An anticancer phototherapy using activation of *E*-combretastatins by two-photon induced isomerization

Kathrin M. Scherer,[a,b] Roger H. Bisby,[b] Stanley W. Botchway,[a] John A. Hadfield,[b] Anthony W.Parker[a]

[a]Research Complex at Harwell, CLF, Rutherford Appleton Laboratory, Harwell Oxford, Didcot, UK, OX11 0FA
[b]Biomedical Research Centre, University of Salford, Salford, Greater Manchester, M5 4WT, UK.

Abstract. The photoisomerization of relatively non-toxic *E*-combretastatins to clinically active *Z*-isomers is shown to occur in solution through both one- and two-photon excitations at 340 and 625 nm respectively. The photoisomerization is also demonstrated to induce mammalian cell death by a two-photon absorption process at 625 nm. Unlike conventional photodynamic therapy (PDT), the mechanism of photoisomerization is oxygen-independent and active in hypoxic environments such as in tumors. The use of red or near-infrared light for two-photon excitation allows greater tissue penetration than conventional UV one-photon excitation. The results provide a baseline for the development of a novel phototherapy that overcomes non-discriminative systemic toxicity of *Z*-combretastatins and the limitations of PDT drugs that require the presence of oxygen to promote their activity, with the added benefits of 2-photon red or near-infrared (NIR) excitation for deeper tissue penetration.

Keywords: two-photon, combretastatin, phototherapy, multiphoton microscopy

Address all correspondence to: Professor R H Bisby Biomedical Research Centre, University of Salford, Salford, Greater Manchester, M5 4WT, UK, telephone +44 161 4764773; E-mail: r.h.bisby@salford.ac.uk

1 Introduction

Photodynamic therapy (PDT) for the treatment of solid tumours combines the use of a photosensitizing drug and light in the presence of oxygen.[1] PDT involves reaction of a photo-excited (usually triplet) state of the sensitizer drug with oxygen either by transfer of excitation energy forming singlet oxygen (type II) or by electron/hydrogen atom transfer forming reactive free radical species such as superoxide (type I). The reactive oxygen species are then capable of damaging critical cellular targets. We have recently suggested an alternative combination of light and pro-drug that relies on oxygen-independent photoisomerization of a combretastatin.[2-4] Combretastatin drugs are based on natural products from an African bush willow that are stilbene derivatives,[5] and which may therefore exist either in *E- (trans)* or *Z- (cis)* configurations (Figure 1). *Z*-Combretastatins such as combretastatin A4 (*Z*-CA4, Figure 1) target microtubule assembly

and ultimately tumour vasculature,[6] and the pro-drug *Z*-combretastatin A4 phosphate (CA4P) has recently been evaluated in clinical trials.[7,8] The *Z*-isomer of CA4 is highly cytotoxic having a nanomolar LD_{50} in the MTT assay whilst the corresponding *E*- isomers of combretastatins are usually less toxic by 2 or 3 orders of magnitude.[9] As with most stilbenes,[10] photoisomerization of combretastatins occurs on illumination within their UV absorption band (ca 320-340 nm)[11] and offers a route to photoactivation via $E \rightleftharpoons Z$ isomerization that is independent of oxygen and therefore potentially useful in a wide range of tissues including hypoxic tumours. Whilst the optical transmission of tissues prevents the use of UV activation via usual one-photon absorption, an optical "window" in the spectra of tissues lies in the range of 600 – 900 nm,[12] equivalent to red or near-infrared wavelengths, permitting 2- or 3-photon absorption and activation of the *E*-combretastatin at approximately 640 nm or 960 nm respectively. Although such multiphoton absorption requires high light intensities, these are now readily achieved with femtosecond or picosecond pulsed lasers that are widely used in multiphoton microscopy.[13] Because multiphoton excitation of phototherapeutic drugs widens the choice of chromophore, considerable effort is now underway to develop new sensitizers with suitably high 2-photon cross-sections in the red/NIR tissue window.[14,15]

