ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2017, 53, 9906

Received 6th July 2017,
Accepted 15th August 2017

DOI: 10.1039/c7cc05196d

rsc.li/chemcomm

Selective photothermal inactivation of cells labeled with near-infrared croconaine dye†

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Photothermal inactivation of cells caused by laser heating of a near-infrared croconaine dye is more effective when the dye is located inside the cell. The cell inactivation is spatially confined – laser irradiation of a mixed population of two different cell lines produces selective inactivation of the cells labeled with croconaine dye and does not harm adjacent unlabeled cells.

Photothermal therapy (PTT) employs an external light source such as a laser to heat and kill diseased tissue.[1] The process is facilitated by the introduction of light absorbing agents to enhance the conversion of light energy into heat. A large number of dyes, materials, and nanoparticles have been investigated for PTT.[2–6] Materials and nanoparticles have the attractive feature of high absorbance cross sections but a potential challenge is the difficulty to fine tune the pharmacokinetic and cell penetration properties.[7,8] Organic dyes are an obvious choice as the light absorbing agent, especially dyes that absorb near infrared light because it penetrates deeper through skin and tissue.[9–12] Early studies focused on free dye molecules such as Indocyanine Green but performance was limited by the poor photostability which motivated research efforts to encapsulate and protect the dyes inside nanoparticles.[13–15] Further development of free dyes for effective PTT requires new classes of high performance molecules with the correct mix of favorable chemical, photophysical, and pharmacokinetic properties. In 2013 we reported that croconaine dyes are very promising photothermal heating agents.[16] They have very high molar absorptivity at 800 nm, short picosecond singlet excited lifetimes, low fluorescence quantum yields, low oxygen photosensitization ability, high chemical stability and high resistance to photobleaching. On a mass basis, free croconaine dyes produce more heat generation than gold nanorods. Subsequent work by us and others have confirmed the attractive photothermal properties of croconaine dyes and used them to produce heat-generating nanoparticles that do not produce singlet oxygen,[17,18] pH activated photothermal nanoparticles,[19] photothermal polymeric films that switch morphology,[20] and self-assembled nanoparticles for tumor ablation in living mouse models of cancer.[21,22]

Our long-term goal is to design molecular croconaine conjugates that target pathological cells and enable selective photothermal inactivation without harming nearby healthy cells. In this report, we test two hypotheses that are central to our objective. The first hypothesis is that laser-induced cell death is more effective when a free croconaine dye is located inside a cell. Supporting this hypothesis is a small number of literature reports that light-absorbing nanoparticles produce enhanced cell death when the nanoparticle associates with the cell.[23–28] But the photothermal inactivation produced by nanoparticles is often attributed to acousto-mechanical disruption by expanding vapor nanobubbles,[23,29] an effect that may not be possible using free dye molecules with small absorption cross-sections and continuous wave lasers. The high stability and low photobleaching properties of croconaine dyes, combined with the negligible singlet oxygen generation, makes them an excellent choice for PTT mechanism studies, and as shown below, we find that cell inactivation is enhanced when the croconaine dye is located inside the cell. This result allowed us to test a second hypothesis that cell death only occurs in cells containing the dye with no harm to neighboring cells. Again the nanoparticle PTT literature offers some support for this hypothesis but very few studies have used a free dye molecule as the light absorbing agent.[26,30,31]

To test the first hypothesis, that laser-induced cell death is more effective when the croconaine dye is inside a cell, we chose to compare the photothermal cell killing efficiencies for the three croconaine dyes shown in Scheme 1. They all have the same central chromophore and thus nearly identical absorption properties, but the structures differ in the flanking units which control molecular lipophilicity. Croconaine **1**, with flanking propargyl groups, is moderately lipophilic ($c\log P = 4.4$), whereas

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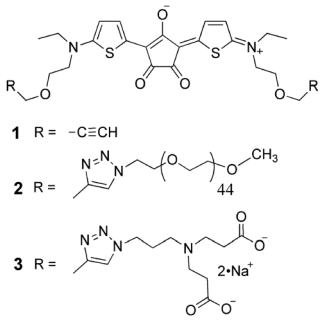
† Electronic supplementary information (ESI) available: Materials and synthesis, cell experiments, and heat shock protein measurements. See DOI: 10.1039/c7cc05196d



Scheme 1 Structures of croconaine dyes **1**, **2**, and **3**.

croconaines **2** and **3** are highly water soluble. Based on our previous experience with structurally similar dyes, we expected lipophilic **1** to readily permeate into cells, whereas hydrophilic **2** and **3** to be impermeable.[32] Thus, the photothermal cell killing efficiency for **1** was predicted to be higher than **2** and **3**.

Uptake of each dye by Chinese Hamster Ovary (CHO-K1) cells was determined by measuring the amount of dye remaining in the supernatant (determined from its absorbance at 785 nm) after incubation with cells for 15 minutes. As shown in Fig. 1A, there was essentially no cell uptake of **2** and less than 10% of **3**; whereas, more than 40% of **1** was taken up by the cells (corresponding to $\sim 10^9$ copies of **1** per cell). The very weak, near-infrared fluorescence of **1** did not allow direct microscopic imaging of its accumulation inside cells, so we examined the cell uptake of a structurally analogous squaraine dye that is highly fluorescent (Fig. S2, ESI†).[32] Fluorescence cell micrographs showed rapid permeation of the squaraine dye into organelle membranes and lipophilic sites throughout the cell interior and we assume a similar intracellular distribution for croconaine **1**.

