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Fluorescence lifetime imaging of *E*-combretastatin uptake and distribution in live mammalian cells.

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ABSTRACT

To investigate within live mammalian cells the uptake and disposition of combretastatins, fluorescence lifetime imaging was used with two-photon excitation (2PE). Combretastatin A4 (CA4) and analogues are potential anticancer drugs due to their ability to inhibit angiogenesis. *E*(*trans)*-combretastatins are considerably less active than the *Z*(*cis)*-combretastatins proposed for clinical use. However the *E*-combretastatins exhibit stronger intrinsic fluorescence with quantum yields and lifetimes that depend markedly on solvent polarity and viscosity. It is proposed that 2PE in the red and near-infrared tissue window may allow *in situ* isomerization of *E*-combretastatins to the more active *Z*-isomer, offering spatial and temporal control of drug activation and constitute a novel form of photodynamic therapy. In the present work we have characterised 2PE of *E*-CA4 and have used fluorescence lifetime imaging with 2PE to study uptake and intracellular disposition of *E*-CA4 and an analogue. The results show that these molecules accumulate rapidly in cells and are located mainly in lipidic environments such as lipid droplets. Within the droplets the local concentrations may be up to 2 orders of magnitude higher than that of the drug in the surrounding medium.

Keywords: Combretastatin, fluorescence, lifetime, multiphoton, live cell, microscopy, drug uptake, lipid droplet, light, photodynamic therapy.

Short Title:- Combretastatin uptake in live cells

Abbreviations:- CA4, combretastatin A4; 2PE, two-photon excitation; TCSPC, time correlated single photon counting; 2APY, 2-aminopyridine; OPO, optical parametric oscillator

INTRODUCTION

The combretastatin drugs are based on the combretastatin A4 molecule isolated by Pettit and co-workers in 1982 from the African bush willow,[1] *Combretum caffrum*. The main variant, combretastatin A4 (*Z*-CA4) (and the water soluble pro-drug phosphate ester CA4P), exert anticancer activity by binding strongly to the colchicine site of tubulin and preventing polymerization to microtubules.[2,3] In regions surrounding developing tumours this leads to inhibition of angiogenesis through interfering with vascular endothelial-cadherin signalling,[3,4] depriving the tumour of the nutrients required for growth. *In vitro* cellular assays of toxicity show *Z*-CA4 to be highly effective and active at nanomolar concentrations in the cell medium.[5]

Combretastatins are substituted stilbenes that are two to three orders of magnitude more active as the *Z*- (*cis*) isomers compared with the corresponding *E*- (*trans*) isomers.[5] This difference results from the geometrical requirements and substitution pattern required for molecular binding at the colchicine site on the tubulin dimer. In room temperature solution, *E*-stilbenes generally have moderate fluorescence quantum yields and sub-nanosecond lifetimes, whereas *Z*-isomers are virtually non-fluorescent. Fluorescence yields and lifetimes of *E*-isomers increase with solvent viscosity as the competing $E \rightarrow Z$ isomerization rate decreases.[6] Electronic excitation of fluorophores using two-photon excitation (2PE) is seen as an attractive method for biological imaging and cellular photochemistry[7] because of the transmission of near infrared light through tissue and readily available ultrafast Ti:sapphire lasers providing MHz 200 fs pulse trains. The concept of using 2PE to isomerise *E*-combretastatins relates to other biological applications of multiphoton excitation such as in photodynamic therapy[8] where tissues are very much more transparent in the

near-infrared region (700 – 900 nm) than in the visible spectrum.[9] For fluorescence microscopy 2PE also has the advantage of pseudo confocality (because of the femtolitre volume targeted where sufficiently high photon densities are obtained within the focussed laser spot) and allows excitation of UV-absorbing fluorophores that is usually prevented by absorbing elements within the microscope optics or the cell itself.[10] In an alternative approach to the development of combretastatins as anti-cancer agents and as probes in identification of the cellular site of action, aryl azide derivatives of *Z*-CA4 have been identified as excellent candidates for photoaffinity labeling studies of tubulin.[11]

In this report we describe real time observation of the intracellular uptake of two *E*-combretastatins in live cells (HeLa and Chinese hamster ovary (CHO) cells) using fluorescence lifetime imaging (FLIM). The compounds used were the *E*-isomers of combretastatin A4 (*E*-CA4) and the analogous fluorinated derivative (*E*-CA4F) (Figure 1). The validity of two photon excitation of *E*-combretastatins in the wavelength range of 560-630 nm is demonstrated by quantifying fluorescence lifetimes and quantum yields and comparing results with conventional one-photon excitation in the ultraviolet. The information obtained from within the cell using fluorescence intensity images is supplemented by the fluorescence lifetime images which, together with co-localization studies using Nile Red, indicating the subcellular regions into which combretastatins accumulate. Furthermore, combinations of intensity and lifetime images allow semi-quantitative measurements of intracellular combretastatin concentrations, demonstrating substantial accumulation of the compounds within both cells types.