We have previously used fluorescence lifetime imaging microscopy (FLIM) with 2-photon excitation (2PE) at 625 nm to study uptake and intracellular accumulation of *E*-combretastatins including CA4 and a fluorinated derivative (CA4F) in live mammalian cells using the native UV fluorescence of the E-isomers.[3,4] Due to their hydrophobic nature, *E*-combretastatins accumulate to intracellular concentrations 2-3 orders of magnitude higher than in solution and are mainly

located in lipidic droplets and membranes. It is now shown that following uptake of *E*-combretastatins into mammalian cells, 2PE is capable of converting the low activity *E*-isomer to the highly cytotoxic Z-isomer to induce cell death.

2 Materials and Methods

2.1 Synthesis of combretastatin derivatives

E- and *Z*- isomers of CA4 (CA4, 1-(3',4',5'-Trimethoxyphenyl)-2-(4''-methoxy-3''-hydroxy-phenyl)ethene) and CA4F (1-(3',4',5'-trimethoxyphenyl)-2-(3''-fluoro-4''-methoxyphenyl)ethene) were synthesized as previously described.[9,16] Structures and purity were determined by TLC and ^{1}H- and ^{13}C-NMR.

2.2 Confocal and multiphoton fluorescence lifetime imaging microscopy (FLIM)

Confocal images, two-photon excited FLIM images and two-photon emission spectra used a combined laser scanning confocal multiphoton FLIM and spectral detection apparatus based on a Nikon TE2000-U inverted fluorescence microscope[3,17] with a x60 water immersion objective (N.A. 1.2) . For lifetime and FLIM measurements with *E*-CA4 and *E*-CA4F, BG3 (Comar) and a narrowband interference (400IU25) filters were used to isolate the light transmitted to the photomultiplier. Excitation for 2PE at 625 nm was provided by a titanium-sapphire laser (Mira, Coherent Ltd (180 fs laser pulses at 76 MHz) pumping an optical parametric oscillator (Coherent Ltd). Samples were irradiated on the motorized and temperature controlled (Peltier heater/cooler) microscope stage. Confocal imaging using 488 and 543 nm excitation was carried out using Nikon eC1-Si or eC2 confocal laser scanning accessories mounted on the same Nikon microscope.

2.3 One- photon photoisomerization quantum yield measurements

Spectroscopic grade solvents (Sigma Aldrich and Alfa Aesar) were used as supplied. Aberchrome 540 was used for actinometry.[18] UV-visible spectra were measured using a PerkinElmer Lambda 25. Steady state fluorescence emission measurements were recorded using a Horiba FluoroMax-3 using the manufacturer supplied spectral correction curves. Irradiations were carried out within the fluorimeter sample cell with a 5 nm excitation slit width. Combretastatin solutions in a 1 cm quartz cuvette were de-aerated by nitrogen bubbling inside an AtmosBag (Sigma Aldrich) to minimize phenanthrene formation. Quantum yields (Φ) for the $E \rightleftharpoons Z$ isomerization of the different combretastatins were determined from the photon flux and the initial changes in absorbance.[19] The concentrations of E- and Z-isomers (c^E and c^Z) at the photostationary state were calculated using extinction coefficients (ε^E and ε^Z) and quantum yields derived from the kinetic measurements (equation 1) and found to be consistent with experimental measurements at the establishment of the photostationary state.[10]

$$\left(\frac{c^E}{c^Z}\right) = \frac{\varepsilon^Z}{\varepsilon^E} * \frac{\phi_{Z\rightarrow E}}{\phi_{E\rightarrow Z}} \qquad \textbf{Equation 1}$$

2.4 Cell culture

Chinese Hamster Ovary (CHO) cells were obtained from the European Collection of Cell Cultures and were grown and maintained in phenol-red free DMEM (Gibco) and MEM (Gibco) respectively supplemented with foetal calf serum FCS (10 %), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cells were seeded at densities of 2 x 10^5 cells/dish on MatTek glass-bottom culture dishes (35 mm ø, No. 1.5, uncoated, γ-irradiated)

(MatTek Corporation) and placed in an incubator under a humidified atmosphere (37 °C, 5 % CO2) for 24 hrs to adhere.

