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Synthesis and evaluation of prodrugs for anti-angiogenic pyrrolylmethylidenyl oxindoles

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Abstract—Potential prodrugs of inhibitors of VEGF-induced angiogenesis have been investigated. The prodrug systems studied were the 4-nitrobenzyl, 2-nitrophenylacetyl and 3-methyl-3-(3,6-dimethylbenzo-1,4-quinon-2-yl)butanoyl groups, readily attached to acidic OH or NH groups in drug molecules, and released upon bioreductive activation. The anti-angiogenic compounds studied were the pyrrolylmethylidenyl oxindole SU5416 (semaxanib) and its novel 6-hydroxy derivative. The potentially pro-anti-angiogenic compounds were assayed for their ability to block VEGF-induced angiogenesis in HUVECS in comparison to the free agents.

There is much current interest in the design of prodrug systems for use in cancer therapy.[1] Such prodrugs have the potential to improve selectivity of chemotherapeutic agents and hence reduce unwanted side effects. A prodrug strategy that uses bioreduction of readily reducible compounds, such as nitroarenes or quinones, to release the active drug from its prodrug has been widely investigated.[2–6] One approach relies on the combination of hypoxia and the upregulation of reductase enzymes to effect a tumour selective reduction that releases the active species. Alternatively, an exogenous activating enzyme is delivered to tumour cells using antibody or gene therapy (ADEPT or GDEPT), with the most common exogenous reductase being a bacterial nitroreductase.

In continuation of our work on bioreductively activated drugs,[7–11] and on anti-angiogenic agents,[12] we have initiated a study of potential prodrugs of inhibitors of (vascular endothelial growth factor) VEGF-induced angiogenesis. The prodrug systems chosen for the initial study were the 4-nitrobenzyl **1**,[13,14] 2-nitrophenylacetyl **2** (R = H and Me) and 3-methyl-3-(3,6-dimethylbenzo-1,4-quinon-2-yl)butanoyl **3**[15–18] groups, readily attached to acidic OH or NH groups in drug molecules (Fig. 1).

A critical aspect of tumour growth is the development of the microvasculature as recognized by Folkman some 35 years ago.[19,20] Angiogenesis is a prerequisite for tumours to grow beyond the minimum volume, and is stimulated by a number of factors. However, there is considerable evidence that VEGF is a major contributor to solid tumour growth by promoting both angiogenesis and vascular permeability.[21,22] Hence the inhibition of VEGF expression, induction or function represents an attractive target for the development of novel therapeutic agents. To date a number of small molecule inhibitors of VEGF receptor tyrosine kinases have been developed and are in clinical trial, although the only FDA-approved drug is Avastin® (bevacizumab), a monoclonal antibody.

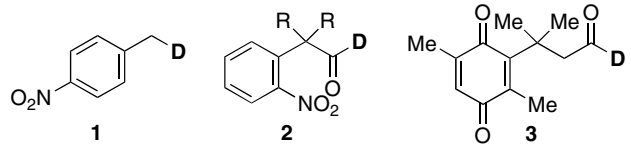

Figure 1. Nitroarene and benzoquinone based prodrug systems; **D**, drug molecule.

Keywords: Oxindole; Angiogenesis; Prodrug; Bioreduction.
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The anti-angiogenic compounds chosen for study were the pyrrolylmethylidene oxindoles SU5416 **4** and its novel 6-hydroxy derivative **5**(Fig. 2). The oxindole SU5416 **4** is a potent inhibitor of the VEGF receptor tyrosine kinase Flk-1/KDR, and a proven anti-angiogenic agent,[23–26] and the compound, now known as semaxanib, is in phase 1 clinical trial.[27–29] Oxindoles are readily functionalized through their NH group, although we also prepared the novel 6-hydroxy derivative **5** to provide an alternative point of attachment of the prodrug moiety.

