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Time-resolved nanosecond fluorescence lifetime imaging and picosecond infrared spectroscopy of combretastatin A-4 in solution and in cellular systems

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Short Title – Spectroscopy and cell imaging of combretastatins

ABSTRACT

Fluorescence lifetime images of intrinsic fluorescence obtained with two-photon excitation at 630 nm are shown following uptake of a series of *E*-combretastatins into live cells, including human umbilical vein endothelial cells (HUVECs) that are the target for the anticancer activity of combretastatins. Images show distribution of the compounds within the cell cytoplasm and in structures identified as lipid droplets by comparison with images obtained following Nile red staining of the same cells. The intracellular fluorescent lifetimes are generally longer than in fluid solution as a consequence of the high viscosity of the cellular environment. Following incubation the intracellular concentrations of a fluorinated derivative of *E*-combretastatin A4 in HUVECs are up to between 2 and 3 orders of magnitude higher than the concentration in the surrounding medium. Evidence is presented to indicate that at moderate laser powers (up to 6 mW) it is possible to isomerize up to 25% of the combretastatin within the femtolitre focal volume of the femtosecond laser beam. This suggests that it may be possible to activate the *E*-combretastatin (with low cellular

toxicity) to the *Z*-isomer with high anticancer drug activity using two-photon irradiation. The isomerization of *Z*- and *E*-combretastatins by 266 nm irradiation has been probed by ultrafast time-resolved infrared spectroscopy. Results for the *E*-isomer show a rapid loss of excess vibrational energy in the excited state with a lifetime of 7 ps, followed by a slower process with a lifetime of 500 ps corresponding to the return to the ground state as also determined from the fluorescence lifetime. In contrast the *Z*-isomer, whilst also appearing to undergo a rapid cooling of the initial excited state, has a much shorter overall excited state lifetime of 14 ps.

Keywords: Fluorescence, lifetime, imaging, microscopy, two-photon, combretastatin, live cell, time-resolved, infrared, spectroscopy, ultrafast

DEDICATION

This paper is dedicated to the memory of Professor Christopher G Morgan (1949-2011). He was a valued colleague and friend at the University of Salford and made significant contributions to the development and applications of fluorescence lifetime imaging.

1. INTRODUCTION

Biophotonics offers opportunities to probe molecular processes that occur within individual living cells in real time and therefore provides insight to the inner working of a cell. Examples include observation of intracellular pH [1] and changing concentrations of important species such as calcium ions [2] and NADH [3]. Such observations become even more informative when the intracellular distributions are observed by imaging techniques. Central to this is confocal microscopy which has good spatial and temporal resolution and which also has the power to detect individual molecular species in living cells based on fluorescence or Raman spectroscopy [4,5]. Fluorescence methods have intrinsically high sensitivity with the ability to report on molecular processes and environmental effects [6] and depend either on the use of covalently attached fluorescent labels (ranging from small fluorophores to fluorescent proteins) or on native intrinsic fluorescence of the molecule to be imaged. Whilst there is extremely widespread use of fluorescence labels in bioimaging, there are comparatively few instances of low molecular weight biomolecules with intrinsic fluorescence, including tryptophan, serotonin and NADH, that allows them to be imaged directly [7]. In the case of tryptophan and serotonin the excitation occurs at wavelengths shorter than 400 nm, resulting in potential problems with transmission of the exciting light through both the sample and microscope optics. These may be avoided through multiphoton excitation. For example Webb and co-workers [8] have used 3-photon excitation at 750 nm of native UV fluorescence from intracellular serotonin to observe granulation in mast cells and measure local concentrations of up to 50 mM. Similarly, two-photon excitation, especially in the region of 630 nm exploiting a spectral window that substantially avoids excitation of cellular tryptophan, allows observation of intracellular serotonin [9,10] and the beta-blocker drug propranolol [11].

Two- and three-photon excitation of cellular fluorophores is most often measured using sub-picosecond pulses provided by a titanium-sapphire laser that also enables simultaneous pixel-by-pixel recording of fluorescence lifetimes using the time-correlated single photon counting method. Fluorescence lifetime imaging (FLIM) microscopy has many applications and benefits [12]. It may be used to quantify fluorophore concentrations by correcting for dynamic quenching according the well-known Stern-Volmer equation. It also enables studies of protein-protein association in cells using fluorescence resonance energy transfer (FRET)

[12], the direct lifetime based imaging of local intracellular viscosity using molecular rotors [13], and the observation of lipid domains by lifetime contrast [14].