2.5 Combretastatin induced apoptosis monitored by confocal imaging of Annexin V AlexaFluor488 conjugate and propidium iodide staining on live cells

Combretastatin induced apoptosis on CHO cell monolayers was determined using Annexin V AlexaFluor 488 conjugate (Invitrogen) as an indicator for the loss of phospholipid asymmetry in the plasma membrane of apoptotic cells in the presence of Ca^{2+}(2.5 mM), and propidium iodide (PI) as a DNA intercalating fluorescent dye that is permeable to membranes of apoptotic or necrotic cells. The cytotoxicity of Z-combretastatins in the CHO cell monolayers was induced by addition of aliquots of a stock solution in DMSO (≤ 5%). Following incubation for 48 hrs the tissue culture medium was aspirated and replaced with $CaCl_2$ and HEPES supplemented medium. The Annexin V conjugate was added at 0.5 % final concentration (5 µl/ml medium). A PI stock solution (1.6 µl/ml) was added and the samples incubated for 15 min in the dark and imaged using confocal laser scanning microscopy (excitation at 543 nm for PI and 488 nm for Annexin V conjugate). For irradiations in the presence of *E*-combretastatins, a 100 x 100 µm field was exposed to 625 nm light by raster-scanning the multiphoton beam for 10 min allowing a total of 20 scans with a pixel dwell time of 2 ms. Samples were incubated for 24 hrs and examined for apoptosis as described above.

3 Results

3.1 Photoisomerization induced by one- and two-photon excitation in vitro

$E \rightleftharpoons Z$ Photoisomerization of *E*-combretastatin A4 (*E*-CA4) (1 mM) in de-aerated methanol on irradiation at 340 nm is illustrated in Figure 2 through changes in absorption spectra. As the irradiation progresses absorbance of the E-isomer at λ_{max} (329 nm) decreases and is replaced by that of the Z-isomer with λ_{max} 287 nm with a lower extinction coefficient. The spectra show good isosbestic points showing that only the two species are involved and that phenanthrene formation was effectively suppressed by de-aeration. A plot of A(340 nm) (inset to Fig 2) indicates progression to the stationary state mixture of *E*- and *Z*- isomers. Quantum yields for photoisomerization were calculated from both the initial rate of absorbance change and the absorbance at the stationary state as detailed in the Materials and Methods and are shown in Table 1 for CA4 and CA4F in methanol solution. The values show that photoisomerization is relatively efficient with quantum yields in the range of 0.27 - 0.48 and are comparable with published data for a wide range of other stilbenes in fluid solution at room temperature.[10]

Table 1 Quantum yields for photoisomerization of *E*- and *Z*- isomers of CA4 and CA4F in methanol solution. The *E*/*Z* ratios at the photostationary state were measured experimentally based on the extinction coefficients and compared with those calculated according to equation (1).

Compound	Irradiation Wavelength (nm)	E-formation Z → E	E-loss E → Z	E/Z meaured at photostationary state	E/Z calculated at photostationary state
E-CA4F	340		0.38		
Z-CA4F	290	0.27			
E-CA4	340		0.48	0.11	0.14
Z-CA4	290	0.41		0.66	0.64