Several new derivatives of SU5416 **4**, obtained from condensation of oxindole with 3,5-dimethylpyrrole-2-caboxaldehyde,[30] were prepared. The *N*-benzyl and 4-nitrobenzyl compounds **6** and **7** were obtained by alkylation reactions. Nitro-substituted phenylacetic acids were attached to SU5416 using Mitsunobu coupling protocols to give compounds **8**–**10**, the dimethyl substituted precursor to **10** being prepared by a literature method.[31] Finally the quinone derivative **11** was obtained by coupling to 3-methyl-3-(3,6-dimethyl-1,4-benzoquinon-2-yl)butanoic acid[32] (Scheme 1). The benzyl and 3-nitrophenylacetyl derivatives were prepared as controls since they cannot participate in reductively activated fragmentations. This is particularly important in the case of the nitrophenylacetate esters since they could also release the free drug by a hydrolytic rather than a reductive process. No attempt was made to optimize the rather poor yields observed in some of these coupling reactions.

The novel oxindole, the 6-hydroxy derivative **5** of SU5416, was also studied. The starting material, 6-hydroxyoxindole,[33] was converted into the corresponding benzyl ether, reaction of which with 3,5-dimethyl pyrrole-2-aldehyde gave the SU5416 derivative **12**. Alternatively, reaction of 6-hydroxyoxindole with the pyrrole aldehyde gave 6-hydroxy SU5416 **5** in good yield. Subsequent reactions of **5** with 4-nitrobenzyl bromide, 2-nitrophenylacetic acid or the aforementioned benzoquinonylbutanoic acid gave the novel derivatives **13**–**15** (Scheme 2).

In order to assess the ability of the various prodrug systems to release their phenolate or oxindole leaving group upon reductive activation, selected compounds were subjected to a simple chemical reduction. Thus, the 4-nitrobenzyl compound **7** was treated with indium powder in ethanolic ammonium chloride solution, an efficient method for the reduction of nitroarenes,[34] and the reaction monitored by NMR spectroscopy. Reduction proceeded cleanly to give the corresponding 4-aminobenzyl derivative that did not fragment further. In contrast, indium reduction of the 2-nitrophenylacetyl derivative **9** did result in release of free SU5416 **4**. In related systems, it was also established that chemical reduction of the 3-methyl-3-(3,6-dimethyl-1,4-benzoquinon-2-yl)butanoyl compounds with sodium dithionite resulted in successful release of the drug (Figs. 1 and 2).