Combretastatins are derived from natural products with significant anticancer activity by virtue of their ability to inhibit the development of blood vessels and so starve growing tumours of their nutritional requirements [15]. Combretastatins are substituted stilbenes and are considerably more active in the *Z-(cis)* configuration (LD_{50} typically 10^{-8} M in cellular assays) compared with the *E-(trans)* configuration (LD_{50} in the region of 10^{-5} M) [16]. Combretastatin A4 in the *Z*-form is the most intensively investigated member of the group and has been tested in preliminary clinical trials. Photoisomerization of stilbenes has been intensively investigated [17] as a model photophysical system. *E*-Stilbenes generally have solvent and viscosity dependent lifetimes of $\sim 10^{-10}$ s for the S_1 state that decays by fluorescence and isomerization. In contrast the S_1 states of *Z*-stilbenes generally decay in a virtually barrier free process with lifetimes of $\sim 10^{-12}$ s and are therefore effectively non-fluorescent. Such processes have been studied using time-resolved fluorescence and Raman spectroscopy [17-20].

Photoisomerization might offer a route to activation of the low activity *E*-combretastatin to the higher activity *Z*-isomer. Light activation of drugs has been proposed to have the advantages of temporal and spatial control [21], and would therefore limit drug activity to diseased parts of the body and avoid adverse effects in normal tissues. Examples include sensitizer drugs that form singlet oxygen for photodynamic therapy (PDT) of cancer [22] and light-controlled release of drugs from photosensitive liposomes [23]. PDT is growing in application, but faces the same limitations as in imaging that are imposed by tissue absorption and scattering at short wavelengths. Consequently in practice the sensitizers used mainly absorb in the region 700-900 nm. Alternatively, 2-photon activation of PDT agents within this tissue window is possible and is attracting current interest [24-25].

MATERIALS AND METHODS

Unless otherwise noted, solvents and reagents were purchased from Sigma Aldrich or Alfa Aesar and used as supplied. Anhydrous tetrahydrofuran (THF) was freshly distilled over sodium and benzophenone under an argon atmosphere as required.

The work described here involved the investigation of combretastatin A-4 (1-(3',4',5'-trimethoxyphenyl)-2-(4''-methoxy-3"-hydroxy- phenyl)ethene, CA4) and four related derivatives (Figure 1). These are 1-(3',4',5'-trimethoxyphenyl)-2-(3"-fluoro-4"-methoxyphenyl)ethene (CA4F), 4-[2-(3,4,5-trimethoxyphenyl)vinyl]benzonitrile (CNCA4), 1-[4-(N,N-dimethylamino)phenyl]-2-(3,4,5-trimethoxyphenyl)ethene (DMACA4) and 1-(3'-Amino-4'-methoxyphenyl)-2-(3",4",5"-trimethoxyphenyl)ethene (ACA4). These were synthesized using published procedures, or adaptations, for CA4 [26], CA4F [27], CNCA4 [28], DMACA4 [29] and ACA4 [30, 31]. All compounds were purified and *E*- and *Z*-isomers separated by flash column chromatography on silica gel (35-70 μm diameter, 60 Å pore size) with petroleum ether : EtOAc 19:1 as solvent, followed by recrystallization from ethanol. Identity and purity of the materials were confirmed using thin layer chromatography and ^{1}H and ^{13}C NMR spectroscopy at 400 MHz and 100 MHz respectively (Bruker AC-400). Log P values were obtained using Molinspiration software (www.molinspiration.com).

HeLa and Chinese hamster ovary (CHO) cells were from the European Collection of Cell Cultures. Human umbilical endothelial cells (HUVECs) were obtained from TCS Cellworks. HeLa cells were grown in MEM medium (Gibco) containing 10% FCS, penicillin (100 units/mL), streptomycin (100 μg/mL) and glutamine (2 mM). HUVECs were sub-cultured in basal medium (TCS Cellworks) supplemented with antibiotic supplement gentamycin/amphotericin-B, following the detailed guidelines provided by TCS Cellworks. For microscopy the adherent cell cultures were grown in 35 mm diameter glass-bottom culture dishes with optical quality glass (MaTek Corporation) and incubated at 37 °C in an atmosphere containing 5 % CO_2.