Photoisomerization of combretastatins by 2PE was inferred from measurements of fluorescence intensity versus laser power on irradiation at 624 nm in the microscope system using femtosecond laser pulses. Under these conditions 2PE excitation takes place within the femtolitre focal volume, from which the product diffuses into bulk solution, away from the detectable zone, resulting in an equilibrium concentration balancing formation and diffusion. The fluorescence intensity (F) from a solution of *E*-CA4F versus laser power (P) (inset to Figure 3) shows the expected quadratic relationship ($F \propto P^n$ with $n = 2.07 \pm 0.05$) at low laser powers up to about 1.5 mW. However a plot of F/P (Figure 3) shows deviation from the expected linear behaviour due to saturation at higher laser powers that is indicative of depletion of the ground state *E*-CA4F molecules as a result of photoisomerization. The saturation threshold of ~ 2 mW laser power is less than that expected (~ 30 mW) from simple excitation and fluorescence decay[20] and strongly suggests that the observed saturation results from conversion of the fluorescent *E*-isomer to the non-fluorescent *Z*-isomer by photoisomerization. For the non-fluorescent *Z*-combretastatins, fluorescence was observed as the laser power was increased and is interpreted as arising from photoisomerization to the fluorescent E-isomer. Through a series of calibrations using solutions of increasing concentrations of *E*-combretastatin, the photoisomerization could be quantified and shown to produce up to 25% conversion within the focal volume at an average laser power of 4.5 mW (Figure 4). These measurements probe only the focal volume, from which photochemical products will rapidly diffuse, and it is concluded that in solution sufficient amounts of *Z*-combretastatins are formed by 2PE photoisomerization of *E*-combretastatins to exert a cytotoxic effect.

3.2 Cell death induced by combretastatin 2-photon isomerization

Z-CA4 induced apoptosis has been reported in human endothelial cells.[21] Staining for cell death (propidium iodide (PI), red fluorescence) and apoptosis (annexin V AlexaFluor488 conjugate, green fluorescence) in cultured Chinese Hamster Ovarian (CHO) cells following exposure to *Z*-CA4F (Figure 5A) shows significant apoptosis at 1 μM drug. The combined effects of laser irradiation at 625 nm and exposure to *E*-CA4 on live cell CHO monolayers are shown in Figure 5B. The central 100 μm^2 area of each filed was illuminated by raster scanning a focused laser beam (625 nm). PI and annexin V staining reveal the effects of increasing laser power and *E*-CA4 concentration. Control cells (no *E*-CA4, Fig. 5B, panels a1-e1) show only occasional staining for cell damage at low laser powers (< 6 mW), but at the higher powers (> 6 mW) there are signs of light-induced cell damage in the central illuminated region. Cells subjected to combined incubation with *E*-CA4 and laser illumination display positive staining for cell death within the laser exposed central regions of the fields shown, whereas the un-exposed peripheral areas remain largely unaffected. These unirradiated border regions represent the control of cells in the presence of drug without light. At *E*-CA4 concentrations of 25 μM and below these peripheral areas show little cell damage, emphasizing the difference in toxicity between the *E*-isomer (Figure 5B) and the *Z*-isomer (Figure 5A). Optimal induction of cell damage by combined effects of light and *E*-CA4 appears at 4.7 mW laser power and 10 μM *E*-CA4 (panel c2). It is noticeable that even in the presence of higher concentrations of *E*-CA4, the highest laser power damages the cells to such an extent that they show less overall staining with both labels (e1-e4). Similar results were obtained with *E*-CA4F.

4 Discussion

The low saturation threshold value of ~ 2 mW observed for the multiphoton-induced fluorescence of *E*-CA4F described in section 3.1 suggests considerable $E \rightarrow Z$ isomerization conversion in fluid solution at higher laser powers. The efficiency of similar isomerization of *E*-CA4 in the cellular experiment may be estimated from an appropriate calculation taking into account the 2PE cross section (σ_2 ~2 x 10^{-50} cm^4 s $photon^{-1}$ at 625 nm,[3] the pulse parameters (76 MHz repetition, 180 fs pulse duration), the focal area (~1 μm diameter) and a typical overall power (5 mW). With these values the peak photon fluence within the pulse (F_p) is ~ 10^{29} photons cm^{-2} s^{-1}.

