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Brief Article

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Re-engineering of the Duocarmycin Structural Architecture Enables Bioprecursor Development Targeting CYP1A1 and CYP2W1 for Biological Activity

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Supporting Information

ABSTRACT: A library of duocarmycin bioprecursors based on the CPI and CBI scaffolds was synthesized and used to probe selective activation by cells expressing CYP1A1 and 2W1, CYPs known to be expressed in high frequency in some tumors. Several CPI-based compounds were pM−nM potent in CYP1A1 expressing cells. CYP2W1 was also shown to sensitize proliferating cells to several compounds, demonstrating its potential as a target for tumor selective activation of duocarmycin bioprecursors.

INTRODUCTION

The cytochromes P450 (CYPs) are responsible for the oxidation of a diverse range of xenobiotic and endogenous compounds. Although CYP 1−3 family members operate mainly to detoxify xenobiotics, isoforms of the CYP1 family are also known to catalyze one of the first steps 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 as well as being associated with the risk of developing cancer. For example, CYP1A1 metabolism of PAHs often generates more reactive intermediates that are capable of binding with DNA and causing genetic mutations.[1] In addition, CYP1A1 has been associated with estradiol metabolism, an event correlated with ovarian cancer pathogenesis.[3] The involvement of CYP1A1 in active metabolite generation and its frequent association with some cancers indicates this enzyme could be a target for locoregionally activated cancer therapeutics.[4−6] CYP2W1 may also be a valuable target because we have shown it to be highly expressed in ∼30% of colon cancers, while the expression in adult nontransformed tissues remains absent.[7] Furthermore, clinical studies show that the extent of CYP2W1 expression represents a prognostic marker for malignancy and survival in colon cancer patients.[8,9]

We have focused our efforts on the natural product family of *seco*-duocarmycins that while showing great promise as ultrapotent cytotoxins have failed to advance clinically due to lack of therapeutic index.[10−12] Their mechanism of action involves spirocyclization of a deep-embedded chloromethylindoline fragment to trigger production of an N3-adenine covalent adduct upon binding to the minor groove of DNA. The spirocyclization event can be halted by blocking the OH group of the *seco*-duocarmycins (i.e., **2**), a strategy that has been employed in tumor-selective prodrug approaches utilizing a bioreduction or deglycosylation to restore activity.[13] Rather than masking this phenolic moiety, our aim is to inactivate the duocarmycins by complete removal of the OH group (i.e., **1**) and to harness the unique capability of selected CYPs to effect regioselective aryl oxidation in order to generate a potent and nonreversible active (hydroxyl) metabolite.[14] Recently, we have demonstrated for the first time that a pharmacophore based on the duocarmycin scaffold is dependent on metabolic hydroxylation by CYP1A1 for biological activity and have shown that the activation pathway is reminiscent of the natural products (Figure 1).[15,16] Here, we report on a library of duocarmycin bioprecursors and their propensity for bioactivation by CYP1A1, 1B1, and 2W1.

RESULTS AND DISCUSSION

Chemistry. The bioprecursors based on the CPI scaffold were synthesized from 5-nitroindole **5** using a version of a synthetic sequence recently reported by us (Scheme 1).[16] Briefly, the indole was protected with Boc and the nitro group reduced to afford protected amino-indole **7**, which was selectively brominated at C-4 by treatment with NBS. The resulting bromoindole **8** was transformed to the bis-Boc protected indole **11** by a high yielding sequence of protecting group manipulations. Alkylation with 1,3-dichloropropene, followed by radical cyclization, gave the protected dehydroxy chloromethylindoline subunit **13**. Deprotection with HCl in

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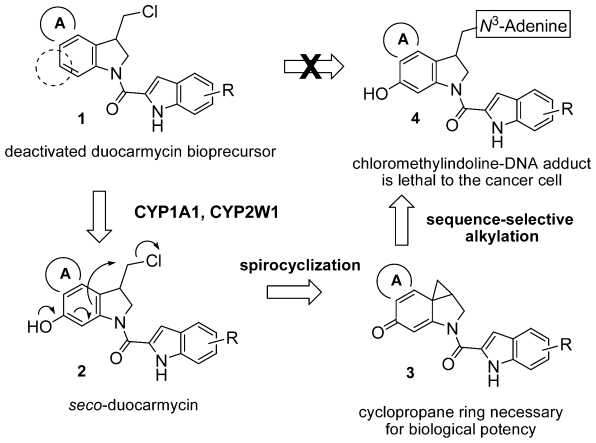

Figure 1. Proposed pathway for CYP activation of duocarmycin bioprecursors (A = any heterocycle or aromatic ring).