MATERIALS AND METHODS

Solvents for spectroscopy (Sigma-Aldrich or Alfa Aesar, analytical or spectroscopic grade) were used as supplied. Anhydrous dimethylformamide was purchased from Sigma-Aldrich, whilst tetrahydrofuran and diethyl ether were freshly distilled over sodium and benzophenone under an argon atmosphere. Nile Red, 2-aminopyridine (2APY) and L-tryptophan were supplied by Sigma-Aldrich and 9-chloroanthracene (9CLA) by Alfa Aesar.

Combretastatins *E*-CA4 and *E*-CA4F were synthesized according to published procedures[12,13] and purified by flash column chromatography on silica gel (35-70 μm diameter, 60 Å pore size) with petroleum ether:EtOAc 19:1 and recrystallized from ethanol. Identity and purity of the materials were confirmed by thin layer chromatography and ^{1}H and ^{13}C NMR spectroscopy at 400 MHz and 100 MHz respectively (Bruker AC-400).

HeLa and Chinese hamster ovary (CHO) cells were from the European Collection of Cell Cultures. HeLa cells were cultured in MEM medium (Gibco) containing 10% foetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL) and glutamine (2 mM). CHO cells were cultured in DMEM (Gibco) with the same additions as for the HeLa cells. For microscopy, adherent cell cultures were grown in 35 mm diameter glass-bottom culture dishes with optical quality glass (MatTek Corporation) and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2.

Absorption spectra were measured in Perkin Elmer Lambda 25 or 950 spectrophotometers. Fluorescence measurements were made with Spex Fluoromax or

Varian Cary Eclipse spectrofluorimeters using the spectral correction curves supplied by the manufacturers. Fluorescence quantum yields with UV (one-photon) excitation were determined using either 2-aminopyridine (Φ_{ref} 0.6)[14] or 9-chloroanthracene (Φ_{ref} 0.50 in dichloromethane, determined here relative to 9,10-diphenylanthracene in cyclohexane (Φ_{ref} 0.97) and anthracene in ethanol (Φ_{ref} 0.28)[15] as standards. Fluorescence quantum yields were determined from the relative slopes of plots of integrated fluorescence intensity versus ($1-10^{-A}$) for samples having absorbance $A \leq 0.1$. The square of the refractive index (n^2) correction was applied for work in different solvents. Sample irradiation was minimized by use of narrow excitation slits to reduce photoisomerization. Repeated fluorescence scans of the sample under these conditions showed almost no change in fluorescence intensity.

The fluorescence lifetime imaging system with multiphoton excitation has been described previously.[16,17] The sources for multiphoton excitation were a Ti:Sapphire laser, tuneable 700 - 1000 nm and producing 180 fs pulses at 76 MHz, and an optical parametric oscillator (OPO) pumped by a second Ti:Sapphire laser. With the doubled idler output of the OPO, useful energies (>200 mW) could be obtained between 550 and 640 nm. Samples were excited on the stage of a Nikon TE2000U inverted microscope using a water immersion x60 objective of NA 1.2. Fluorescence was measured with either a spectrometer (Acton 275) and CCD setup (Andor iDUS), enabling emission spectra and intensities to be determined, or with a time-correlated single photon counting (TCSPC) system (Becker and Hickl SPC-830 and software) enabling fluorescence lifetime measurements and fluorescence lifetime imaging (FLIM). For the E-combretastatin lifetimes measured in solution, data was collected to 10^4 counts in the peak channel and good fits to a single exponential decay were

obtained using the Becker and Hickl software with typical values of $\chi^2 \leq 1.4$. For FLIM fluorescence was isolated by combined narrowband interference (400IU25) and BG3 (Comar) filters. The sample could also be imaged using a confocal imaging system (Nikon eC1-Si) on the same microscope using 488 and 543 nm excitation. Cell images were overlaid using Adobe Photoshop CS3. 2PE cross sections were determined as described by Mathai *et al.*,[18] using 9-chloroanthracene as a reference fluorophore.[19]