The microscope system used for the multiphoton experiments has been described previously [9, 32]. Briefly, it consists of an inverted Nikon TE2000U microscope with a water-immersion objective (NA 1.2, x60). For multiphoton microscopy the sample could be excited with pulses (ca 180 fs, 76 MHz) from either a titanium:sapphire laser (710-1000 nm) or an optical parametric oscillator (OPO) pumped by the titanium:sapphire laser (560-630 nm). Fluorescence lifetime images were obtained using a Becker and Hickl time-correlated single photon counting system (SPC830) and software (SPCImage). Alternatively, fluorescence spectra could be obtained through a second microscope port using a spectrograph (Acton

275) and Andor CCD camera. Also fitted to the same microscope was a scanning confocal microscopy system (Nikon eC1-Si) with either 488 nm or 543 nm excitation.

The ultrafast time-resolved infrared (TRIR) experiments were performed on the "ULTRA" laser system at the Science and Technology Facilities Council Rutherford Appleton Laboratory. The capabilities of the ULTRA instrument are described in detail elsewhere [33]. Briefly, a chirped pulse amplified titanium:sapphire laser operating at 10 kHz repetition rate produced 40–80 fs duration pulses at a wavelength of 800 nm. The UV excitation pulse was generated from third harmonic generation of the 800 nm output of the amplifier with ~50 fs duration at 266 nm, irradiating samples with a 1 μJ, 100 μm diameter beam. The mid-IR probe pulse was generated using ~0.4 mJ of the 800 nm femtosecond output to pump an optical parametric amplifier (OPA), and difference frequency mixing of the signal and idler components. The mid-IR probe output pulses had ~500 cm^{-1} bandwidth and~50 fs pulse duration. The UV pulses were polarized at a magic angle to the IR probe laser polarization. The timing between the pump and probe was performed using a PC controlled optical delay line. The sample in solution was placed between two 25 mm diameter CaF_2 plates in a raster-scanned Harrick cell to limit irradiation of the sample by multiple pulses. The optical path of the sample between the two windows was controlled using a 100 μm PTFE spacer and the solution flowed using a peristaltic pump. The intensity of the transmitted mid-IR light was dispersed by a grating onto a pair of 128-element mercury-cadmium telluride array detectors (IR Associates), providing full (i.e., ~500 cm^{-1}) spectral coverage with every laser pulse. The overall instrument response time was ~200 fs. A reference spectrum of the laser pulse (before the Harrick cell) was accumulated on a third, 64-element array detector and was used to remove the effects of laser fluctuations from the transient absorption data. Ground state FTIR spectra were recorded using a Nicolet Avatar 360 spectrometer.

RESULTS AND DISCUSSION

1. *E*-combretastatin fluorescence and power dependence of fluorescence from intracellular *E*-CA4F

All of the *E*-combretastatin derivatives shown in Figure 1 have absorption and fluorescence spectra in the ultraviolet, with typical data for solutions in DMSO in Figure 1 showing that

E-CA4 and *E*-CA4F have excitation and emission maxima at 332-334 nm and 390-392 nm respectively that exhibit little dependence on solvent. The other compounds absorb at similar UV wavelengths but have emission maxima at longer wavelengths in DMSO and are more sensitive to solvent polarity. Fluorescence lifetimes of *E*-CA4 and *E*-CA4F were good single exponentials, with that of *E*-CA4 ranging from 230 ps in methanol through to 1.18 ns in glycerol, an effect due to hindrance by viscosity of the competing isomerization [32]. In DMSO solutions *E*-CNCA4, *E*-ACA4 and *E*-DMACA4 exhibited single exponential decay with lifetimes of 0.71, 4.8 and 0.36 ns respectively, but showed more complex behaviour in other solvents. In solution all the compounds in Figure 1 could be induced to fluoresce by 2-photon excitation in the region of 600-630 nm. As usual, this was demonstrated by a quadratic dependence of fluorescence intensity (FI) on laser power (P), i.e. $FI \propto P^2$. This was also demonstrated for *E*-combretastatin fluorescence after uptake in live mammalian cells. Figure 2 shows three images recorded after incubation of HeLa cells with *E*-CNCA4 at 37 °C using laser powers (at the sample) of between 0.32 and 0.97 mW. The laser wavelength used for two-photon excitation was 625 nm, providing slightly more energy than at the one-photon absorption maximum at 343 nm. Excellent contrast between *E*-CNCA4 fluorescence and cell autofluorescence was obtained in these images, and for all studies laser power was limited to ≤ 1 mW in order to minimize photodamage to the sample. Figure 2D shows that the plot of the total image fluorescence intensity versus laser power is a good fit to the expected quadratic relationship. For *E*-CNCA4 in HeLa cells, these FLIM images indicate that the drug accumulates mainly within the cell cytoplasm and is excluded from the cell nucleus.