At a concentration c of solute, equation 2 gives the rate of excitation per cm^3 (N_{ex}) as 6 x 10^{25} molecules cm^{-3} s^{-1} in a 1 mM solution. The concentration of *E*-CA4 within cells is not uniform, but may readily exceed 1 mM locally within lipid droplets and other hydrophobic regions according to our FLIM data.[3]

$$\frac{d(N_{ex})}{dt} = \frac{1}{2}\sigma_2 \, c \, F_p^2 \quad \text{.....} \quad \textit{Equation} \;\; 2$$

The number of excitations ***per molecule*** within a single 200 fs laser pulse is 2 x 10^{-5}. In our cell experiments each voxel was exposed to the laser for 40 ms (2 ms dwell time and 20 scans) equivalent to 32 x 10^5 laser pulses. Therefore the total number of excitations per molecule in the experiment was 64. Allowing for the quantum yield for photoisomerization (ca 0.4) this shows

sufficient energy is deposited at each voxel to convert a significant fraction of *E*-CA4 to the active *Z* isomer, although at present we do not know the composition of the photostationary state since σ_2 for the non-fluorescent *Z*-isomer is unknown, but is expected to be slightly less than that of the *E*-isomer based on the one-photon absorption cross section in the UV (section 3.1). Since the laser beam was scanned in the XY plane with a step size of ~0.8 μm, this resulted in deposition of a fairly uniform plane of converted drug with a depth of about 1.25 μm (twice the wavelength) within the cell monolayer.

The results demonstrate that using a combination of *E*-CA4 and red light leads to effective cell killing attributable to two-photon induced isomerization of *E*-CA4 to *Z*-CA4. The findings provide evidence for the development of a new form of phototherapy based on light-induced oxygen independent pro-drug activation. Activation of the *E*-combretastatins occurs via a singlet excited state with sub-nanosecond lifetime; it is oxygen-independent as demonstrated for one-photon isomerization and therefore goes beyond current PDT limitations to treat hypoxic cancerous tissue. Recently *Z*-CA4 has been studied in clinical trials[8] and whilst proven to have good anticancer activity it has indiscriminate systemic toxicity that limits wide clinical application.[22] Locally targeted two-photon pro-drug activation is a promising route to overcoming this problem: drug activation is limited to the area of illumination (i.e. tumour targeted to the single cell level) which can be precisely controlled using well established features of two-photon illumination. The use of 2PE overcomes the problem of competitive background absorption of UV light by other cellular/tissue constituents that would naturally prevent sufficient light penetration to activate the drug. The extent of penetration of focused light for effective two-photon activation depends on the extent of scattering and wavelength,[1] but there

are several reports in the literature of effective tumour and vascular control using two-photon PDT.[15,23-26] Starkey et al.[15] show that effective PDT might be attained at up to 4 cm depth in tissue models with unfocussed lasers. Development of more effective combretastatins for the purpose described here would benefit from being able to use longer wavelengths (> 700 nm) to activate the isomerization process and take advantage of reduced scattering and greater transmission than at the wavelength presently used (625 nm) with thee-photon activation (at ~960 nm) being a further option. *E*-Combretastatins with higher two-photon absorption cross-sections are also seen as desirable and we are currently investigating new molecules with this in mind.[27]

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References

1. P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette and B.C. Wilson, "Photodynamic therapy of cancer: an update," *CA Cancer J. Clin*. **61,** 250–281 (2011) [doi:10.3322/caac.20114].
2. R. H. Bisby, S. W. Botchway, J. A. Hadfield, A. T. McGown and K. M. Scherer, "Multi-photon isomerisation of combretastatins and their use in therapy," UK Patent Application WO2013021208 (A2) (2013)

