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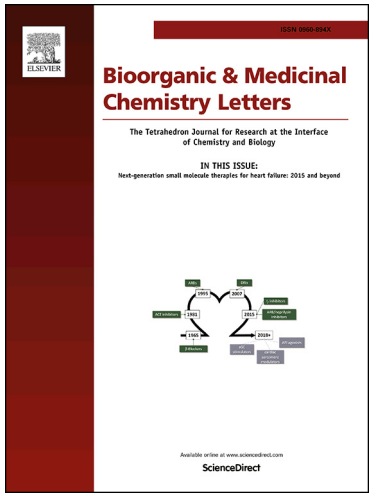

The synthesis of 4,6-diaryl-2-pyridones and their bioactivation in CYP1 expressing breast cancer cells

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The synthesis of 4,6-diaryl-2-pyridones and their bioactivation in CYP1 expressing breast cancer cells

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ABSTRACT

As part of a programme to develop anticancer prodrugs which are activated by cytochrome P450 (CYP)1B1, a library of 4,6-diaryl-2-pyridones was synthesised in yields of 6-60% from the corresponding chalcones. A number of these derivatives showed promising antiproliferative activities in human breast cancer cell lines which express CYP1B1 and CYP1A1, while showing little toxicity towards a non-tumour breast cell line with no CYP expression. Metabolism studies provided evidence supporting the involvement of CYP1 enzymes in the bioactivation of these compounds.

Keywords: 4,6-diaryl-2-pyridones, chalcones, antiproliferative, prodrug, CYP1B1

Cytochrome P450 (CYP) expression has been studied in a variety of human tumours and it is clear that many have distinct CYP profiles overexpressing specific CYP sub-families [1, 2]. The therapeutic action of a number of clinically used anticancer drugs has been shown to involve metabolism by CYPs and several anticancer prodrugs have been designed to be activated by these enzymes [3]. Although the expression levels of many CYPs differ between tumour and corresponding normal tissue CYP1B1 is one of the few CYP subfamilies which is significantly and consistently overexpressed in tumours and is capable of metabolising a structurally diverse range of anticancer drugs leading to drug resistance in certain cancer cell lines [4]. Because of this, and its role in the activation of procarcinogens, CYP1B1 is seen as an important target for anticancer drug development both in terms of inhibitors for this enzyme and in prodrug activation [5-7]. We have designed several compounds based on the chalcone and stilbene structures to be activated by CYP1B1 [8-10]. A number of these have also shown potential as chemopreventative agents including the chalcone DMU-135 (Fig. 1) which was found to inhibit Apc(Min/+) mouse

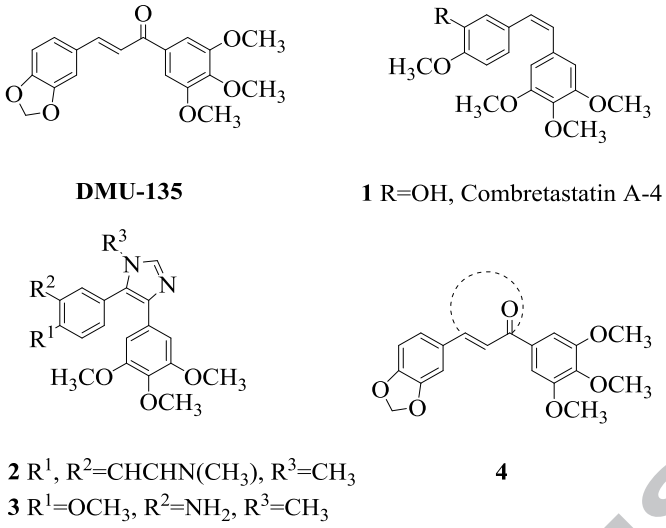
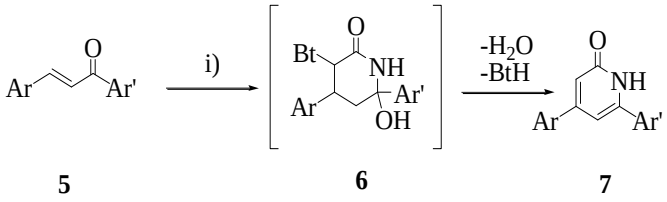


Figure 1. Incorporation of a hererocyclic ring into chalcone and stilbene structures.

gastrointestinal adenoma formation, reducing adenoma multiplicity by 46% compared to controls. DMU-135 was well tolerated and induced no systemic side-effects [11-13]. Our group has also demonstrated that CYP1B1 and CYP1A1 catalyse the conversion of certain naturally occurring methoxylated flavones with good bioavailability but relatively little biological activity into hydroxylated metabolites with strong antiproliferative activity [14-16].