2. FLIM of *E*-CA4F intracellular distribution in HUVEC cells and co-localisation with Nile red

Whilst studies of intracellular drug uptake in tumour-derived cell lines such as HeLa and CHO was readily observed [32], the mode of action of *Z*-combretastatins suggests that the true target cells are the endothelial cells that line the blood capillaries [15, 34]. *Z*-CA4 and analogues exert their anti-cancer activity by preventing the process of angiogenesis in which a growing tumour is supplied with new vasculature for the supply of nutrients via the bloodstream. We have therefore studied interactions between combretastatins and cultured human umbilical vein endothelial cells (HUVECs). These non-immortalised cells were found to be more difficult to work with and, unlike most tumour cells, required to be constantly maintained at 37 °C in medium with a CO_2 atmosphere on the microscope stage.

Nevertheless it was possible to obtain images of cells after uptake upon *E*-combretastatin addition to the supporting medium at relatively low concentrations. Fluorescence intensity (Figure 3A) and lifetime images (Figure 3C) of a HUVEC cell after incubation with *E*-CA4F (5 μM) at 37 °C for 60 minutes show that the combretastatin is taken up into the cytoplasmic region of the cell, but excluded from the cell nucleus. In addition there are some punctate structures apparent in the images that appear to correlate with lipid droplets identified by Nile red staining of the same cell. At 37 °C exact co-localisation of the sequential images of combretastatin fluorescence (Figure 3A) and Nile red labelled lipid droplets (Figure 3B), obtained using the two different imaging systems attached to the microscope, is not possible because of the Brownian motion and the occasional rapid saltation of these structures that occur as a result of their transport upon the cellular microtubule network [35]. The fluorescence lifetime distribution of intracellular *E*-CA4F observed in this cell simultaneously labelled with Nile red peaks at ~950 ps. A similar experiment undertaken in the absence of Nile red (not shown) shows a peak in the lifetime distribution of 1,100 ps, indicating lifetime quenching of combretastatin by Nile red through energy transfer, enabled by weak overlap of the fluorescence spectrum of *E*-CA4F (λ_{max} 390 nm) and the excitation spectrum of Nile red (λ_{max} 450-500 nm) [36]. This confirms that Nile red and *E*-CA4F exist in the same space and are able to come close enough to allow FRET. The long lifetime of *E*-CA4F fluorescence in the HUVEC cell indicates a viscous environment for *E*-CA4F within the cell which is consistent with a lipidic location such as a membrane or lipid droplet [32]. Since the fluorescence lifetimes and quantum yields of *E*-CA4F are directly related [32], it is possible to combine the fluorescence intensity and lifetime images to calculate the concentration distribution, using a series of standard solutions (DMSO was the chosen solvent with τ 810 ps). The result is shown in Figure 3E which shows that intracellular concentrations in many parts of the cell reach 500 μM, or 100 times that of *E*-CA4F added to the surrounding medium, whilst in the lipid droplets the concentration peaks at over 1 mM. It is therefore clear that *E*-CA4F (and other compounds in Table 1) are taken up by their target endothelial cell and accumulated by up to 200 times the extracellular concentration of the compound. Similar images have been obtained that show corresponding uptake of *E*-CA4F within HUVECs from solutions containing as little as 1 μM *E*-CA4F.