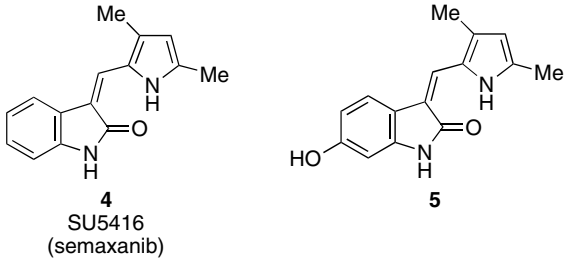
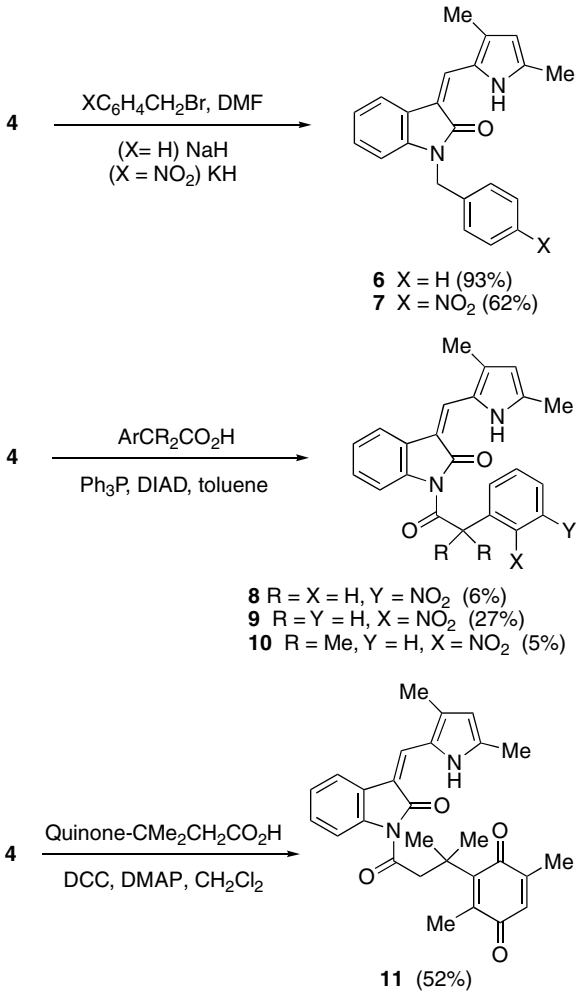
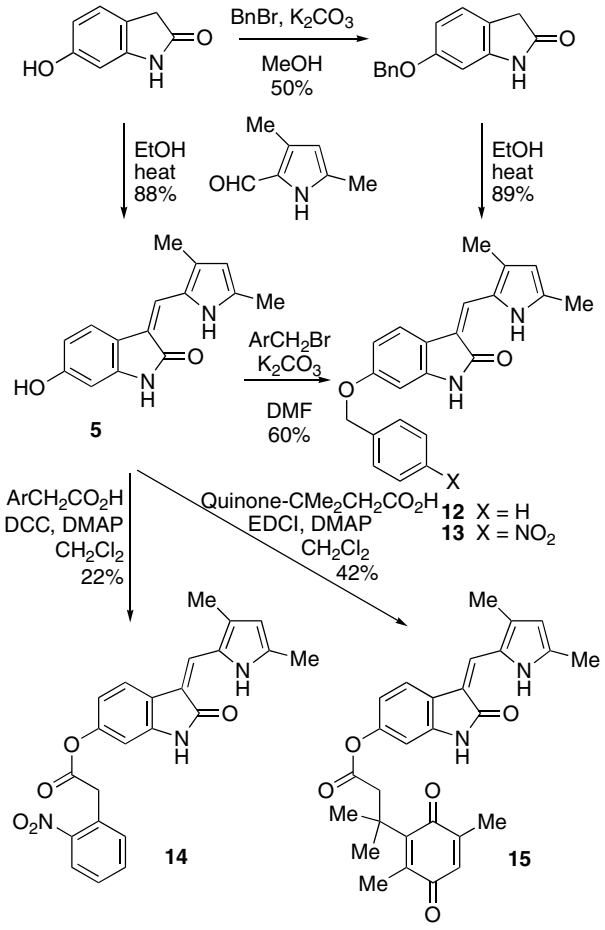


Figure 2. Pyrrolylmethylidene oxindoles SU5416 **4** and its 6-hydroxy derivative **5**.



Scheme 1. Synthesis of SU5416 derivatives **6**–**11**.

All the novel potentially pro-anti-angiogenic compounds **6**–**15** were assayed for their ability to inhibit VEGF-induced angiogenesis in human umbilical vein endothelial cells (HUVECs) in comparison to the free agents **4** and **5**. This assay examines the ability of endothelial cells grown on a fibrin matrix to form 3-dimensional structures when stimulated by VEGF. The ability of each compound to inhibit this angiogenesis at a range of concentrations was then examined. HUVECs have been reported to express a range of bioreductive enzymes including NQO1[35,36] and cytochrome c reductase (unpublished data from this laboratory) indicating that the cellular systems for bioreduction of the prodrugs are present.



Scheme 2. Synthesis of 6-hydroxy SU5416 **5** and derivatives **12**–**15**.

The results are summarized in Figures 3 and 4. The SU5416 (semaxanib) series of compounds are generally quite potent, with free SU5416 **4** itself causing 94% inhibition of VEGF-stimulated angiogenesis at 1 μM (Fig. 3). The benzyl derivative **6** that cannot undergo bioreductive fragmentation is considerably less potent than the free drug, consistent with our earlier study,[12] whilst its 4-nitrobenzyl analogue **7**, a potential prodrug candidate, not only exhibits a dose response but is also significantly more potent than **6**, suggesting that bioreductive release does occur. The similarity between the 3-and 2-nitrophenylacetyl compounds **8** and **9** suggests that the compounds may be undergoing hydrolytic rather than reductive fragmentation. The most potent compound is the benzoquinone derivative **11**, the compound most likely to undergo efficient reductive fragmentation, although hydrolytic release cannot be ruled out.