3. R. H. Bisby, S. W. Botchway, J. A. Hadfield, A. T. McGown, A. W. Parker and K. M. Scherer, "Fluorescence lifetime imaging of *E*-combretastatin uptake and distribution in live mammalian cells," *Eur. J. Cancer* **48,** 1896-1903 (2012) [doi:10.1016/j.ejca.2011.11.025].
4. R. H. Bisby, S. W. Botchway, G. M. Greetham, J. A. Hadfield, A. T. McGown, A. W. Parker, K. M. Scherer and M. Towrie, "Time-resolved nanosecond fluorescence lifetime imaging and picosecond infrared spectroscopy of combretastatin A-4 in solution and in cellular systems," *Meas. Sci. Technol.* **23,** 084001 (10pp) (2012) [doi:10.1088/0957-0233/23/8/084001].
5. G. G. Dark, S. A. Hill, V. E. Prise, G. M. Tozer, G. R. Pettit and D. J. Chaplin, "Combretastatin A-4 , an agent that displays potent and selective toxicity toward tumor vasculature," *Cancer Res.* **57,** 1829-1834 (1997).
6. E. L. Schwartz, "Anti-vascular actions of microtubule-binding drugs," *Clin. Cancer Res.* **15,** 2594-2601 (2009) [doi: 10.1158/1078-0432.CCR-08-2710].
7. J. H. Bilenker, K. T. Flaherty, M. Rosen, L. Davis, M. Gallagher, J. P. Stevenson, W. Sun , D. Vaughn, B. Giantonio, R. Zimmer, M. Schnall and P. J. O'Dwyer, "Phase I trial of combretastatin A-4 phosphate with carboplatin," *Clin. Cancer Res.* **11,** 1527-1533 (2005) [doi:10.1158/1078-0432.CCR-04-1434].
8. G. J. Rustin, G. Shreeves, P. D. Nathan, A. Gaya, T, S, Ganesan, D. Wang, J. Boxall, L. Poupard, D. J. Chaplin, M. R. L. Stratford, J. Balkissoon and M. Zweifel, "A phase Ib trial of CA4P (combretastatin A-4 phosphate), carboplatin, and paclitaxel in patients with advanced cancer," *Br. J. Cancer* **102,** 1355-1360 (2010) [doi:10.1038/sj.bjc.6605650].

9. K. Gaukroger, J. A. Hadfield, N. J. Lawrence, S. Nolan and A. T. McGown, "Structural requirements for the interaction of combretastatins with tubulin: how important is the trimethoxy unit?" *Org. Biomol. Chem.* **1,** 3033–3037 (2003) [doi: 10.1039/b306878a].
10. H. Görner and H. J. Kuhn, "Cis-trans photoisomerization of stilbenes and stilbene-like molecules," *Adv. Photochem*. **19**, 1–117 (1995).
11. J. A. Hadfield, A. T. McGown, S. P. Mayalap, E. J. Land, I. Hamblett, K. Gaukroger, N. J. Lawrence, L. A. Hepworth and J. Butler, "Substituted stilbenes and their reactions," US Patent US2013023663 (A1) (2007).
12. A. Torricelli, A. Pifferi, P. Taroni, E. Giambattistelli and R. Cubeddu, "In vivo optical characterization of human tissues from 610 to 1010 nm by time-resolved reflectance spectroscopy," *Phys. Med. Biol.* **46**, 2227–2237 (2001) [doi:10.1088/0031-9155/46/8/313].
13. A. Ustione and D. W. Piston, "A simple introduction to multiphoton microscopy," *J. Microscopy* **243**, 221-226 (2011) [doi: 10.1111/j.1365-2818.2011.03532.x].
14. M. Pawlicki, H. Collins, R. G. Denning and H. L. Anderson, "Two-photon absorption and the design of two-photon dyes," *Angew. Chem. Int. Edit.* **48,** 3244-3266 (2009) [doi:10.1002/anie.200805257].
15. J. R. Starkey, A. K. Rebane, M. A. Drobizhev, F. Meng, A. Gong, A. Elliott, K. McInnerney and C. W. Spangler, "New two-photon activated photodynamic therapy sensitizers induce xenograft tumor regressions after near-IR laser treatment through the body of the host mouse," *Clin. Cancer Res.* **14,** 6564-6573 (2008) [doi:10.1158/1078-0432.CCR-07-4162].
16 N. J. Lawrence, L. A. Hepworth, D. Rennison, A. T. McGown and J. A. Hadfield, "Synthesis and anticancer activity of fluorinated analogues of combretastatin A-4," J. Fluorine Chem. **123,** 101-108 (2003) [doi: 10.1016/S0022-1139(03)00117-9].