3. Intracellular FLIM of *E*-ACA4 and *E*-DMACA4

The other two compounds indicated in Figure 1, E-ACA4 and E-DMACA4, and not discussed so far have also been shown to be taken up into live cells as shown by the images in Figure 4. Whilst both *E*-ACA4 and *E*-DMACA4 exhibited pronounced non-exponential fluorescence decays in some solvents that remains to be investigated further and is suggestive of excited charge transfer processes, the differences in lifetimes (Figure 4) and emission spectra (Figure 5) of the intracellular fluorescence from these compounds is further confirmation that the fluorescence observed does originate from these combretastatins after cellular uptake. The intensity image for *E*-DMACA4 in CHO cells (Figure 4a1) shows a cytoplasmic distribution similar to that for *E*-CA4F, with a few bright regions resembling lipid droplets. This appears consistent with the logP (P is the octanol/water partition coefficient indicative of polarity) for *E*-DMACA4 being similar to that for *E*-CA4F (Figure 1). The similarity with *E*-CA4F extends to the fluorescence lifetime of *E*-DMACA4 in DMSO (0.36 ns) which increases to a peak in the lifetime image in CHO cells at 1.07 ns (Figures 4a2 and 4a3). For *E*-ACA4 the logP value is slightly less than for *E*-CA4F and closer to that for *E*-CA4. However as a primary amine, it is likely to act as a weak base and protonates to some extent in aqueous solution becoming more polar. Indeed we observe that *E*-ACA4 is more soluble in water than the other combretastatins studied here. The fluorescence lifetime is longer than those of the other combretastatins (4.8 ns in DMSO) and although reduced to a peak at 2.15 ns in the FLIM image of CHO cells (Figure 4b3) remains longer than the lifetime of the other intracellular combretastatins observed here. The fluorescence images of *E*-ACA4 in CHO cells also appear rather different, with fluorescence intensity mainly confined to regions that appear to be intracellular vesicles, reminiscent of the distribution of serotonin in mast cells [8] or propranolol in rat aorta cells [11]. In these instances vesicular uptake is driven by the accumulation of the weak base into acidic compartments in cells such as lysosomes and mitochondria.

Figure 5 shows the fluorescence spectra of intracellular combretastatins recorded using the spectrograph and CCD camera attached to the microscope. The spectrum recorded for *E*-DMACA4 in HeLa cells has a maximum at 430 nm and is very similar to that in solution in dichloromethane, and compares with a peak at 450 nm in the more polar DMSO. Figure 5

also shows the expected emission from *E*-CA4 in HeLa cells at shorter wavelengths. However the spectrum reflects the spectral sensitivity of the microscope system that diminishes rapidly below about 380 nm.

4. Isomerization of combretastatin A-4 within the focal volume of two-photon excitation

The ultimate objective of this project is to use 2-photon activation of a low activity *E*-combretastatin to the active *Z*-isomer with high drug activity within the target site *in vivo*. At present experiments are focussed on demonstrating effective $E \rightleftharpoons Z$ interconversion of a combretastatin with red or near-infrared laser 2-photon excitation (2PE), initially in solution and in cultured cells. There can be no doubt that such isomerization occurs, since it originates from the same excited state as produces fluorescence; however what is at issue is whether such isomerization is sufficiently efficient as to deliver useful amounts of drug capable of exerting a toxic effect on cells and showing a therapeutic effect. Using 2PE the photochemical effects occur within the femtolitre volume of excitation [37]. Although our previous attempts to measure chemical change within a bulk sample have been unsuccessful, the femtolitre volume is significant in comparison with cellular dimensions and converted drug is expected to diffuse from the illuminated femtolitre volume into surrounding regions of the cell. More widely dispersed drug delivery would also be enabled by scanning the beam as in the imaging experiments. At any one instant the concentration of isomerized compound in the focal volume will represent a steady state achieved through the balance of diffusion from the focal volume and the rate of photochemical formation.

Direct demonstration of the desired $E \rightarrow Z$ isomerization of CA4 is difficult, as in terms of fluorescence it would involve a loss of fluorescence intensity that may also occur for several reasons. However an indication that this does occur within solutions is demonstrated by Figure 6A which shows the observed dependence of 2-photon excited fluorescence from *E*-CA4 on laser power up to 6 mW incident on the sample. (This is rather higher power then would normally be used in imaging, when we prefer to use less than 1 mW in order to limit cell damage). Whilst a good quadratic power dependence (indicated by the solid line) is observed below 1mW, saturation occurs at higher powers and may at least in part be ascribed to isomerization and the desired formation of *Z*-CA4. In contrast, the demonstration of *E*-CA4 as a fluorescent product from 2-PE excitation of non-fluorescent

Z-CA4 is expected to be much easier to demonstrate. Calibration curves were constructed of fluorescence intensity versus *E*-CA4 concentration at each laser power. These were then compared with the fluorescence intensity from the two photon experiment with *Z*-CA4 to estimate the concentration of *E*-CA4 formed within the focal volume. The results in Figure 6B indicate that about 250 μM *E*-CA4 is formed within the focal volume. This corresponds to the conversion of 25% of the initial concentration of *Z*-CA4 (1 mM) in solution.

