mmol) in anhyd·THF (56.4 mL) was added dropwise over 30 min to DIBAL-H (84.6 mL, 0.10 mol), under N_2 at 0 °C and stirred for 1 h. A cold solution of HCl (2 M, 100 mL) was added at 0 °C to quench the reaction and the mixture was subsequently extracted with EtOAc (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography (50% EtOAc in hexane) gave **22** (1.54 g, 43%) as a dark golden oil; R_f 0.13 (50% EtOAc in hexane); IR (neat) υ_{max} 3238, 2946, 2889, 2355, 1609, 1513, 1481, 1352, 1229, 1057, 979, 853, 744 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.79 (d, 1H, J = 8.0 Hz, ArH), 7.59–7.52 (m, 2H, ArH), 7.39 (ddd, 1H, J = 8.3, 6.8, 1.5 Hz, ArH), 4.0 (m, 4H, CH_2), 3.57 (quin., 1H, J = 6.3, 5.9 Hz, CH), 2.85 (br s, 2H, OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 150.6 ($ArCNO_2$), 134.2 (ArC), 132.7 (ArCH), 129.1 (ArCH), 127.7 (ArCH), 124.4 (ArCH), 64.7 (2C, CH_2OH), 43.8 (CH); MS (ES −) m/z calcd for $C_9H_{11}NO_4$ [M] 197.1. Found [M^{-1} + 2Na]$^+$ 242.1; Anal. calcd for $C_9H_{11}NO_4$: C, 54.82; H, 5.62; N, 7.10. Found: C, 54.64; H, 5.46; N, 7.18.

2-(5-Fluoro-2-nitrophenyl)propane-1,3-diol (**23**): Compound **23** was synthesized and purified as described above for **22**, starting from compound **21** (2.0 g, 7.38 mmol). Yield: 28%; R_f 0.32 (50% EtOAc in hexane); mp = 79.9–82.2 °C; IR (neat) υ_{max} 3251 (br), 3087, 2954, 2874, 1618, 1586, 1522, 1479, 1345, 1245, 1055, 1003, 926, 878, 837, 699 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.95 (dd, 1H, J = 9.0, 5.2 Hz, ArH), 7.42 (dd, 1H, J = 10.1, 2.8 Hz, ArH), 7.21 (ddd, 1H, J = 9.1, 7.6, 2.8 Hz, ArH), 4.93 (br s, 2H, OH), 3.91 (m, 4H, CH_2OH), 3.61 (m, 1H, CH); ^{13}C NMR (CD_3OD, 100 MHz) δ 165.7 (d, J_{CF} = 252 Hz, ArCF), 149.0 ($ArCNO_2$), 140.1 (d, J_{CF} = 9 Hz, ArC), 128.1 (d, JCF = 10 Hz, ArCH), 117.6 (d, J_{CF} = 24 Hz, ArCH), 115.5 (d, J_{CF} = 24 Hz, ArCH), 64.2 (2C, OCH_2), 46.1 (CH); MS (ES −) m/z calcd for $C_9H_{10}FNO_4$ [M] 215.1. Found [M + HCOO] 260.1; Analysis calcd for $C_9H_{10}FNO_4$: C, 50.24; H, 4.68; N, 6.51. Found: C, 50.07; H, 4.68; N, 4.52.

Methanesulfonic acid 3-methanesulfonyloxy-2-(2-nitrophenyl)-propyl ester (**24**[35]): A solution of **22** (796.6 mg, 3.9 mmol) in anhyd. CH_2Cl_2 (8 mL) was treated with Et_3N (1.62 mL, 11.7 mmol) and cooled to 0 °C. A solution of MsCl (0.9 mL, 11.7 mmol) in anhyd. CH_2Cl_2 (7.5 mL) was prepared and slowly added to the reaction mixture and subsequently stirred for 1 h at 0 °C. The reaction was quenched with H_2O (25 mL) and extracted with EtOAc (3 × 25 mL). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Column chromatography (35% EtOAc in hexane) gave **41** (0.98 g, 71%) as an off-white crystalline solid; R_f 0.30 (50% EtOAc in hexane); mp = 95.9–97.2 °C; IR (neat) υ_{max} 3076, 3033, 2937, 1608, 1519, 1337, 1171, 982, 941,

832, 788 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.93 (dd, 1H, $J = 8.6$, 1.5 Hz, ArH), 7.67 (ddd, 1H, $J = 7.7$, 7.6, 1.3 Hz, ArH), 7.54–7.50 (m, 2H, ArH), 4.59 (m, 4H, CH_2), 4.09 (quin., 1H, $J = 5.8$, 5.7 Hz, CH), 3.02 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 150.0 ($ArCNO_2$), 133.4 (ArCH), 130.2, 129.5 (ArCH), 129.2 (ArCH), 125.3 (ArCH), 67.8 (2C, CH_2), 39.0 (CH), 37.5 (2C, CH_3); MS (ES +) *m/z* calcd for $C_{11}H_{15}NO_8S1$ [*M*] 353.6. Found $[M + Na]^+$ 376.0; Analysis calcd for $C_{11}H_{15}NO_8S1$: C, 37.39; H, 4.28; N, 3.96. Found: C, 37.16; H, 4.20; N, 3.76.

