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Short communication

Enhanced fluorescence detection of *cis*-combretastatins by post-column photolysis

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Abstract

A method is described to enhance the sensitivity of fluorescence detection of *cis*-combretastatins using a short post-column photolysis coil with a mercury lamp, by inducing the rapid conversion to the *trans* isomer. Although all the compounds studied showed enhanced fluorescence after photolysis, there were large differences in the absolute level, with the inherent response of the catechol CA1 being much lower than the corresponding phenolic CA4. Brief exposure to the deuterium lamp in a photodiode array detector also resulted in significant enhancement.

Keywords: Fluorescence detection; Combretastatin; Post-column; Photolysis; *cis–trans* isomerisation; Zybrestat

1. Introduction

The colchicine analogues *cis*-combretastatin A4 (CA4) (administered as the phosphate ester prodrug (Zybrestat, CA4P) [1,2] and *cis*-combretastatin A1 phosphate (CA1P, OXi4503) (Fig. 1) are currently in clinical trials. We previously published a validated method for the determination of plasma CA4 and CA4P by HPLC utilising either absorbance (murine) or fluorescence (human) detection [3]. In this assay, we acquired data for both detection modes serially, with the diode array detection (DAD) system first. Subsequently, this method was developed for the determination of the pharmacokinetics of CA4(P) in Phase I studies with this drug [1,2], and it was planned to use fluorescence detection, as the most sensitive and specific detection mode in man. Without the DAD system between column and fluorescence detector, we found that the sensitivity was much lower. We had already noted that the *trans* isomer of CA4(P), present as a trace impurity in the drug, and readily increased by exposure of the *cis* parent to light, was much more fluorescent than the active *cis* isomer. We hypothesised that the loss of sensitivity was because the photolytically induced conversion of the weakly fluorescent *cis* to the highly fluorescent *trans* isomer, induced by brief exposure to the deuterium lamp in the DAD system, was no longer occurring. High sensitivity for CA4(P) was needed for the clinical trial, and therefore this observation was further investigated.

This paper describes the construction of a simple post-column photolytic reactor constructed from a short length of narrow-bore polytetrafluoroethylene (PTFE) tubing and a low-pressure mercury lamp, which leads to a further improvement in sensitivity. Other photodiode array-induced changes in second detector response are also described.

2. Experimental

2.1. Chemicals

Structures of the drugs studied are shown in Fig. 1. *cis*-CA1P (dipotassium salt) and *cis*-CA1 (OXi4500) were from Evotec (Abingdon, UK), *cis*-CA4P and *cis*-CA4 were from Pharm-Eco Labs. (Devens, MA, USA) and *trans*-CA4 was a gift from Professor G.R. Pettit, Arizona State University. Not shown are the glucuronides of *cis*-CA1, designated CA1G1 and CA1G2 (Oxigene, Boston, MA, USA), where either one of the two OH groups is glucuronidated, and the two possible monophosphates CA1MP1 and CA1MP2, generated by prolonged standing of an aqueous solution of CA1P, which were also included in this study. For both the glucuronides and the monophosphates, the position of each

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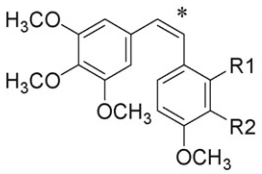

	CA1	CA1P	CA4	CA4P
R1	OH	$OHPO_3K$	H	H
R2	OH	$OHPO_3K$	OH	OPO_3Na_2

For *trans*-CA4, the bond marked * is *trans*

Fig. 1. Structures of the compounds studied.

substitution is not known at present. Methanol, acetonitrile (far UV grade), dipotassium hydrogenorthophosphate and tetra-*n*-butylammonium hydrogensulfate (TBA) (ECD grade) were from Fisher (Loughborough, UK), ascorbic acid and dimethyl sulfoxide (DMSO) were from Sigma (Poole, UK). Formic acid (BDH Aristar) was from VWR (Lutterworth, UK). Stock solutions of the lipophilic CA1 and CA4 (0.1–1 mM) were prepared in DMSO (including 5 mM ascorbic acid for CA1) while the hydrophilic prodrugs and glucuronides were dissolved in water.

2.2. Construction of the photolysis coil

A 75 cm length of PTFE tubing, 1/16 in. O.D., 0.006 in. I.D. (Thames Restek, High Wycombe, UK) (total volume 14 μL) was cut, and the central section wound (∼8 turns) in a single layer round a mercury lamp, length 19 mm, diameter 7 mm, designed for a Waters 440 detector. The coil was held in place by a tight-fitting slotted aluminium sleeve, and rendered light-tight with a second, concentric aluminium cylinder. The lamp power supply from a 440 detector was switched so that the lamp could only operate when solvent was flowing. Fig. 2 shows the components of the coil.