RESULTS AND DISCUSSION

1. Fluorescence properties – spectra, quantum yields and fluorescence lifetimes

The absorption and fluorescence emission spectra of *E*-CA4 and *E*-CA4F in dichloromethane are similar (Figure 2) with *E*-CA4 and *E*-CA4F having ultraviolet absorption maxima at 332 and 329 nm respectively. In contrast, *Z*-CA4 has a less intense absorption maximum at shorter wavelength (298 nm). The fluorescence quantum yields (ϕ) for *E*-combretastatins with UV excitation (290-300 nm) were found to strongly depend on solvent polarity and viscosity, ranging from 0.07 to 0.42 with lower values in low viscosity, polar protic solvents (methanol and ethanol) and higher values in non-polar or viscous polar solvents (hexane and glycerol). Fluorescence quantum yields for *E*-CA4F were generally higher than for *E*-CA4.

Fluorescence lifetimes were determined using 2PE at 630 nm with the microscope TCSPC setup (overall time response of 25 ps fwhm) and produced the results shown in Table 1. All the fluorescence decays were fitted well by a single exponential. The fluorescence lifetime of *E*-CA4 exhibited a strong solvent dependence, ranging from 230 ps in a polar solvents such as methanol to 860 ps in hexane. In the very viscous

environment provided by glycerol the lifetime increases further to 1180 ps. In the range of alkanols studied the lifetime is closely correlated with solvent viscosity as shown in the inset to Figure 3. The data from all solvents investigated indicates that fluorescence lifetime is a complex function of solvent properties including polarity and viscosity. However it is clear that the measured lifetime has the potential to report on cellular environments in FLIM studies. For *E*-CA4F similar trends are observed, but the overall differences in both lifetimes and quantum yields over the range of solvents are rather less than for *E*-CA4. The fluorescence lifetimes of *E*-CA4F are confined to a narrower range having values ranging from 440 ps in methanol through 750 ps in hexane to 1070 ps in glycerol. The more pronounced solvent dependence for *E*-CA4 reflects the more polar nature of the molecule compared with *E*-CA4F. The relative solute polarities may be judged from the partition coefficients (P) calculated according to the method of Brown *et al.*.[20] Log P values of for *E*-CA4 and *E*-CA4F are 3.81 and 4.62 respectively, confirming *E*-CA4F to be the more lipophilic of the two compounds. A plot of fluorescence quantum yields versus fluorescence lifetimes for both *E*-CA4 and *E*-CA4F is shown in Figure 3. The results show the anticipated linear relation[21] between quantum yield (Φ) and lifetime (τ) in the form

$$\Phi/\Phi_o = \tau/\tau_o \quad .....(1)$$

Furthermore both compounds behave similarly indicating a similar natural lifetime (τ_o). This is a very useful result for FLIM studies described below since even though the intracellular fluorophore lifetime may vary between (and within) the imaged sample and a calibrating fluorophore solution, the observed intracellular intensities may be adjusted by the use of the lifetime image through equation (1) to obtain quantitative estimates of the intracellular fluorophore concentration.

Compared with the commonly used fluorescent labels and probes with absorption and emission maxima in the visible spectral region and with quantum yields approaching unity, these properties of *E*-CA4 and *E*-CA4F are considerably less favourable for imaging with fluorescence microscopy. Nonetheless it has previously been shown[16,22,23] that it is possible to excite UV fluorescence in the region of 340-400 nm from intracellular 5-hyroxytryptophan, serotonin and propranolol using 2PE at 630 nm. At this excitation wavelength background interference from intracellular fluorophores such as tryptophan and cofactors is minimised.[24] Log-log plots of fluorescence intensity (I) versus laser power (P) for E-combretastatins in solution all demonstrated a slope of 2.0 ± 0.2, consistent with the anticipated quadratic power dependence for 2PE (equation (2)).