EtOAc followed by EDCI-mediated amide bond formation with 2-carboxylic acid indoles, either commercially obtained or prepared using previous published procedures,[17] afforded *seco*-duocarmycin bioprecursor analogues **15**−**23** in moderate yields (Scheme 1). The chloromethylbenzoindoline (CBI) analogue **29** was synthesized from 1-bromo-naphthalen-2-ylamine **24** using previously described conditions (Scheme 1).[18,19]

Chemosensitivity. The duocarmycin bioprecursors were evaluated for their ability to inhibit the growth of a panel of CHO and HEK293 cell lines that were naturally devoid of the CYPs of interest and isogenic variants that were engineered to be proficient in the target enzymes CYP1A1 or 2W1 as previously described.[7,16] In agreement with the literature, the authentic *seco*-CPI-MI and *seco*-CBI-MI compounds were shown to possess IC_{50} values in the pM range, consistent with the functional requirement of the hydroxyl group for spirocyclization of the CBI and CPI pharmacophores. In contrast, all CPI-based bioprecursors displayed low antiproliferative activity in the 1−25 μM range against CHO cells not expressing the target CYPs (Table 1). This is consistent with our previous report[16] that showed **15** to possess only a weak association with the DNA minor groove and poor antiproliferative activity in ovarian cancer cells. In the CYP1A1-expressing CHO cells, it was evident that the CBI-based **29** was not activated to the same extent compared with its direct CPI-based analogue **15**. This indicates that the replacement of an indole with a naphthyl moiety, as in the CBI scaffold, removes a nitrogen-center possibly critical for coordinating the CYP1A1 catalytic heme toward hydroxylation at the alkylating subunit. The data obtained from the CHO-1A1 cells revealed an interesting structure−activity relationship (SAR), with 5-Cl or $-OCH_3$ monosubstituted analogues possessing sub-nM (**15**, **21**) antiproliferative activity, whereas the 5-OH monosubstituted analogue (**22**) in comparison was significantly less potent (IC_{50} = 300 nM). This is unlikely to be due to differences in cell uptake and the associated availability for oxidation by CYP1A1 because we previously showed that cell accumulation of **15** and *seco*-CPI-MI, which differ only by a hydroxy group, was similar.[16] However, it may be a result of the diminished lipophilicity of **22** in comparison to **15** or **21** with reduced DNA affinity as a result. Disubstitution of OCH_3 at positions 5 and 6 (**16**) did not lead to any loss in activity when compared with the most potent compound (**15**) although the disubstituted fluorine analogue (**18**) was significantly less potent (IC_{50} = 0.23 μM). The natural duocarmycin DNA recognition motif (DNA-RM) comprising 5,6,7-trimethoxy substitution and a trimethoxy derivative (**17**) was also investigated here, but no significant bioactivation in the presence of CYP1A1 was evident. Furthermore, compounds with substitution at positions 5 and 7 (**19**, **23**) were also inactive in the CYP1A1-transfected cells, suggesting that functionalization at position 7 may pose steric hindrance and poor interaction with CYP1A1. The compounds were also assessed in RT112 bladder cancer cells, which naturally express moderate levels of CYP1A1.[15] Although the compounds were less potent than in the CHO-1A1 cell line, the SAR pattern was similar, with analogues **15**, **16**, and **21** being the most potent.