5. Investigation of excited states of *E*- and *Z*-CA4 by picosecond time-resolved infrared (TRIR) spectroscopy

Figures 7 and 8 show the TRIR spectra of both isomers of CA4 following ultrafast electronic excitation using 266 nm, with the ground state FTIR spectra shown beneath the time-resolved data for comparison. To date there has been little characterisation of combretastatins by infrared spectroscopy [38]. However, the ground state FTIR spectra have obvious similarities with stilbene and on this basis we assign the 1586/1581 and 1510/1509 cm^{-1} bands in *E*-CA4/*Z*-CA4 to the ν_{8a} ring stretches of the phenyl groups and ν_{19a} ring stretch with additional contributions from C-H in-plane bending. In the excited state spectra there are negative absorption bands corresponding to bleaching of the ground state absorptions and both the *E*- and Z-isomers are dominated by transient bands between 1350 and 1500 cm^{-1}. At early times the transient bands show rapid shifting over ca. 10 cm^{-1} to higher wavenumbers associated with rapid "cooling" of excess vibrational energy following electronic excitation as commonly observed in other molecular systems including DNA [39] and for the C=C stretching band of *E*-stilbene observed by time-resolved resonance Raman spectroscopy [20]. It is also notable that for *E*-CA4 at pump-probe delays up to 20 ps the intensities of the transient bands increase and this is most likely due to molecular rearrangements taking place lowering the overall symmetry of the molecule and increasing the cross-sections of these IR active modes. The converse is true for *Z*-CA4 and the intensities of the ground state bleached bands are equal to or greater than than those of the excited state transient bands and whilst spectral shifting, as for the *E*-isomer, occurs due to rapid cooling we do not see any increases in intensity. This indicates that the molecular frame is likely becoming more symmetric in this case. Furthermore, whilst the ground state FTIR spectra are similar for both the *E*- and *Z*- forms, the TRIR spectra are very different

suggesting that the *E*- and the *Z*- forms do not share a common intermediate structure. As with stilbene the symmetry of the molecule in the ground and excited states of both *E*- and *Z*-isomers means that the central C=C double bond, whilst having an extremely high Raman intensity [40] is not observable in the FTIR/TRIR spectra. This is also the case for diphenylacetylene (DPA) and indeed in their TRIR study Ishibashi *et al.* [41] observe that in the excited state TRIR spectrum the out of phase ν_{8a} and ν_{19a} modes shift to lower wavenumber from their ground state positions by 45 and 46 cm^{-1} respectively. On this basis we tentatively assign the transient bands at 1481 and 1459 cm^{-1} to correspond to the ν_{8a} and ν_{19a} modes of S_1 respectively. The kinetics of formation and decay of the S_1 states of *E*-CA4 and the *Z*-CA4 are vastly different and again reflect those of the parent stilbene in so far as the rate of excited state decay of *Z*- isomer is an order of magnitude faster than for the *E*-isomer. The kinetics were fitted to a biexponential decay, see inserts in Figures 7 and 8, for all the transient and recovery (bleach) bands giving average values for *E*-CA4 in dichloromethane of 6.5 ± 0.46 ps and 503 ± 56 ps and for *Z*-CA4 1.5 ± 0.15 ps and 10.1 ± 0.71 ps. The shorter lifetime is the rapid cascading from S_1 ($\upsilon > 1$) and the longer lifetime the relaxation time of the equilibrated upper electronic state. This lifetime of the relaxed excited state of *E*-CA4 measured by TRIR agrees well with the fluorescence lifetime of 500 ps shown in Table 1. Furthermore, the lifetime of the relaxed excited state measured by TRIR for *E*-CA4 in methanol (230 ± 14 ps) is also the same as the fluorescence lifetime in methanol (230 ps). On close inspection of the TRIR spectra for *Z*-CA4 (Figure 8) one is able to directly observe the *Z*- to *E*-isomerization via the small positive growing bands at 1512 and 1586 cm^{-1}.