$$I = \sigma_2 P^2 \qquad \text{.....(2)}$$

Published cross sections for two photon absorption (σ_2, the probability of an absorption process or photon interaction) show large variations depending on the method of measurement and the power range and pulse duration of the laser used for excitation.[18] Values of σ_2 were measured using a 9-chloroanthracene (9ClA) as a reference fluorophore[18] with an appropriate fluorescence spectrum and measured 2PE cross-sections.[19] The values of σ_2 at 620 nm for *E*-CA4 and *E*-CA4F in DCM were determined to be 2.1 ± 0.5 and 2.75 ± 0.6 GM units respectively. The combination of Ti-sapphire laser and OPO allowed investigation of decreasing wavelength to 560 nm, whereupon *E*-CA4 showed a substantial increase in cross section (Figure 2), similar to that reported for bis(diphenylamino)-stilbene by Makarov *et al.*.[19]

2. Cell uptake and intracellular fluorescence of *E*-combretastatins

Intracellular distributions of *E*-combretastatins in cell monolayers were obtained using fluorescence lifetime imaging, in which the rastered excitation laser beam dwells on each pixel for a few milliseconds and at each position records a nanosecond fluorescence lifetime decay curve using TCSPC. The intensity image is constructed from the integrated photon count, which may then be modified using the lifetime information. Typically the microscopic field of observation contained between 15 and 20 live cells. Figure 4 shows fluorescence intensity images of CHO cells before and after incubation with either *E*-CA4 or *E*-CA4F, using 2PE at 628 nm and measuring fluorescence at 400 nm. The micrographs show clear images of the intracellular distribution of both compounds within the cells minutes after addition. Excellent contrast is obtained with negligible cell autofluorescence from unlabelled cells under the conditions used. Plots of fluorescence intensity versus incubation time (Figure 4) show biphasic uptake with an initial unresolved rapid phase within the first 100 seconds and a slower phase lasting over several minutes. The initial rise in fluorescence in Figure 4C cannot be ascribed to the fluorescence of *E*-combretastatin added to and remaining in the extracellular medium. On the basis of the intensity from calibration solutions, the added *E*-combretastatin (10 μM) would be expected to contribute an additional 0.56 counts per pixel in these images, compared with an initial rise of 3.6 counts per pixel in Figure 4C. Furthermore, the longer (>900 ps) fluorescence lifetimes in the intracellular FLIM image indicate an intracellular viscous or hydrophobic environment for *E*-CA4F even at this early stage (see below). Both *E*-CA4 and *E*-CA4F are excluded from the cell nucleus, and *E*-CA4 appears to be located mainly within the cytoplasmic region of the cell. In contrast *E*-CA4F shows a more punctuate distribution in CHO cells.

Fluorescence intensity and lifetime images of *E*-CA4 in HeLa cells are shown in Figure 5A. The lifetimes range between 750 and 1200 ps, with a peak in the distribution at 900 ps. *E*-CA4F in HeLa and CHO cells has a peak in the lifetime distribution at 1.1 ns (Figure 5B and 5C). These intracellular fluorescence lifetimes are longer than in hexane (0.86 ns, Table1) but similar to that in glycerol (1.18 ns) and suggest that the *E*-combretastatins occupy a non-polar and/or viscous environment within the cells. The microviscosities of the hydrophobic interiors of lipid bilayer membranes[25] and triglyceride lipid emulsion droplets[26] are between approximately 0.5 and 1 poise and similar to that of glycerol (0.95 poise at 25 ºC). Further evidence for the locations of *E*-combretastatins in these cells was obtained from labelling with Nile Red, a dye known to localise in lipid droplets and membrane systems in cells. [27] Nile Red staining of cells previously loaded with *E*-combretastatins was imaged using the confocal scanning facility on the multiphoton microscope using an excitation wavelength of 488 nm to preferentially observe lipid droplets over membranes.[28] Examples of Nile Red stained images are shown in comparison with fluorescence intensity and lifetime images in Figures 5, together with overlaid intensity/lifetime and Nile Red images. Figure 5C shows the results for *E*-CA4F uptake in CHO cells. The intensity images show a low background level of fluorescence intensity within the cell cytoplasm and intense staining in localised regions identified as lipid droplets by co-localisation with Nile Red fluorescence. Slight discrepancies in the co-localization result from the use of two different imaging systems (the multiphoton intensity/lifetime images and the confocal image of Nile Red fluorescence). The higher uptake of *E*-CA4F into lipid droplets in CHO cells compared with that of *E*-CA4 (Figures 4 and 5) may arise from the higher hydrophobicity of *E*-CA4F noted

above. Figures 5A and 5B show uptake of *E*-CA4 and *E*-CA4F into HeLa cells. Compared with the CHO cells, there are fewer lipid droplets within the HeLa cells and the compounds are preferentially located within the cytoplasmic regions.