The therapeutic use of most chalcones and stilbenes is hampered by their high lipophilicity which results in low water solubility and poor pharmaco-kinetics [17]. In addition, solutions of some chalcones are susceptible to light induced isomerisation about the carbon–carbon double bond resulting in mixtures of isomers [18]. One potential solution to these problems is to integrate the key structural features of the molecule into a heterocyclic system. This approach has been used with some success by Wang *et al* who prepared analogues of the cytotoxic stilbene combretastatin A-4 **1** (Fig. 1) in which the key cis double bond was replaced by a 4,5-disubstituted imidazole ring. The incorporation of this ring prevented isomerisation about the double bond and the resulting analogues **2** and **3** (Fig. 1) had greatly improved pharmacokinetics, longer half-lives and excellent bioavailability [17]. For DMU-135 a heterocyclic ring could be incorporated into the chalcone structure as shown in **4** (Fig 1), where the dotted line represents the heterocyclic ring. This would fix the important geometry about the 2-propenone portion of the molecule and, depending on the nature of the ring system, could enable formulation as a pharmaceutically acceptable water soluble addition salt.

As a starting point, it was decided to incorporate a 2-pyridone ring into the chalcone structure. The 2-pyridone nucleus occurs widely in biologically important natural alkaloids and is a useful intermediate for the generation of other heterocyclic systems which will allow for further elaboration of analogues generated [19]. Chalcones are readily accessible in high yield via the Claisen-Schmidt



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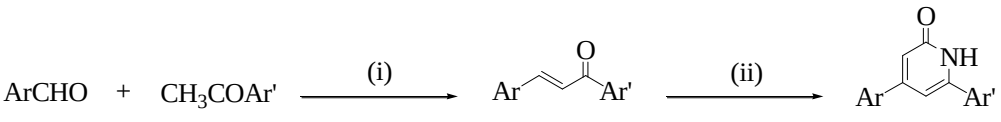
Scheme 1. Reagents and conditions: i) $BtCH_2CONH_2$, NaOH, EtOH, reflux.

condensation reaction and are therefore an attractive starting material [10]. Several methods have been reported for the preparation of 3-unsubstituted 2-pyridones from chalcones, notable among these is the benzotriazole-assisted preparation developed by Katritzky *et al* (Scheme 1) [19]. The chalcone **5** undergoes a tandem reaction with 2-(benzotriazol-1-yl)acetamide to yield a 3-(benzotriazol-1-yl)-4,6-diaryl-2-piperidinone **6** as an intermediate. Loss of benzotriazole (BtH) and water generates the pyridone ring **7**. A modification of this approach using 2-acetamidoacetamide (*N*α-acetylglycinamide) in place of 2-(benzotriazole-1-yl)acetamide offers advantages of commercially available starting reagents and shorter reaction time while retaining high regioselectivity and good yields [20].

A series of chalcones **5a-q** were prepared in yields of 51-94% as described by us previously [10] by reacting the appropriate benzaldehyde and acetophenone with aqueous sodium hydroxide solution in methanol at room temperature (Scheme 2). Conversion of the chalcones into the corresponding 4,6-diaryl-2-pyridones **7a-q** was achieved using a modification of the method of Wang *et al* [20]. A mixture of the chalcone, 2-acetamidoacetamide and cesium carbonate in DMF was heated at 150˚C for 1 h (Scheme 2). The crude materials were purified by either flash chromatography or trituration with ethyl acetate affording the desired products in yields of 6-60%. Analytical data were fully consistent with the proposed structures. A combinatorial chemistry approach was adopted for these syntheses with no effort being made to optimise individual yields.

The antiproliferative activities of the pyridones **7a-r** were investigated using a human breast cell line panel which has been characterised for CYP expression in our laboratories [14-16]. This panel consists of MCF-7, an ER positive tumour cell line with very low levels of CYP expression, MCF-7 induced with TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) which expresses high levels of CYP1A1, MDA-MB-468, an ER negative tumour cell line which constitutively expresses both CYP1B1 and



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Scheme2. Reagents and conditions: (i) NaOH(aq), MeOH, rt, 51-94% yield. (ii) $CH_3CONHCH_2CONH_2$, Cs_2CO_3, DMF, 150^0C, 1h, 6-60% yield.