Methanesulfonic acid 2-(5-fluoro-2-nitrophenyl)-3-methane-sulfonyloxy-propyl ester (**25**): Compound **25** was synthesized and purified as described above for **24**, starting from compound **23** (390 mg, 1.81 mmol). Yield: 35%; R_f 0.30 (50% EtOAc in hexane); mp = 99.4–102.1 °C; IR (neat) υ_{max} 3093, 3023, 2938, 1525, 1347, 1330, 1172, 978, 949, 833, 846, 751, 702 cm^{-1}; ^1H NMR (CD_3COCD_3, 400 MHz) δ 8.01 (dd, 1H, $J = 9.1$, 5.2 Hz, ArH), 7.48 (dd, 1H, $J = 9.8$, 2.8 Hz, ArH), 7.28 (ddd, 1H, $J = 9.1$, 7.5, 2.8 Hz, ArH), 4.57 (d, 4H, $J = 6.1$ Hz, CH_2), 4.12 (m, 1H, CH), 3.01 (s, 6H, CH_3); 13C NMR (CD_3COCD_3, 100 MHz) δ 165.4 (d, $J_{CF} = 254$ Hz, ArCF), 147.8 ($ArCNO_2$), 135.6 (d, $J_{CF} = 9$ Hz, ArC), 129.0 (d, $J_{CF} = 10$ Hz, ArCH), 117.9 (d, $J_{CF} = 25$ Hz, ArCH), 116.9 (d, $J_{CF} = 24$ Hz, ArCH), 69.2 (2C, CH_2), 40.2 (CH), 37.2 (2C, CH_3); MS (ES +) *m/z* calcd for $C_{11}H_{14}FNO_8S1$ [*M*] 371.0. Found $[M + Na]^+$ 393.9; Analysis calcd for $C_{11}H_{14}FNO_8S1$: C, 35.58; H, 3.80; N, 3.77. Found: C, 35.77; H, 3.72; N, 3.95.

tert-Butyl 3-methanesulfonyloxymethyl-2,3-dihydroindole-1-carboxylate (**26**): Et_3N (0.05 mL, 0.38 mmol), Boc_2O (85.5 mg, 0.38 mmol) and Pd/C (6.3 mg, 9.4% *w/w*) were added to a solution of **24** (66.9 mg, 0.19 mmol) in anhyd·THF (3.4 mL). The mixture was stirred for 16 h at RT under a positive pressure of H_2. The reaction mixture was filtered through Celite and concentrated *in vacuo*. Column chromatography (15% EtOAc in hexane) gave **26** (43.4 mg, 70%) as a clear, colourless, viscous oil; R_f 0.55 (35% EtOAc in hexane). Characterisation consistent with reference.[36] similar in all respects to the previously reported compound.

tert-Butyl 5-fluoro-3-methanesulfonyloxymethyl-2,3-dihydro-indole-1-carboxylate (**27**): Compound **27** was synthesized and purified as described above for **26**, starting from compound **25** (183.0 mg, 0.49 mmol). Yield: 34% (oil); R_f 0.46 (35% EtOAc in hexane); IR (neat) υ_{max} 3020, 2979, 2938, 1690, 1490, 1394, 1354, 1175, 1143, 961, 846, 814, 783, 736 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.57 (br d, 1H, ArH), 6.92 (m, 2H, ArH), 4.34 (dd, 1H, $J = 9.9$, 5.9 Hz, CH_2), 4.24 (dd, 1H, $J = 9.7$, 7.7 Hz, CH_2), 4.11 (dd, 1H, $J = 11.2$, 9.6 Hz, CH_2), 3.87 (dd, 1H, $J = 11.0$, 3.6 Hz, CH_2), 3.70 (m, 1H, CH) 2.99 (s, 3H, CH_3), 1.55 (br s, 9H, $(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.5 (d, $J_{CF} = 241$ Hz, ArCF), 152.2 (C═O), 115.8 (d, $J_{CF} = 8$ Hz, ArCH), 115.4 (d, $J_{CF} = 22$ Hz, ArCH), 112.1 (ArCH), 81.3 ($C(CH_3)_3$), 70.4 (CH_2OMs), 50.9 (CH_2), 39.3 (CH), 37.6 (CH_3), 28.4 (3C, $C(CH_3)_3$); MS (ES +) *m/z* calcd for $C_{15}H_{20}FNO_5S$ [*M*] 345.1. Found $[M + Na]^+$ 368.1; Analysis calcd for $C_{15}H_{20}FNO_5S$: C, 52.16; H, 5.84; N, 4.06. Found: C, 52.09; H, 5.53; N, 4.25.