Quantification of fluorophore concentration from intensity measurements is aided by the simultaneous recording of fluorescence lifetime images and the observation that fluorescent lifetimes for *E*-CA4 and *E*-CA4F are linearly related to quantum yield. Dynamic quenching of fluorescence, may be taken into account and sample fluorophore concentration (C_S) determined quantitatively by comparison of the sample fluorescence intensity (I_S) and lifetime (τ_S) with those of a standard reference solution (I_R, τ_R) of known concentration (C_R):-

$$C_S = I_S\left(\frac{C_R}{I_R}\right)\left(\frac{\tau_S}{\tau_R}\right) \qquad .....(3)$$

A series of solutions containing increasing concentrations of *E*-CA4F in DMSO were used to produce a linear calibration curve. DMSO was chosen since it produces a reasonably long lifetime and is more easily handled than glycerol. Using this procedure, concentration maps of intracellular combretastatin were obtained and examples shown in Figures 6A – 6C. Initial studies with HeLa cells and *E*-CA4 added to the serum at 100 μM led to the result in Figure 6A. Substantial regions of the cell cytoplasm contain in excess of 2 mM *E*-CA4 whilst smaller regions contain in excess of 4 mM, or more than 20 - 40 times the concentration of *E*-CA4 in the medium. This demonstrates that the compound is rapidly taken up and concentrated within the cell, making it available as a drug to interfere with cell function such as microtubule activity. Further investigations used only 10 μM combretastatin in the medium

(Figures 6B and 6C). Figure 6C shows that the concentration of *E*-CA4F within the lipid droplets in CHO cells reaches peaks of almost 10 mM whilst substantial regions of the cells contain around 1 mM of the compound. For the less lipophilic *E*-CA4 (Figure ~~9B~~6B) the peak concentrations are less but substantial regions of the cell cytoplasm contain between 250 and 750 μM of the drug. This indicates a concentrating of the drug by a factor of 25-75 times that in the surrounding medium.

CONCLUSIONS

Overall these results show that the *E*-combretastatins are rapidly (within minutes) accumulated by live mammalian cells and reach intracellular concentrations many times those in the medium. This is likely to be a passive process depending mainly on the lipophilicity of these compounds. In typical toxicity assays the concentrations of *Z*- and *E*-combretastatin A4 required to kill these cells are determined to be in the nanomolar and micromolar regions respectively.[5] The present results suggest that the true intracellular concentrations are much higher and for *Z*-CA4 may correspond more closely to the micromolar concentrations at which inhibition of microtubule assembly is observed.[29] Since the active *Z*-combretastatins exhibit extremely weak fluorescence, only with the less active but more fluorescent *E*-combretastatins has it been possible here to observe the rapid intracellular accumulation. Since the *E*- and *Z*-isomers are likely to differ very little in terms of lipophilicity, it may be expected that *Z*-combretastatins are similarly accumulated within mammalian cells.

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Conflict of interest statement

There are no conflicts of interest arising from this work.

References

1. Pettit GR, Cragg GM, Herald DL, Schmidt JM, Lohavanijaya P. Anti-neoplastic agents. 84. Isolation and structure of combretastatin. *Can J Chem* 1982;**60:**1374-76.
2. Woods JA, Hadfield JA, Pettit GR, Fox BW, McGown AT. The interaction with tubulin of a series of stilbenes based on combretastatin A-4. *Br J Cancer* 1995;**71**:705-11.
3. Hadfield JA, Ducki S, Hirst N, McGown AT. Tubulin and microtubules as targets for anticancer drugs. *Prog Cell Cycle Res* 2003;**5**:309-25.
4. Vincent L, Kermani P, Young LM, et al. Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signalling. *J Clin Invest* 2005;**115**:2992-3006.
5. Lawrence NJ, Rennison D, Woo M, McGown AT, Hadfield JA. Antimitotic and cell growth properties of combretastatin A4-like ethers. *Bioorg Med Chem Lett* 2001;**11:**51-54.
6. Görner H, Kuhn HJ. *Cis-trans* photoisomerization of stilbenes and stilbene-like molecules. *Adv Photochem* 2005;**19**:1-117.
7. Xu C, Webb WW. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J Opt Soc Am B* 1996;**13**:481-91.
8. Pawlicki M, Collins HA, Denning RG, Anderson HL. Two-photon absorption and the design of two-photon dyes. *Angew Chem Int Ed* 2009;**48**:3244–66.
9. Torricelli A, Pifferi A, Taroni P, Giambattistelli E, Cubeddu R. *In vivo* optical characterization of human tissues from 610 to 1010 nm by time-resolved reflectance spectroscopy. *Phys Med Biol* 2001;**46**: 2227–37.