The novel 6-hydroxy derivatives of SU5416 **5** and **12**–**15** all appear equipotent with similar dose response profiles (Fig. 4). Although the benzyl compound **12** cannot be readily cleaved under reductive conditions, on the basis of the aforementioned studies, we believe that compounds **13**–**15** do release the free drug. However, at present we cannot differentiate between the planned reductive fragmentation and the alternative hydrolytic cleavage of the ester bonds in prodrugs **14** and **15**. The fact that such phenyl esters hydrolyse readily is a possible limitation in their use. A small number of 6-substituted derivatives of pyrrolylmethylidenyl oxindoles have been investigated previously; in the SU5416 series, the 6-fluoro-analogue is reported to be slightly more potent than its 6-unsubstituted counterpart,[23] whereas in the closely related SU6668 series of compounds that contain an additional 2-carboxyethyl group at the pyrrole 4-position, introduction of a large aryl substituent at the oxindole 6-position appears to increase potency.[24] Interestingly, the 5-hydroxy derivative of SU5416, a metabolite of the drug, has also been investigated in multiple sclerosis model systems.[37]

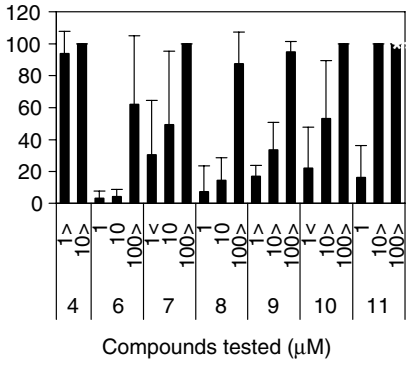


Figure 3. Inhibition of VEGF-stimulated angiogenesis in HUVECs by SU5416 **4** and derivatives **6**–**11**. HUVECs were seeded onto preformed fibrin matrices and treated with 100 ng/ml VEGF ± anti-angiogenic compounds at 1, 10, or 100 μM. After a maximum of 5 days, the formation of tubular structures was quantified. At each concentration the results are presented only for those assays in which cell growth was unaffected by the compound as assessed visually. Results are expressed as percentage inhibition of a VEGF control and show means ± SD. [∧ signifies $P \leqslant 0.005$, ∨ signifies $P < 0.05$, $n = 5$].

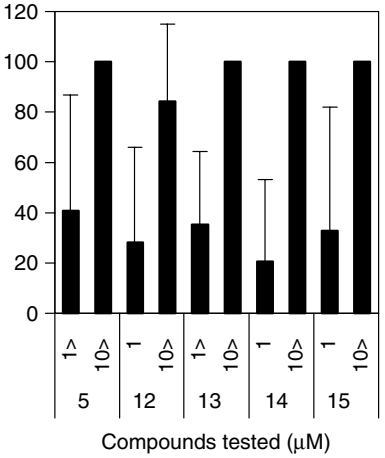


Figure 4. Inhibition of VEGF-stimulated angiogenesis in HUVECs by 6-hydroxy SU5416 **5** and derivatives **12**–**15**. Results are expressed as percentage inhibition of a VEGF control and show means ± SD. [∧ signifies $P \leqslant 0.005$, ∨ signifies $P < 0.05$, $n = 6$].

We have synthesized and evaluated novel derivatives of the anti-angiogenic compound SU5416 (semaxanib) as potential prodrug systems that can be bioreductively activated. Under chemical reducing conditions, the 3-methyl-3-(3,6-dimethyl-1,4-benzoquinon-2-yl)butanoic

acid based prodrugs appear to fragment most efficiently, followed by the 2-nitrophenylacetate esters with the 4-nitrobenzyl ethers being the least efficient. In cellular systems all of the compounds that fragment rapidly by bioreduction significantly inhibited VEGF-stimulated angiogenesis at concentrations comparable to their parent compound (SU5416 10 μM; 6-hydroxy SU5416 10 μM) suggesting that in all cases the active drug is being released in a biological system. Several of the compounds have useful anti-angiogenic activity, and form the basis of further study.

Acknowledgments

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