4.2. Growth inhibition assays

The human bladder carcinoma cell lines, RT112 and EJ-138, were obtained from the European Collection of Cell Cultures (Salisbury, UK) and were authenticated morphologically. CHO lines were a gift from the late Dr. T. Friedberg, University of Dundee. Cell lines were grown as monolayers in either RPMI 1640 (RT112 and EJ-138) supplemented with 10% (*v/v*) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM of L-glutamine or DMEM (CHO and CHO-1A1) at 37 °C in 5% CO_2. Compounds were dissolved in DMSO and then diluted in complete cell culture medium to give a broad range of concentrations (0.001–100 μM), such that the final DMSO concentration was not greater than 0.1%. Medium was removed from each well and replaced with compound or control solutions, and the well plates were then incubated for a further 96 h before the MTT assay was performed as previously described.[16] Results were expressed in terms of IC_{50} values (concentration of compound required to kill 50% of cells), and all experiments were performed in triplicate.

4.3. Role of CYP in chemosensitivity of CI-based bioprecursors

4.3.1. CYP bactosomes studies

Involvement of specific CYP isoforms in the activation of **16** and **17** was determined by evaluating the chemosensitivity of CYP-generated metabolites of **16** and **17**. Metabolites were created via incubation of **16** and **17** (50 μM) in the reaction mixture (2 mM NADPH, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 20 pmol of CYP1A1, 1A2, 1B1, or 3A4 bactosomes. The latter are human CYP isoforms co-expressed with CYP-reductase in *Escherichia coli*. Concentrations in pmol CYP/mg protein varies depending on isoform used and specific information can be obtained from the manufacturer's protocols (Cypex). Control reactions were carried out using CYP-null bactosomes. Following 1 h incubation at 37 °C, metabolites were extracted using acetonitrile and centrifugation at 10000 *g* for 10 min. The resultant supernatant was removed, dried using vacuum evaporation (Genevac), and the resultant pellet resuspended in DMSO, and the antiproliferative activity was assessed by the MTT assay following 96 h exposure to EJ138 cells as described above.

4.3.2. Human recombinant CYP1A1 studies

A reconstituted protein system (RPS) was created by mixing 200 pmol P450 with 400 pmol hPORG3H6-delta27 and incubated for 10mins at room temperature. A freshly prepared DLPC (240 μM) in assay buffer (100 mM KPi pH 7.4) was added and incubation continued for additional 10 mins at room temperature. The RPS was then added to an eppendorf tube containing assay buffer and substrate (50 μM), at a total volume of 480 μl prior to the addition of NADPH. The mixture was incubated at 37 ºC for 3 mins in a block heater (Grant Block Heater QBD4, UK). NADPH (20 μl of 25 mM stock in assay buffer) was added to the incubating mixture at a final concentration 1 mM. Reaction aliquots (100 μl) were then removed over a 60-minute period into labelled eppendorf tubes containing dichloromethane (200 μl), gently mixed and placed on ice. Tubes were centrifuged (4500 g, 2 mins) with 200 μl of the bottom organic layer carefully removed into separate tubes, and dried using SP Genevac EZ-PLUS evaporator for 30 mins. The dried reaction was dissolved in 50 μl of 90% acetonitrile, 10% H_2O, 0.1% formic acid and transferred into HPLC vial for LC-MS analysis.

LC-MS was carried out using a gradient method with solvent A (90% H_2O, 10% MeOH, 0.1% FA) and solvent B (90% MeOH, 10% H_2O, 0.1% formic acid) and flow rate = 0.30 mL/min; t = 0 (60% A, 40% B), t = 10 min (30% A, 70% B), t = 20 min (10% A, 70% B), t = 25 min (0% A, 100% B) and t = 26 min (60% A, 40% B). Metabolites were run using a HiChrom RPB column (25 cm × 2.1 mm id; HIRPB-250AM; R6125) and a Waters Alliance 2695 HPLC (Micromass, Manchester, UK) with a photodiode array detector and connected in series with Waters Micromass ZQ quadrupole mass spectrometer in ESI^+ mode. Bioprecursors and their respective metabolites were analysed using UV absorbance at 315 nm with their associated masses identified as singularly charged ions on the MS.