10. König K, Multiphoton microscopy in life sciences. *J Microsc* 2000;**200**:83-104.
11. Pinney KG, Mejia MP, Villalobos VM, et al. Synthesis and biological evaluation of aryl azide derivatives of combretastatin A-4 as molecular probes of tubulin. *Bioorg Med Chem* 2000;**8**:2417-2425.
12. Pettit GR, Singh SB, Boyd MR, et al. Antineoplastic Agents. 291. Isolation and synthesis of combretastatins A-4, A-5, and A-6. *J Med Chem* 1995;**38**:1666-72.
13. Lawrence NJ, Hepworth LA, Rennison D, McGown AT, Hadfield JA. Synthesis and anticancer activity of fluorinated analogues of combretastatin A-4. *J Fluorine Chem* 2003;**123**:101-8.
14. Rusakomicz R, Testa AC. 2-Aminopyridine as a standard for low-wavelength spectrofluorimetry. *J Phys Chem* 1968;**72:**2680-81.
15. Suzuki K, Kobayashi A, Kaneko S, et al. Reevaluation of absolute luminescence quantum yields of standard solutions using a spectrometer with an integrating sphere and a back-thinned CCD detector. *Phys Chem Chem Phys* 2009;**11**:9850–60.
16. Botchway SW, Parker AW, Bisby RH, Crisostomo AG. Real-time cellular uptake of serotonin using fluorescence lifetime imaging with two-photon excitation. *Microsc Res Tech* 2008;**71**:267–73.
17. Bisby RH, Crisostomo AG, Botchway SW, Parker AW. Nanoscale hydroxyl radical generation from multiphoton ionization of tryptophan. *Photochem Photobiol* 2009;**85**:353-57.
18. Mathai S, Bird DK, Stylli SS, Smith TA, Ghiggino KP. Two-photon absorption cross sections and time-resolved imaging using phorphyrin photosensitisers. *Photochem Photobiol Sci* 2007;**6**:1019-26.

19. Makarov NS, Drobizhev M, Rebane A. Two-photon absorption standards in the 550-1600 nm excitation wavelength range. *Opt Express* 2008;**16**:4029-47.
20. Brown ML, Rieger JM, Macdonald TL. Comparative molecular field analysis of colchicine inhibition and tubulin polymerization for combretastatins binding to the colchicine binding site on β-tubulin. *Bioorgan Med Chem* 2000;**8**:1433-41
21. Lakowicz JR *Principles of Fluorescence Spectroscopy* (2nd edition). New York: Kluwer; 1999.
22. Balaji J, Desai R, Maiti S. Live cell ultraviolet microscopy: a comparison between two- and three-photon excitation. *Microsc Res Tech* 2004;**63**:67-71
23. Bisby RH, Crisostomo AG, Botchway SW, Parker AW, Watts SW. Intracellular imaging of serotonin and propranolol in rat aorta cells. Central Laser Facility Annual Report 2008/2009, RAL-TR-2009-025 (ISBN 978-0-9556616-5-5) pp150-152; 2009.
24. Li C, Pitsillides C, Runnels JM, Cote D, Lin CP. Multiphoton microscopy of live tissues with ultraviolet autofluorescence. *IEEE J Sel Top Quant Elect* 2010;**16**:516-23.
25. Cogan U, Shinitzky M, Weber G, Nishida T. Microviscosity and order in the hydrocarbon region of phospholipid and phospholipid-cholesterol dispersions determined with fluorescent probes. *Biochemistry* 1973;**12:**521-28.
26. Zhang X, Kirsch LE. The physical stability of thermally-stressed phospholipid-based emulsions containing methyl, propyl and heptyl parabens as model drugs. *Int J Pharm* 2003;**265**:133-40.
27. Greenspan P, Mayer EP, Fowler SD. Nile Red: A selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 1985;**100**:965-73.