CYP1A1 and MCF-10A, a non-tumour 'normal' cell line which has no basal CYP expression. IC_{50} values were determined using a standard MTT cytotoxicity assay [10] and are shown in Table 1. To enable rapid characterization of compounds, two selectivity ratios are included in this table. Activation factor (AF) quantifies the change in activity in the MCF7 cell line on treatment with TCDD (AF = IC_{50} (MCF7)/ IC_{50} (MCF7 + TCDD)) with values above one indicating an increase in activity. Tumour selectivity (TS) characterizes the difference in activity between the non-tumour MCF-10A cells and the MDA-MB-468 breast cancer cell line (TS = IC_{50} (MCF-10A)/ IC_{50} (MDA-MB-468)). TS values above one indicate selectivity for the CYP1 expressing cancer cell line.

Compound **7a**, which has the same aromatic substitutions as DMU-135, showed little antiproliferative activity in the MCF-10A and MCF-7 cell lines both of which have low levels of CYP expression. In the MDA-MB-468 cell line, which constitutively expresses both CYP1B1 and CYP1A1, a slightly higher activity was observed. Treatment of the MCF-7 cells with TCDD, to induce CYP1A1 expression, resulted in a significantly increased potency, the IC_{50} for **7a** decreasing to 0.2 μM with an AF >500. Moving the methoxy groups to the 2,3,4 positions, as in **7b**, had the effect of increasing toxicity to both the cancer cell lines. However, treatment of the MCF-7 cells with TCDD did not produce the same increase in potency as was observed with **7a** suggesting that the location of the methoxy substituents on the B-ring is key for activity. Little toxicity was seen with the non-tumour MCF-10A cells. Pyridone **7c**, with a 3,5-dimethoxyphenyl B-ring, was highly toxic to all the cell lines tested. The 3,4-dimethoxy derivative **7d** showed little activity in the MCF-7 and MCF-10A cell lines but relatively high potency in the MDA-MB-468 cells. As this cell line constitutively expresses CYP1B1 this result could suggest selective activation by this enzyme possibly indicating that a 3,4-dimethoxy substitution on the B-ring confers selectivity. Compound **7e** was highly active in the MDA-MB-468 cancer cell line with an IC_{50} of 0.04 μM. In the non-tumour cell line this 2,4-dimethoxy B-ring analogue gave an IC_{50} of 40 μM, a TS of 1000. Some antiproliferative activity was also seen in the MCF-7 cells, particularly when these were treated with TCDD. The 2,5-dimethoxy derivative **7f** showed similar activity to **7e** in the MDA-MB-468 cell line but was more toxic to the non-tumour cells. In the MCF-7 cell line an IC_{50} of 2 μM was reduced to 0.02 μM on treatment of these cells with TCDD. Thus, as with our trimethoxy analogues, the location of the two methoxy groups on the B-ring appears to influence both activity and selectivity within the cell lines. The 4-methoxy derivative **7h** showed similar selectivity to **7d** for the MDA-MB-468 cell line. The 2-methoxy analogue **7g** was much less selective for the MDA-MB-468 cells being more comparable to **7e** in activity. Replacing the methoxy groups in the B-ring of **7e** with chloro groups, as in **7i**, resulted in a derivative with little activity thus confirming the importance of the methoxy groups. The unsubstituted B-ring analogue **7j** showed relatively high toxicity to all the cells tested. The 3,4-dimethoxy A-ring analogue **7k** was prepared to investigate the effect of A-ring substitution on activity. **7k** was less active than the comparable 3,4-methylenedioxy analogue **7e** in the MDA-MB-