Next, we investigated CYP2W1 because there is justification that this enzyme would share a similar substrate affinity profile with CYP1A1 based on their active site sequence homology.[20] CYP2W1 does have a propensity for xenobiotic metabolism[20] including indoles[21] and may contribute to the pharmacological activity of the 2-arylbenzothiazole-based Phortress and GW-610 compounds.[22,23] Using 2W1-transfected HEK293 cell line in the chemosensitivity screen, the antiproliferative activity of three compounds (**15**, **20**, **21**) was shown to be potentiated in the presence of CYP2W1 (Table 1). **16**, one of the most potent CYP1A1-activated bioprecursors, was not a good substrate for

Scheme 1. Synthesis of Duocarmycin Bioprecursors Based on the CPI and CBI-Based Pharmacophores (See Table 1 for Specific Structures)

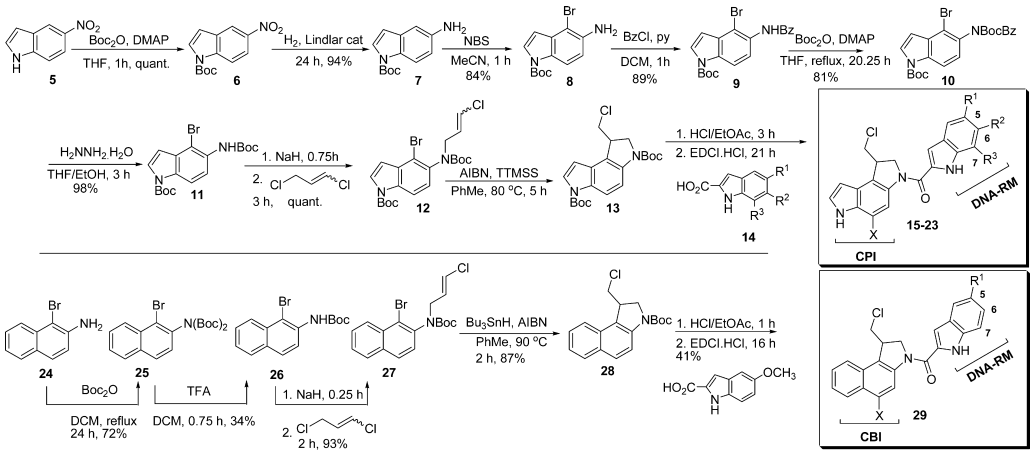

Table 1. Growth Inhibition of Chloromethylindolines against a Panel of Parental CHO and HEK293 Cell Lines and Their CYP1A1 and 2W1-Transfected Variants

	substitution					CHO			RT112	HEK293		
compd ID	unit	X	R^1	R^2	R^3	wt	1A1	PF[b]	1A1	wt	2W1	PF
15	CPI	H	OCH_3	H	H	3.2 ± 0.3[a]	<0.001	>3200	0.2 ± 0.0	8.8 ± 2.6	0.6 ± 0.6	14.7
16	CPI	H	OCH_3	OCH_3	H	3.0 ± 0.4	<0.001	>3000	0.3 ± 0.1	7.7 ± 1.9	6.1 ± 2.5	1.3
17	CPI	H	OCH_3	OCH_3	OCH_3	7.5 ± 1.6	3.1 ± 2.9	2.4	7.0 ± 3.9	11.8 ± 3.6	11.1 ± 3.1	1.1
18	CPI	H	F	F	H	18.4 ± 4.8	0.23 ± 0.1	80	1.8 ± 0.1	9.5 ± 3.2	3.7 ± 2.8	2.6
19	CPI	H	F	H	F	21 ± 5.2	>1	<21	12.3 ± 0.8	10.1 ± 2.8	9.1 ± 3.2	1.1
20	CPI	H	F	H	H	22.6 ± 5.8	0.09 ± 0.0	251	11.1 ± 0.9	10.5 ± 3.4	0.5 ± 0.02	21
21	CPI	H	Cl	H	H	23.2 ± 3.3	<0.001	>23200	0.7 ± 0.5	12.5 ± 3.6	0.3 ± 0.02	41.7
22	CPI	H	OH	H	H	3.3 ± 0.7	0.30 ± 0.3	11	32.7 ± 12.4	16.6 ± 4.1	20.5 ± 3.6	0.8
23	CPI	H	F	H	SO_2CH_3	3.2 ± 0.5	4.1 ± 1.2	0.8	7.7 ± 1.0	11.5 ± 3.0	12.2 ± 3.1	0.9
seco-CPI-MI	CPI	OH	OCH_3	H	H	167 ± 18.9	624 ± 178.6	0.2	125 ± 11.4	453 ± 0.3	376 ± 287.7	1.2
29	CBI	H	OCH_3	H	H	>50[c]	0.96 ± 0.7		>50[c]	>50[c]	>50[c]	
seco-CBI-MI	CBI	OH	OCH_3	H	H	194 ± 30.0	102 ± 1.9	1.9	151 ± 13.9	137 ± 71.4	641 ± 123.3	0.2