In conclusion, this study has shown that the intracellular concentrations of *E*-combretastatin derivatives within representative drug target cells (HUVECs) may be estimated by FLIM. The compounds are accumulated within the cell, residing mainly within lipid structures, and reach several hundred times the concentration of that in the extracellular medium. Evidence is provided to demonstrate *E*⇌*Z* isomerization of combretastatin A4 by two photon absorption within the focal volume of a near-infrared femtosecond laser beam, suggesting this may provide an *in vivo* method of drug activation. Further studies are underway in an attempt to observe the cellular effects of *Z*-combretatstatins generated intracellulary in this

manner. Although 2-photon induced photodynamic therapy involving the intermediacy of singlet oxygen has already been demonstrated [25], we believe this is the first approach using isomerization of stilbenes derivatives. Finally the dynamics of excitation and isomerization have been investigated by ultrafast time-resolved infrared spectroscopy, showing initial ultrafast cooling of the initial state and subsequent isomerization, with the excited state of *Z*-CA4 being much shorter lived than that of the *E*-isomer.

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FIGURE LEGENDS

Figure 1 Structures of the combretastatin analogues used in this work. Photophysical properties including fluorescence quantum yields (ϕ) and fluorescence lifetimes (τ) were measured in dimethyl sulfoxide (DMSO) solution.

Figure 2 A series of fluorescence intensity images from HeLa cells incubated with *E*-CNCA4 (50 μM) for 30 minutes measured with increasing laser powers of 0.32 (A), 0.65 (B) and 0.97 (C) mW at the sample. Panel D shows the quadratic dependence of image intensity (average number of counts per pixel) on laser power. Each image shows a 70x70 μm field.

Figure 3 Images of a HUVEC cell after incubation with *E*-CA4F (5 μM) for 60 minutes and colocalisation with Nile red. Shown are the intensity of *E*-CA4F fluorescence (A), the *E*-CA4F fluorescence lifetime (C) images and the *E*-CA4F fluorescence lifetime distribution (D) measured with 625 nm 2-photon excitation. Also shown is Nile red fluorescence (B) excited at 488 nm. Panel E shows the concentration profile of *E*-CA4F within the cell calculated from intensity and lifetime data as described in the text. Scale bar 20 μm.

Figure 4 Fluorescence intensity (left) and lifetime (middle) images of CHO cells incubated with *E*-DMAC (top, a1 to a3) and *E*-ACA (bottom, b1 to b3) and measured with 2-photon excitation at 628 nm. The frames on the right show the lifetime distribution correspond to the images. Both compounds were added to the cell medium at a final concentration of 10 μmol dm^{-3} and the images acquired 10 minutes (*E*-ACA) and 55 minutes (*E*-DMAC) after addition. The bar represents 20 μm.

Figure 5 Fluorescence spectra of *E*-CA4 (a) and *E*-DMACA4 (b) in DCM solutions (dotted curves) measured using one photon excitation in the Spex Fluoromax fluorimeter compared with 2-photon excited (628 nm)

fluorescence spectra of the same compounds after uptake into HeLa cells (full curves).

Figure 6 Evidence for isomerization of *E*- and *Z*-CA4 induced by 2-photon absorption at 590 nm in DMSO solution. A:- Power dependence of fluorescence intensity from *E*-CA4 (0.4 mM) versus laser power at the sample. B:- Percentage conversion of *Z*-CA4 to the *E*-isomer versus laser power at the sample, calculated from the measured fluorescence intensity from a solution of Z-CA4 (1 mM) and a series of calibration curves using solutions of *E*-CA4 at each laser power.

Figure 7 TRIR spectra of *E*-CA4 (10 mM) in dichloromethane (DCM) measured with 266 nm (150 nJ $pulse^{-1}$) excitation. The upper series shows spectra recorded with pump-to-probe delays between 1 ps and 3000 ps. The spectra are colour coded up to 20 ps delay. Thereafter the spectra decay. Below the transient spectra are shown the FTIR ground state spectra of *E*-CA4 and *Z*-CA4 in DCM for comparison. Inset: kinetics for formation and decay of the positive bands at 1471 cm^{-1} (□) and 1490 cm^{-1} (●) and the negative band at 1511 cm^{-1} (■).

Figure 8 TRIR spectra of *Z*-CA4 (10 mM) in dichloromethane (DCM) measured with 266 nm (150 nJ $pulse^{-1}$) excitation. The upper series shows spectra recorded with pump-to-probe delays between 1 ps and 100 ps. Below the transient spectra are shown the FTIR ground state spectra of *E*-CA4 and *Z*-CA4 in DCM for comparison. Inset: kinetics for formation and decay of negative band at 1512 cm^{-1} (■).