2.3. HPLC

The HPLC system for most of the study consisted of a 2695 separations module, a 474 fluorescence detector (5 μL flow cell), and, where appropriate, a 2996 DAD system, all from Waters (Watford, UK). The column (ACE 3 μm C18, 150 mm × 3 mm) (Hichrom, Reading, UK) was maintained at 35 °C. Buffered solvents were filtered (0.45 μm Pall polypropylene) (VWR). Solvents were: (A) 20% methanol, 8 mM dipotassium hydrogenorthophosphate, 5 mM TBA; (B) 75% methanol/water; (C) 75% acetonitrile/water. Initial conditions were 73% A, 27% B, with a linear gradient to 45% A, 55% B over 9 min, then to 5% A, 55% B, 40% C over 5 min, and finally to 100% C over 0.5 min, held for 1 min before returning to the initial conditions, flow rate 0.5 mL/min. Either the photolysis coil or the DAD system was placed between the column and fluorescence detector (excitation 320 nm, emission 390 nm, bandwidth 18 nm). Data was acquired using Waters Millennium software.

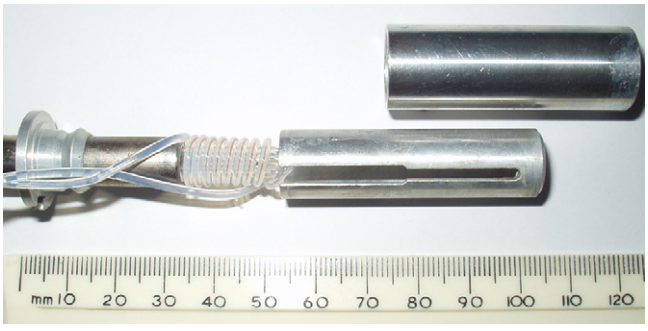

Fig. 2. Photo of a disassembled photolysis coil showing the lamp, tubing, slotted sleeve and outer cylinder.

The LC–MS study used a 2695, 2996 and a ZQ mass detector (Waters). Chromatography was on an RPB C18 column, 100 mm × 3.2 mm (Hichrom), maintained at 30 °C. Solvents were: (A) 10 mM formic acid; (B) acetonitrile, with a linear gradient from 20 to 70% B over 5 min. The flow rate was 1 mL/min, of which 0.2 mL/min was fed to the mass detector. The capillary was set at +2.5 kV, the cone voltage 18 V, source temperature 120 °C, desolvation temperature 425 °C, desolvation gas flow 450 L/h and cone gas flow 140 L/h.

3. Results and discussion

An HPLC method was developed to separate the prodrug and active agent for both combretastatins, and in addition resolve the two positional isomer monophosphates and the corresponding monoglucuronides for CA1P. Analytical methods for both drugs are of interest as they are in early clinical trials. The *trans* isomer of CA4 was included to show the difference in the effect of photolysis on *cis* and *trans* isomers. The two potential monophosphates are not available as standards, but are seen *in vivo* [4], and their identity has been confirmed by mass spectrometry (data not shown).

Fig. 3 shows the separation with fluorescence detection using either the photolysis coil with the mercury lamp off (A), the lamp switched on (C), or, in place of the coil, the DAD system in series with the fluorimeter (B). The response for the two monophosphates is shown in the inset panel (D), with and without photolysis. The concentration of the latter (40–50 μM) is approximate, calculated assuming the same absorbance as CA1P. The corresponding peak area data, normalised to *cis*-CA4 = 100, is given in Table 1 and together they illustrate the large enhancements in peak areas for the *cis* isomers provided by the two photolysis techniques (with a corresponding smaller peak area following photolysis of *trans*-CA4). In the absence of photolysis (A), the fluorescence response for 10 μM *trans*-CA4 is almost fivefold higher than for 20 μM *cis*-CA4. The peak area for the *trans* is reduced by nearly 50% when the photolysis lamp is switched on, while the *cis* isomers are all significantly increased (mean 6.1), although there are sizeable differences between compounds, with the enhancement ranging from 2.5 for CA1 to over eightfold for CA1G1 and CA1MP1. The DAD system lamp (B) is able to substitute to some extent for the custom built assembly, although the enhancement is about threefold less. There are some notable differences in fluorescence response both with and without photolysis. The drugs for which the assay was originally devised, *cis*-CA4/CA4P, show the highest fluo-