28. Greenspan P, Fowler SD. Spectrofluorometric studies of the lipid probe Nile Red. *J Lipid Res* 1985;**26**:781-89.

29. Lin CM, Ho HH, Pettit GR, Hamel E. Antimitotic natural products combretastatin A-4 and combretastatin A-2: Studies on the mechanism of their inhibition of the binding of colchicine to tubulin. *Biochemistry* 1989;**28**:6984-91.

Table 1 Fluorescence lifetimes (in ns) and quantum yields for combretastatin fluorescence in a range of solvents at 20 ºC. The lifetimes were measured using two-photon excitation (620-630 nm). The fluorescence quantum yields were obtained with 290-300 nm excitation with 2-aminopyridine in H_2SO_4 (0.2 mol dm^{-3}) as the fluorescence standard (ϕ 0.60). The quantum yields are estimated to be ± 10%. Fluorescence decays were all good exponentials with estimated lifetimes ± 5%.

	***E*- CA4**		***E*- CA4F**	
	τ	ϕ	τ	ϕ
MeOH	0.23	0.071	0.44	0.15
EtOH	0.28	0.093	0.48	0.17
Ethylene glycol	0.58	0.19	0.76	0.29
Glycerol	1.18	0.35	1.07	0.42
MeCN	0.29	0.099	0.59	0.2
DCM	0.50	0.19	0.61	0.23
DMSO	0.49	0.15	0.81	0.29
Hexane	0.86	0.36	0.75	0.31

FIGURE LEGENDS

Figure 1 Structures of *E*- and *Z*-combretastatin A4 and fluorinated analogue used in this study.

Figure 2 Normalised one-photon absorption and fluorescence spectra of the *E*-CA4 (full curves) and *E*-CA4F (dotted curves). The spectrum of *Z*-CA4F (dashed curved) is shown for comparison with the absorbance at the correct ratio to that of *E*-CA4F. Also shown are the 2-photon cross sections for *E*-CA4 (■-■) on a wavelength scale (top axis) at twice that used for the one-photon spectra. All spectra were measured in dichloromethane.

Figure 3 Correlation between fluorescence quantum yield and fluorescence lifetime (both measured at 20 ºC) for *E*-CA4 (♦) and *E*-CA4F (◇) in the range of solvents shown in Table 1. **Inset:** Effect of solvent viscosity on fluorescence lifetimes and quantum yields for the alkanols in Table 1.

Figure 4 Fluorescence images of CHO cells before (images A1 and B1) and at 30 seconds (A2, B2), 3 minutes (A3, B3) and 6 minutes (A4, B4) after addition of *E*-CA4F (series A) or *E*-CA4 (series B) at 22 ºC. Both combretastatins were added to a final concentration of 10 μmol dm^{-3}. Fluorescence at 400 nm was excited by two-photon absorption at 628 nm. Panel C shows the overall average number of counts per pixel (•) and maximum (peak) pixel intensity within the image (□) after addition of *E*-CA4F (series A).

Figure 5 Fluorescence lifetime imaging of *E*-CA4 and *E*-CA4F in cells and co-localization with Nile Red. A: *E*-CA4 (100 μmol dm^{-3} in medium) with HeLa cells; cells; B: *E*-CA4F (50 μmol dm^{-3} in medium) with HeLa cells; C: *E*-CA4F (50 μmol dm^{-3} in medium) with CHO. Frames show (a) intensity images of combretastatin fluorescence at 400 nm with 628 nm 2-photon excitation, (b) Nile Red fluorescence (515 – 535 nm) excited at 488 nm, and (c) fluorescence lifetime images. Below are (d) the overlaid images of the drug and Nile Red images (a and b) and (e) the fluorescence lifetime distributions derived from the lifetime images. The bar represents 20 μm.

Figure 6 Contour plots showing intracellular concentrations of combretastatins in cells at room temperature after incubation for approximately 10 minutes: A: *E*-CA4 (100 μM added) in HeLa cells; B: *E*-CA4 (10 μM added) in CHO cells (the image corresponds to that in Figure ~~8A~~4A); C: *E*-CA4F (10 μM added) in CHO cells. The concentrations are

calculated using the intensity and lifetime at each pixel as described in the text. Each plot represents an area of 70 x70 μm. The vertical scales show the combretastatin concentration in μM.