17. S. W. Botchway, P. Reynolds, A. W. Parker and P. O'Neill, "Laser-induced radiation microbeam technology and simultaneous real-time fluorescence imaging in live cells," *Methods Enzymol.* **504,** 3–28 (2012) [doi: 10.1016/B978-0-12-391857-4.00001-X].
18. P. J. Darcy, H. G. Heller, P. J. Strydom and J. Whittall, "Photochromic heterocyclic fulgides. Part 2. Electrocyclic reactions of (E)-α-2,5-dimethyl-3-furylethylidene(alkyl-substituted methylene)succinic anhydrides," *J. Chem. Soc. Perkin Trans. 1* 202-205 (1981) [doi: 10.1039/p19810000202]
19. M. Bayda, G. L. Hug, J. Lukaszewicz, M. Majchrzak, B. Marciniec and B. Marciniak, "Kinetics of reversible photoisomerization: determination of the primary quantum yields for the E-Z photoisomerization of silylenephenylenevinylene derivatives," *Photochem. Photobiol. Sci*. **8,** 1667–75 (2009) [doi: 10.1039/b907242j].
20. C. Xu, W. Zipfel, J. B. Shear, R. M. Williams and W. W. Webb, "Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy," *Proc. Natl. Acad. Sci. USA* **93**, 10763-10768 (1996) [doi: 10.1073/pnas.93.20.10763].
21. S. Iyer, D. J. Chaplin, D. S. Rosenthal, A. H. Boulares, L. Y. Li and M. E. Smulson, "Induction of apoptosis in proliferating human endothelial cells by the tumor-specific antiangiogenesis agent combretastatin A-4," *Cancer Res.* **58**, 4510-4514 (1998).
22. M. Busk, A. B. Bohn, M. Skals, T. Wang and M. R. Horsman, "Combretastatin-induced hypertension and the consequences for its combination with other therapies," *Vasc. Pharmacol.* **54**, 13-17 (2011) [doi: 10.1016/j.vph.2010.10.002].
23. M. Khurana, H. A. Collins, A. Karotki, H. L. Anderson, D. T. Cramb and B. C. Wilson, "Quantitative in vitro demonstration of two-photon photodynamic therapy using photofrin

and visudyne," *Photochem. Photobiol.* **83**, 1441–1448 (2007) [doi:10.1111/j.1751-1097.2007.00185.x].

24. H. A. Collins, M. Khurana, E. H. Moriyama, A. Mariampillai, E. Dahlstedt, M. Balaz, M. K. Kuimova, M. Drobizhev, V. X. D. Yang, D. Phillips, A. Rebane, B. C. Wilson and H. L. Anderson, "Blood-vessel closure using photosensitizers engineered for two-photon excitation," *Nat. Photonics* **2**, 420-424 (2008) [doi: 10.1038/nphoton.2008.100].
25. M. Gary-Bobo, Y. Mir, C. Rouxel, D. Brevet, I. Basile, M. Maynadier, O. Vaillant, O. Mongin, M. Blanchard-Desce, A. Morere, M. Garcia, J-O. Durand and L. Raehm, "Mannose-functionalized mesoporous silica nanoparticles for efficient two-photon photodynamic therapy of solid tumors," *Angew. Chem. Int. Edit.* **50,** 11425–11429 (2011) [doi: 10.1002/anie.201104765].
26. J. R. Starkey, E. M. Pascucci, M. A. Drobizhev, A. Elliott and A. K. Rebane, "Vascular targeting to the SST2 receptor improves the therapeutic response to near-IR two-photon activated PDT for deep-tissue cancer treatment," *Biochim. Biophys. Acta* **1830**, 4594–4603 (2013) [doi: 10.1016/j.bbagen.2013.05.043].
27. K. M. Scherer, R. H. Bisby, S. W. Botchway, G. M. Greetham, J. A. Hadfield, A. W. Parker and M. Towrie, "Spectroscopy and fluorescence lifetime imaging in live cells of a cyano-substituted combretastatin," *Biomed. Spectrosc. Imaging*, **3**, 211-218 (2014) [doi: 10.3233/BSI-140067].

