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

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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, including polycyclic aromatic hydrocarbons (PAHs), nitroaromatics, and arylamines. 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. In addition, CYP1A1 has been associated with estradiol metabolism, an event correlated with ovarian cancer pathogenesis. 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. 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. Further, clinical studies show that the extent of CYP2W1 expression represents a prognostic marker for malignancy and survival in colon cancer patients.

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. 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 bioreduction or deglycosylation to restore activity. 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. Recently, we have demonstrated for the first time that a pharmacophore based on the duocarmycin scaffold is dependent on metabolic hydroxylaton by CYP1A1 for biological activity and have shown that the activation pathway is reminiscent of the natural products. 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). 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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Supporting Information

Supporting Information
EtOAc followed by EDCI-mediated amide bond formation with 2-carboxylic acid indoles, either commercially obtained or prepared using previous published procedures, 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 using previously described conditions (Scheme 1).

**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 express the target CYPs (Table 1). This is consistent with the functional requirement of the hydroxyl group for 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. 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. CYP2W1 does have a propensity for xenobiotic metabolism including indoles and may contribute to the pharmacological activity of the 2-arylbenezothiazole-based Phortress and GW-610 compounds. 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). One of the most potent CYP1A1-activated bioprecursors, was not a good substrate 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. 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 OCH₃ 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 (IC₅₀ = 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. 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. CYP2W1 does have a propensity for xenobiotic metabolism including indoles and may contribute to the pharmacological activity of the 2-arylbenezothiazole-based Phortress and GW-610 compounds. 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). 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)**
Brief Article

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

<table>
<thead>
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<th>compd ID</th>
<th>substitution</th>
<th>CHO</th>
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<th>HEK293</th>
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<tr>
<td></td>
<td></td>
<td>wt</td>
<td>IA1 M</td>
<td>IA1 M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>CPI H OCH3 H H</td>
<td>3.2 ± 0.3 &lt;0.001</td>
<td>&gt;3200</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>16</td>
<td>CPI H OCH3 OCH3</td>
<td>3.0 ± 0.4 &lt;0.001</td>
<td>&gt;3000</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>17</td>
<td>CPI H OCH3 OCH3</td>
<td>7.5 ± 1.6 3.1 ± 2.9</td>
<td>2.4</td>
<td>7.0 ± 3.9</td>
</tr>
<tr>
<td>18</td>
<td>CPI H F F H</td>
<td>18.4 ± 4.8 0.23 ± 0.1</td>
<td>80</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>19</td>
<td>CPI H F H F</td>
<td>21 ± 5.2 &gt;1</td>
<td>21</td>
<td>12.3 ± 0.8</td>
</tr>
<tr>
<td>20</td>
<td>CPI H F H H</td>
<td>22.6 ± 5.8 0.09 ± 0.0</td>
<td>25</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>21</td>
<td>CPI H Cl H H</td>
<td>23.2 ± 3.3 &lt;0.001</td>
<td>&gt;23000</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>22</td>
<td>CPI H OH H H</td>
<td>3.3 ± 0.7 0.30 ± 0.3</td>
<td>11</td>
<td>32.7 ± 12.4</td>
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<tr>
<td>23</td>
<td>CPI H F H SO3CH3</td>
<td>3.2 ± 0.5 4.1 ± 1.2</td>
<td>0.8</td>
<td>7.7 ± 1.0</td>
</tr>
<tr>
<td>src-CPI-MI</td>
<td>CPI OH OCH3 H H</td>
<td>167 ± 18.9 624 ± 178.6</td>
<td>0.2</td>
<td>125 ± 11.4</td>
</tr>
<tr>
<td>24</td>
<td>CBI H OCH3 H H</td>
<td>&gt;50</td>
<td>96 ± 0.7</td>
<td>&gt;50</td>
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<tr>
<td>src-CBI-MI</td>
<td>CBI OH OCH3 H H</td>
<td>194 ± 30.0 102 ± 1.9</td>
<td>1.9</td>
<td>151 ± 13.9</td>
</tr>
</tbody>
</table>

*IC50 values for bioprecursors (μM), CPI (μM), and CBI (pM) are the mean ± SD of at least three independent assays; dose–response curves for 15, 16, 17, CPI, and CBI are shown in SI Figures S1 and S2. Potentiation factor (IC50 in mock-transfected cells/CYP1A1 or 2W1-transfected cells). 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 suggest 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 trypsin 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. 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 spirocycled 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). This resulted in 100-fold potentiation by CYP1A1, corroborating the CHO-1A1 cell data. Neither CYP1A2, 1B1, 2D6, nor 3A4 potentiates 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...
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 associated with patient subgroups provides a basis for personalized therapy. CYP1A1 and 2W1 are examples of CYP isoforms in the activation 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 NAPDH, 1 mM MgCl₂, 50 mM Tris-HCl (pH 7.4), 20 pmol of CYP1A1, 1A2, 2B1, 2D6, or 2A4 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 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.

Role of CYPs in Chemosensitivity of 29. 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 bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine or RPMI 1640 (RT112 and EJ-138) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 2 mM ofL-glutamine. Role of CYPs in Chemosensitivity of 29.

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

<table>
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<tr>
<th>compd ID</th>
<th>EJ-138</th>
<th>control bactosomes</th>
<th>EJ-138 + 1A1</th>
<th>EJ-138 + 1A2</th>
<th>EJ-138 + 1B1</th>
<th>EJ-138 + 2D6</th>
<th>EJ-138 + 3A4</th>
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<tbody>
<tr>
<td>29</td>
<td>IC₅₀</td>
<td>2.88</td>
<td>1.99</td>
<td>0.02</td>
<td>100</td>
<td>2.23</td>
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<td>1.91</td>
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</table>

"IC₅₀ = μM, bPotentiation factor (IC₅₀ in EJ-138 + control bactosomes/IC₅₀ in EJ-138 + CYP isoform metabolites)."
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; TT MSS, tris(trimethylsilyl)-silane

REFERENCES