Table 1. Antiproliferative activities of 4,6-diaryl-2-pyridones

No	Ar (A-ring)	Ar' (B-ring)	Cell line/ IC_{50} μM[a]		AF	Cell line/ IC_{50} μM[a]		TS
			MCF-7	MCF-7 + TCDD		MDA-MB-468	MCF-10A	
7a	3,4-OCH_2O-Ph	3,4,5-$(CH_3O)_3$Ph	>100	0.2	>500	50	>100	>2
7b	3,4-OCH_2O-Ph	2,3,4-$(CH_3O)_3$Ph	12	10	1.2	8	>100	>12.5
7c	3,4-OCH_2O-Ph	3,5-$(CH_3O)_2$Ph	0.4	0.2	2	0.35	0.7	2
7d	3,4-OCH_2O-Ph	3,4-$(CH_3O)_2$Ph	>100	>100	1	0.41	>100	>244
7e	3,4-OCH_2O-Ph	2,4-$(CH_3O)_2$Ph	9	1.8	5	0.04	40	1000
7f	3,4-OCH_2O-Ph	2,5-$(CH_3O)_2$Ph	2	0.02	100	0.02	5	250
7g	3,4-OCH_2O-Ph	2-CH_3OPh	7	3.5	2	0.7	28	40
7h	3,4-OCH_2O-Ph	4-CH_3OPh	>100	>100	1	1	>100	>100
7i	3,4-OCH_2O-Ph	2,4-$(Cl)_2$Ph	85	70	1.2	60	>100	>1.7
7j	3,4-OCH_2O-Ph	Ph	1.6	0.8	2	0.8	2.5	3.1
7k	3,4-$(CH_3O)_2$Ph	2,4-$(CH_3O)_2$Ph	1	0.4	2.5	0.5	50	100
7l	2,4-$(CH_3O)_2$Ph	2,4-$(CH_3O)_2$Ph	9	4.5	2	5	5	1
7m	3,4-$O(CH_2)_2O$-Ph	2,4-$(CH_3O)_2$Ph	2.5	3	0.8	0.5	6	12
7n	4-CH_3OPh	2,4-$(CH_3O)_2$Ph	0.5	0.2	2.5	0.08	1.6	20
7o	4-PrOPh	2,4-$(CH_3O)_2$Ph	45	9	5	0.4	50	125
7p	4-PrOPh	3,4-$(CH_3O)_2$Ph	60	20	3	0.18	70	389
7q	2,4-$(CH_3O)_2$Ph	3,4-OCH_2O-Ph	18	1	18	6	80	13
7r	3,4-$(HO)_2$Ph	2,4-$(CH_3O)_2$Ph	1.5	0.7	2.1	0.5	1.5	3
Combretastatin A-4			0.002	0.002	1	0.003	0.0016	0.53

[a]Values are means two independent experiments in quadruplicate.

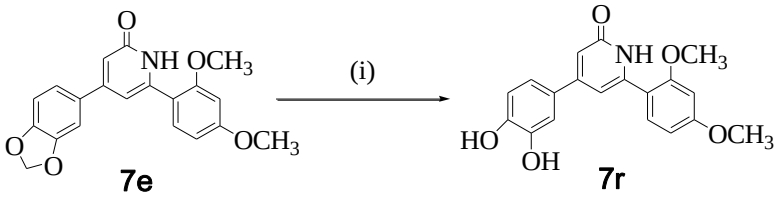
468 cell line, but more active in the MCF-7 cells. Changing to a 2,4-dimethoxy substitution on the A-ring (**7l)** reduced activity in the cancer cell lines while increasing toxicity in the non-tumour cells. This could indicate that a 3,4-substitution on the A-ring is important for activity. The 3,4-ethylenedioxy analogue **7m** was significantly less active in the cancer cell lines than its methylenedioxy counterpart and more toxic to the non-tumour MCF-10A cells. The 4-methoxy A-ring analogue **7n**, with a 2,4-dimethoxyphenyl B-ring, had comparable activity to **7e** in the MDA-MB-468 cell line but was significantly more toxic to the MCF-10A cells. It was also more active in the MCF-7 cell line. Interesting results were obtained when the 4-methoxy group on the A-ring was replaced

Table 2. Effect of α-naphthoflavone on the activity of **7e**

αNF/ μM	Cell line/ IC_{50} μM			
	MCF-7	MCF-7 + TCDD	MDA-MB-468	MCF-10A
0	9	1.8	0.04	40
1	12	9	>100	>100

with a propoxy group. These derivatives **7o** and **7p** showed good selectivity for the MDA-MB-468 cell line with TS values of 125 and 389 respectively. The latter, like **7d,** has a 3,4-dimethoxy substitution on the B-ring again indicating that this substitution may result in selectivity for the CYP1B1 expressing cell line. To investigate if the position of the nitrogen atom in the pyridone ring had an influence on antiproliferative activity a derivative of **7e** was prepared in which the aromatic groups were "reversed". This compound **7q**, showed much lower activity in the MDA-MB-468 cell line when compared with **7e** thus indicating that the location of the nitrogen in the pyridone ring is important.