[a] IC_{50} values for bioprecursors (μM), CBI (pM), and CPI (pM) are the mean ± SD of at least three independent assays; dose−response curves for **15**, **16**, **17**, CBI, and CPI are shown in SI Figures S1 and S2. [b] Potentiation factor (IC_{50} in mock-transfected cells/CYP1A1 or 2W1-transfected cells). [c] The compound precipitated above 50 μM.

CYP2W1-activation, while **22**, which possessed moderate CYP1A1-mediated activity, was not active in CYP2W1-expressing cells. Given that the 5-monosubstituted bioprecursors **15**, **20**, and **21** were the only analogues potentiated in CYP2W1-transfected cells, the results suggests that functionalization at position 6 and 7 is likely to cause steric hindrance and poor interaction with CYP2W1.

Nonetheless, the results obtained here suggest that selectivity for targeting CYP2W1 over CYP1A1 can be achieved.

DNA Damage and Cell Death. To obtain a valuable SAR between DNA damage and cell death, we decided to evaluate the methoxy-functionalized bioprecursors **15**−**17**. RT112, HEK293-mock, and 2W1-transfected cells were treated with **15**−**17** for 48 h, and the appearance of phosphorylated H2A.X histone (γH2A.X), a positive indicator of DNA damage, was analyzed by immunodetection, while trypan blue uptake assay was used as a measure of cell death. The data obtained (Figure 2) revealed a clear trend showing that **15** was the most potent bioprecursor of DNA damage and cell death followed by the di- and trisubstituted methoxy analogues. Significantly, no accumulation of γH2A.X was detectable in the HEK293-mock transfected cells. To demonstrate the capacity of CYP2W1 in the bioactivation of **15**, the compound was incubated with recombinant CYP2W1 and cumene hydroperoxide, a commonly used constituent employed to reconstitute an electron donor system.[24] As illustrated in Figure 3, CYP2W1 was capable of oxidizing **15** to a product with m/z 360.1, which is consistent with the formation of the active spirocyclized CPI-MI molecule.

CYP Metabolism of the CBI Pharmacophore. Given that **29** is potently activated by CYP1A1, we further evaluated the CYP selectivity of this CBI-based member of the duocarmycin family. Bioprecursor **29** was incubated with several bactosomes (CYP null, 1A1, 1A2, 1B1, 3A4, and 2D6) for 1 h at 37 °C. Any metabolites of **29** produced following incubation with specific CYP bactosomes were extracted and added to EJ-138 bladder cancer cells (null for CYP1−3 family members).[15] This resulted in 100-fold potentiation by CYP1A1, corroborating the CHO-1A1 cell data. Neither CYP1A2, 1B1, 2D6, nor 3A4 potentiated the antiproliferative activity of **29**, indicating CYP1A1 selectivity even over CYP1A2, a hepatic CYP1 family member with over 70% sequence homology (Table 2). Next, we showed