Kathrin M. Scherer received her BSc (2009) and PhD (2013) from the University of Salford. Since November 2012 she has been a postdoctoral researcher within the Central Laser Facility at

the Rutherford Appleton Laboratory STFC, using advanced fluorescence imaging techniques including single-molecule TIRF and super-resolution STED imaging to study the structure and function of the epidermal growth factor receptor (EGFR) family. She has authored 4 peer-reviewed publications and 1 patent.

Roger H. Bisby received his BSc (1969) and PhD (1973) from the University of Nottingham. He is emeritus professor at the University of Salford and a Fellow of the Royal Society of Chemistry. He has authored over 90 scientific publications that reflect his lifelong interest in fast reactions and biochemical spectroscopy.

Stanley W. Botchway received his BSc in Chemistry (1990) and PhD (1996) from the University of Leicester followed by a Fellowship at Harvard Medical School. He is currently visiting professor at Oxford Brookes University and STFC senior scientist. He has developed laser applications including instruments for ultrafast microbeam irradiation of cellular DNA and multiphoton FLIM. He has authored over 70 papers and is a member of the Royal Society of Chemistry and Radiation Research Society.

John A. Hadfield is Senior Lecturer in Medicinal Chemistry at the University of Salford. He received his BSc in Chemistry from the University of Nottingham (1979) and his PhD in Organic Chemistry from Trent Polytechnic (1984). He is the author of more than 50 journal papers and was Chairman of Onco-NX (2011-2014). His research interests include the development of anticancer drugs, especially antivascular agents.

Anthony W. Parker received his PhD (University of Warwick, photochemistry of DNA) and was postdoctoral fellow at the Royal Institution of Great Britain. He joined the Rutherford Appleton Laboratory in 1986. He is now STFC Fellow applying Raman spectroscopy to bone disease, co-inventor of Spatially Offset Raman Spectroscopy with 5 patents and 250 publications. He has honorary chairs at UCL and Universities of Salford and Stellenbosh and is Fellow of the Royal Society of Chemistry.

Caption List

Fig 1 Structures of combretastatins referred to in the text showing photo-reversible isomerization between *E*- (*trans*, upper) and *Z*- (*cis*, lower) isomers.

Fig 2 Changes in the absorption spectrum of *E*-CA4 (67 μM) in N_2-saturated methanol on irradiation at 340 nm. The inset shows the change in absorbance at 340 nm with irradiation time.

Fig 3 Effect of average laser power (P) on fluorescence intensity (F, at 390 nm) from a solution of *E*-CA4F (0.5 mM) in ethanol on illumination with the femtosecond laser beam at 624 nm. The main figure shows a plot of F/P versus P with the solid line showing the expected linear plot for two-photon excitation. The inset shows a direct plot of F versus P data showing the expected quadratic relation at low laser powers confirming the 2PE process under these conditions.

Fig 4 Percentage conversions to the *E*-isomers deduced from fluorescence intensities produced by a focused laser beam excitation at 590 nm in 1 mM DMSO solutions of *Z*-CA4 (■) and *Z*-CA4F (□).

Fig 5 Apoptosis and cell permeability induced in CHO cell monolayers by combretastatins assessed by staining with Annexin V AlexaFluor488 conjugate (green) and propidium iodide (red) respectively. A: effect of increasing concentration of *Z*-CA4F (0, 0.1 and 1 μM) after 48 h. B: effects of *E*-CA4 (0, 10, 25 and 50 μM) added 2 h before irradiation of a 100 100 μm field for 10 min at 625 nm with increasing laser powers at the sample (1.6, 3.1, 4.7, 6.3 and 9.4 mW).