As a preliminary investigation into the role played by CYP1 enzymes in the mechanism of action of the 4,6-diaryl-2-pyridones the cytotoxicity assay for **7e** was repeated with the addition of the known CYP1 inhibitor α-naphthoflavone (αNF) [21]. If CYP1 enzymes are involved in the bioactivation of this derivative, then their inhibition should result in a reduction in antiproliferative activity. The analogue **7e** was selected because it shows activity in both the cancer cell lines and low toxicity to the non-tumour MCF-10A cells. In addition, we have previously carried out the same experiment with the parent chalcone **5e** [22]. With **7e** in the MDA-MB-468 cell line addition of αNF produced a significant reduction in activity, the IC_{50} increasing from 0.04 to over 100 μM (Table 2). With the TCDD treated MCF-7 cells, which expresses high levels of CYP1A1, a more modest 5 fold reduction in activity was observed, the IC_{50} value obtained being comparable to that of the untreated MCF7 cells. Both these results are consistent with CYP1 enzymes being involved in the bioactvation of this compound. The greater activity seen with **7e** in the MDA-MB-468 cell line, which expresses both CYP1B1 and CYP1A1, could suggest that CYP1B1 is playing a more significant role than CYP1A1 in the activation of this compound. To investigate this further the pyridone **7e** was incubated with Supersomes™ expressing either CYP1A1 or CYP1B1 and NADPH reductase (20pmol.mL^{-1} of human CYP expressed in insect cells). Control incubations were carried out using non-CYP expressing insect Supersomes™ or with the omission of either test compound, NADPH or the active enzyme. Samples were taken at 5 min intervals in duplicate with the enzymatic reaction being terminated by the addition of ice-cold acetonitrile. Following centrifugation, aliquots of the supernatant were analysed by reverse phase HPLC. As can be seen (Fig. 1, Supporting info.), the pyridone was metabolised more rapidly by CYP1A1 than CYP1B1 with half-lives of 12.9 and 30.1 mins respectively. With CYP1A1 the concentration of **7e** appeared to plateau at around 15 mins which may indicate that this compound is a mechanism based inhibitor of this enzyme.



Scheme 3. Reagents and conditions: (i) BCl_3, CH_2Cl_2, -5^{0}C, 1h, 59%.

CYP1A1 produced a single metabolite with **7e** which eluted at 10.48 mins (Fig. 2, Supporting info.). CYP1B1 gave two metabolites, one with the same retention time as that obtained with CYP1A1 and a second, more polar, metabolite with a retention time of 9.22 mins (Fig. 2, Supporting info.). Due to the increased polarity of the metabolites compared to **7e**, and our previous work with the corresponding chalcone **5e** [22], we anticipated that these metabolites resulted from either: i) dealkylation of the methylenedioxy group to give a catechol or, ii) demethylation of one of the methoxy groups to give an alcohol. The catechol metabolite **7r** was prepared from **7e** in 59 % yield by reaction with boron trichloride and purified by flash chromatography (Scheme 3) [23]. When supernatants from each of the enzyme reactions were spiked with this authentic standard co-elution of **7r** with the peaks at 10.48 mins provided evidence that this is the major metabolite produced by both CYP1A1 and CYP1B1. The second metabolite produced by CYP1B1 is more polar than **7r** and therefore unlikely to result from the demethylation of **7e**. It is possible that this metabolite arises from demethylation of the previously formed catechol **7r**. To investigate whether the metabolite **7r** showed antiproliferative activity, it was tested against our human breast cell line panel where it was found to be toxic to all the cell lines tested, including the non-tumour MCF-10A cells with an IC_{50} of 1.5 μM (Table 1). This result provides further evidence that metabolism by CYP1 enzymes is involved in the mechanism of action of **7e**, with **7r** being an active metabolite. The reduced activity seen with **7r** in the MDA-MB-468 cell line when compared to **7e** is probably due to its increased polarity, the catechol group making it more difficult for this molecule to permeate the cell membrane.

In summary, certain 4,6-diaryl-2-pyridones show promising and selective antiproliferative activities in human breast cancer cell lines which express CYP1B1 and CYP1A1, while showing little toxicity towards a non-tumour breast cell line with no CYP expression. Metabolism studies have provided supporting evidence for the involvement of CYP1 enzymes in the bioactivation of these compounds.

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