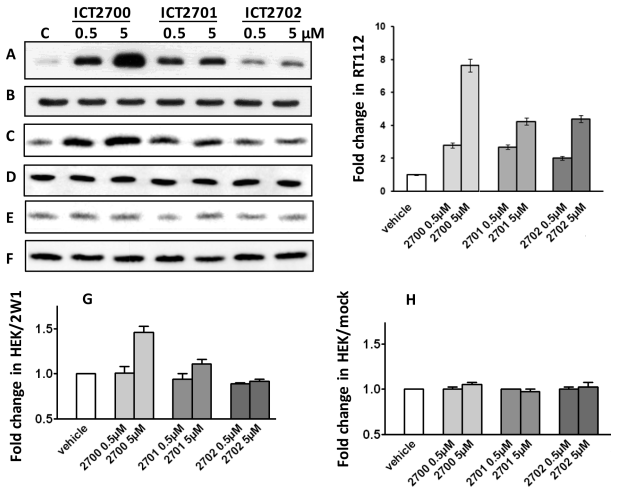

Figure 2. γ-H2AX induction and cell death after 48 h treatment with **15**−**17**. γ-H2AX induction in RT112 (A), HEK/2W1 (C), or HEK-mock cells (E) as determined by Western blotting. β-Actin and ERp29 were used as loading control in RT112 (B), HEK/2W1 (D), and HEK/mock cells (F), respectively. V is vehicle (DMSO). Cell death was measured by the trypan blue assay in RT112 (F), HEK/2W1 (G), or HEK/mock cells (H).

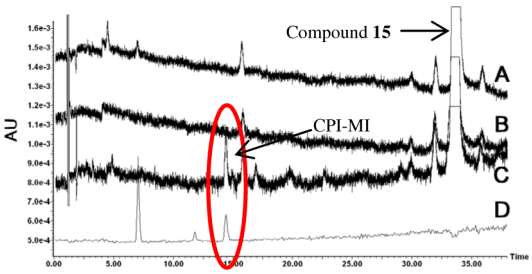

Figure 3. Metabolite profiles following incubation of **15** with CYP2W1 and CuOOH. (A) **15** (100 μM) and CuOOH (0.5 mM); (B) **15** (100 μM) and CYP2W1 (20 pmol); (C) **15** (100 μM), CuOOH (0.5 mM), and CYP2W1 (20 pmol); (D) channel D when extracted using specific SIR channels at m/z 360.1 (the toxic spirocyclized metabolite).

Table 2. Growth Inhibition of Bioprecursor 29 after Incubation with CYP Bactosomes against EJ-138 Bladder Cancer Cell Line

	EJ-138	control bactosomes	EJ-138 + 1A1		EJ-138 + 1A2		EJ-138 + 1B1		EJ-138 + 2D6		EJ-138 + 3A4	
compd ID	IC_{50}[a]	IC_{50}	IC_{50}	PF[b]	IC_{50}	PF[a]	IC_{50}	PF[a]	IC_{50}	PF[a]	IC_{50}	PF[a]
29	2.88	1.99	0.02	100	2.23	0.9	1.53	1.30	2.53	0.79	1.04	1.91

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

that the hydroxylated *seco*-CBI precursor could be observed directly by LCMS detection after incubation of **29** in the presence of CYP1A1 bactosomes for 1 h at 37 °C (Supporting Information (SI) Figure S3). Although the use of a panel of recombinant CYP proteins was a useful indicator of potential CYP involvement in activation of **29**, it does not fully address the multiple CYP interactions present in the liver, the major site of drug metabolism. Clearly the opportunity for tumor selective activation of duocarmycin bioprecursors must avoid activation by the liver because this could result in systemic toxicity. Accordingly, **29** was incubated in the presence of mouse liver homogenate or microsomal fraction, both representing a rich source of drug metabolizing CYP enzymes. Both homogenate and microsomes produced a similar metabolite profile indicating extensive metabolism (see SI Figures S4 and S5) although fewer metabolites were observed in the homogenate, which is likely to be a result of the lower CYP content per mg of homogenate. Importantly, the active metabolite of **29** was not produced by either homogenate or microsomes and provides evidence to support tumor selective activation.