MS ESI + source parameters used: Desolvation gas; 650 l/hr, cone gas; 50 l/hr, capillary voltage; 3 kV, extraction voltage; 5 V, cone voltage; 20 V, Rf voltage; 0.2 V, source block temperature; 120 °C and desolvation temperature; 350 °C.

4.4. Preparation of microsomes

4.4.1. Human liver

Approximately 5 g of liver was snap-frozen in liquid nitrogen and ground in a percussion mortar and pestle. Powdered tissue was transferred into an ice-cold glass homogeniser along with 1 mL of homogenisation buffer (50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl, 0.25 M sucrose and two tablets of Complete Protease inhibitor cocktail) for every 0.1 g of ground tissue. The resulting mixture was homogenised with 30 S using a Teflon pestle with the first five strokes at greater

pressure than the rest. The tubes were kept ice-cold during the homogenisation process. The homogenate was diluted to 5 volumes of liver weight (approximately 5 mL) and centrifuged at 2,400 *g* in a Sigma 6 *K*10 centrifuge for 10 min which sediments the cell debris, nuclei and unbroken cells. The supernatant from the first centrifugation was transferred into 70 mL Beckman centrifuge tubes, which were filled to the top with homogenisation buffer. The supernatants were centrifuged at 9,000 *g* in a Beckman L8-60 M centrifuge with a Beckman 45 TI rotor for 20 min to sediment the mitochondrial fraction and any broken fragments. After centrifugation, the supernatant was transferred to ultracentrifuge tubes and centrifuged at 180,000 *g* for 60 min in the Beckman L8-60 M centrifuge with a Beckman 70.1-TI rotor. The upper lipid layer and the cytosolic supernatant were removed and the microsomal pellets were resuspended in microsomal buffer (0.1M Tris-HCl, pH 7.4 containing 15% glycerol, one tablet of Complete Protease inhibitor cocktail) and brought to a final volume of 1 mL. Samples were stored at − 80 °C.

4.4.2. Rat liver

Fresh liver (10 g) from (7–8 week old) female *ACI* rats was washed with cold isotonic saline (0.9% NaCl, 4 °C) to remove blood and connective tissue was excised. The liver was homogenised using an Ultra Turax T25 (Janke and Kunkel, IKA Labortecnik, Staufen, Germany) in 0.01 M Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose, 15% glycerol and 0.67 mM phenylmethanesulphonyl fluoride (PMSF). Having obtained a tissue homogenate, it was centrifuged at 4 °C isolate subcellular fractions. The tissue homogenate was centrifuged at 2,400 *g* for 10 min to pellet intact cells, cell debris, nuclei and unbroken cells. The resultant supernatant was transferred to tubes and centrifuged at 12,000 *g* for 20 min at 4 °C. After centrifugation, the supernatant was transferred to ultracentrifuge tubes and centrifuged at 180,000 *g* for 1 h at 4 °C. The supernatant was then discarded and the microsomal pellet was re-suspended in 0.1 M Tris-HCl buffer, containing 15% glycerol and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4 and stored at − 80 °C. Protein concentration of the resulting rat liver microsomes was determined using the Bradford reagent with bovine serum albumin as the standard.

4.5. Incubation of agents with human and rat liver microsomes

Fresh liver (9–10 g) from male and female outbred 10-week-old Wistar rats (Bantin and Kingman, Hull, UK) was washed with cold isotonic saline (0.9% sodium chloride, 4 °C) to remove blood. Connective tissue was excised and rat liver microsomes were stored on ice. To an ice-cold mixture of 50 mM Tris-HCl buffer, 1 mM $MgCl_2$ and human or rat liver microsomes (approximately 1 mg/mL final protein concentration) at pH 7.4 was added 2 µl of drug solution (for CI-MI drug concentrations = 0.5, 0.9, 1.4, 2.4, 5.6, 11.8, 23.5 µM). The incubation mixture was pre-warmed at 37 °C for 5 min in a water bath after which the reaction was started by adding 2.5 mM NADPH so that the final incubation volume was 200 µl. After 30 min of incubation, the reaction was cooled on ice and centrifuged at 2,000 *g* for 5 min. The resulting supernatant was then used for HPLC-fluorescence detector analysis.

The same procedure was followed using human liver microsomes. The protocol for this study was approved by the Ethics Committee of the Royal Free Hospital and University College School of Medicine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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