Discussion. 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 tumor tissue. Several clinically used cancer chemotherapeutics, notably the DNA alkylating oxazaphosphorines and nitrosoureas, are known to be metabolized by CYPs to reactive intermediates, however primarily in the liver.[5,25] This systemic CYP metabolism is associated with profound tissue toxicity and lack of tumor selectivity. The continued importance of cytotoxic chemotherapy in management of cancer has identified a need for more tumor-selective treatments. Furthermore, the increasing ability to identify the expression of specific targets associated with patient subgroups provides a basis for personalized therapy. CYP1A1 and 2W1 are examples of monooxygenases that are highly expressed in bladder[15] and colon[7,9,20] cancer respectively and hence provide a rational basis for discovery of CYP-selective chemotherapeutic activation and the ultrapotent duocarmycins are ideal candidates for CYP mediated bioprecursor drug development.[13] In our previous work,[16] we demonstrated CYP1A1-mediated activation of a duocarmycin analogue capable of generating biological activity via DNA damage and showed that no hepatic CYP was contributing to this activity.[15] Here, data obtained for the naphthalene-based analogue **29** revealed that the propensity for CYP1A1-activation was at least 500-fold less with regard to antiproliferative potentiation compared with **15**. Importantly, although **29** was shown to undergo hepatic metabolism, no cytotoxic metabolite was observed to be generated in mouse liver microsomes or homogenate, demonstrating its potential for tumor-specific activation. We also prepared CPI analogues of **15** that differed in their substitution pattern of the DNA-RM and have shown that subtle changes to the substitution pattern can result in alteration of the CYP-mediated increase in the cytotoxicity observed. CYP1A1-mediated bioactivation seemed to be affected by functionalization at position 7, while CYP2W1-bioactivation was intolerant to substitution in positions 6 and 7, indicating that these enzymes do not metabolize and/or activate these bioprecursors to the same extent. These results are important in targeting CYP2W1 over 1A1 (or vice versa) and suggest that further re-engineering of the *seco*-duocarmycin bioprecursors could lead to even greater selectivity and ultimately tumor CYP-specific targeting.

■ EXPERIMENTAL SECTION

All compounds were analyzed by ^{1}H NMR. Compounds that had not previously been reported and the final target compounds (**15**−**23** and **29**) were analyzed by ^{13}C NMR, LRMS, and HRMS. The purity of all compounds was ≥95% as measured by HPLC. Full experimental protocols can be found in the SI.

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. All three 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-1A1) at 37 °C in 5% CO_2. The Flp-In system (Invitrogen) was used for the generation of the human embryonic kidney HEK293-2W1 cell line, which was transfected as previously described.[7] 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.

Role of CYPs in Chemosensitivity of 29. Involvement of specific CYP isoforms in the activation of **29** was determined by evaluating the chemosensitivity of CYP-generated metabolites of **29**. Metabolites were created via incubation of **29** (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, 2D6, or 3A4 bactosomes (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 EJ-138 cells as described above.

γH2A.X Phosphorylation and Cell Death. RT112, HEK293-mock, and 2W1-transfected cells were lysed at 4 °C for 15 min in 1% Triton X-100 in PBS with addition of a protease inhibitors cocktail (Roche) or in RIPA buffer with subsequent centrifugation at 13000*g*. Cell lysates were centrifuged at 13000*g* for 10 min. The aliquots of supernatants were subjected to SDS-PAGE and subsequent immunoblotting using CYP2W1, γH2A.X, β-actin, or ERp29 antibodies. Cell death was measured by use of the trypan blue assay according to manufacturer's protocol (Sigma Aldrich).

CYP2W1 Metabolism of 15. CYP2W1 (20 pmol) was incubated with **15** (25 or 50 μM) in sodium phosphate buffer (pH 7.4) in a total volume of 400 μL. After 3 min preincubation at 37 °C, the catalytic

reactions were initiated by the addition of CuOOH (125 μM). After 30 min, the reactions were stopped by snap freezing. Control incubations included (i) **15**, CYP2W1 but no CuOOH, and (ii) **15**, CuOOH but no CYP2W1.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of synthetic methods used to prepare target compounds and metabolism data associated with evaluation of bioprecursor **29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CBI, chloromethylbenzoindoline; CHO, Chinese hamster ovary; CPI, chloromethylpyrroloindoline; CYP, cytochrome P450; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HEK, human embryonic kidney; TTMSS, tris(trimethylsilyl)-silane

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