Photothermal cell inactivation efficiency for each croconaine dye was measured using a microwell plate that was equilibrated to 37 °C. Each microwell was loaded with the same number of CHO-K1 cells and one of the three dyes. With each dye, the absorbance at 808 nm in the microwell was adjusted to be 0.025 ($\sim$1.25 μM of dye) to ensure equivalent laser heating. Each microwell in the plate was sequentially irradiated for 125 seconds with a diode laser operating at 808 nm (2 W cm^{-2}) which steadily raised the temperature in the well (measured using a near-infrared camera) from 37 °C to 41.5 °C (Fig. S3, ESI†). The laser irradiation was stopped, the supernatant immediately replaced with fresh media, and the wells incubated overnight in the dark at 37 °C.‡ The cell viability in each well was then determined by a standard MTT assay that measured mitochondrial activity as an indicator of cell viability. As shown in Fig. 1 photothermal heating of microwells containing **2** or **3** had little effect on cell viability which remained >90%, whereas the same photothermal heating of microwells containing **1** dropped cell viability to 20%. As expected, control MTT assays that incubated CHO-K1 cells under dark conditions with **1** at concentrations up to 25 μM showed little toxicity (Fig. S1, ESI†). The photothermal inactivation of cells labeled with croconaine **1** was also evaluated using cell microscopy. After laser treatment, the supernatant was replaced with fresh media and the cells incubated overnight. The following day, the cells were treated with a binary mixture of green fluorescent live-cell stain Calcein AM (3 μg mL^{-1}) and red fluorescent dead-cell stain PSVue643 (10 μM) for 20 minutes. The stained cells were washed and viewed using an epi-fluorescence microscope with the appropriate green and red filter channels. As expected, micrographs of control microwells that lacked the dye treatment or laser irradiation showed that all the cells were healthy (Fig. S4, ESI†), whereas micrographs of cells that were labeled with **1** and laser irradiated showed extensive photothermal-induced cell death. Moreover, the spatially localized nature of the photothermal inactivation was demonstrated by an experiment that irradiated a sub-section of the labeled cells in a microwell with a narrow laser beam (808 nm, 3 W cm^{-2}) and then treated the cells with live and dead cell stains. In Fig. 2 and Fig. S5 (ESI†) are representative micrographs showing a clear boundary between red-emitting dead cells that were directly illuminated by the laser and green-emitting healthy cells that were located outside the beam. Even though the entire supernatant in the microwell was heated evenly by the photothermal process, only the dye-labeled cells that directly absorbed the light were killed, further supporting the hypothesis that photothermal cell inactivation is greatly enhanced when the dye is located inside the cells.

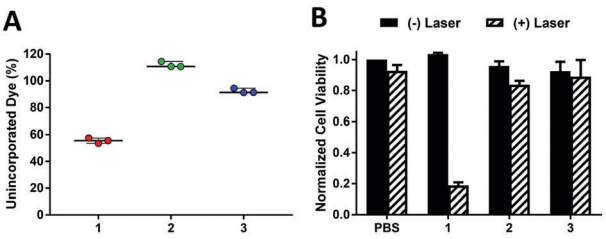


Fig. 1 (A) Percentage of croconaine dyes **1–3** not taken up by CHO-K1 cells after 15 min incubation at 37 °C. (B) Viability of CHO-K1 cells after treatment with croconaine dyes **1–3**, followed by laser irradiation at 2 W cm^{-2} for 125 seconds that raised the temperature from 37 °C to 41.5 °C, washing with fresh media, and overnight incubation at 37 °C. Error bars represent mean ± standard error of the mean. N = 3.

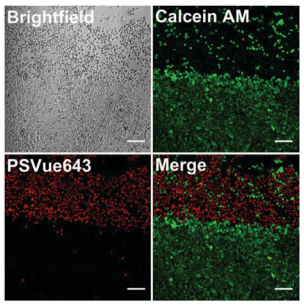


Fig. 2 Spatially localized photothermal inactivation of CHO-K1 cells that were labeled with **1** (5 μM). Micrographs showing cells after a sub-section of the microwell area was irradiated with a narrow laser beam (808 nm, 3 W cm^{-2}) for 10 minutes. After irradiation the cells were incubated overnight in fresh media at 37 °C, then treated with a mixture of red fluorescent live-cell stain Calcein AM (3 μg mL^{-1}) and red fluorescent dead-cell stain PSVue643 (10 μM; red) for 20 minutes with a PBS buffer wash. The images clearly show a border between living cells and laser-induced dead cells. Scale bar = 100 μm.

The second hypothesis addressed by this study was whether cell death only occurs in cells containing croconaine **1** with no harm to neighbouring cells. A series of experiments were conducted that mixed populations of two different cell lines; adherent CHO-K1 cells, and non-adherent Jurkat cells (Human T Lymphocytes), and measured cell selective photothermal inactivation. The two cell lines were chosen in part because they could be readily distinguished in cell micrographs of a mixed population. Before each photothermal experiment one of the cell populations was labeled by preliminary incubation with croconaine **1**. In the first set of studies, adherent CHO-K1 cells were grown to near confluency and then labeled by incubating with **1** (5 μM) for 45 minutes. The supernatant was removed and the cells washed with buffer. Unlabeled Jurkat cells in media were added to the same microwell and the cell mixture illuminated with an 808 nm diode laser at 2 W cm^{-2} until the average temperature of the well reached 41.5 °C (~125 seconds). The supernatant was immediately replaced with fresh media, the cells incubated overnight in the dark then treated with a binary mixture of fluorescent live and dead cell stains and imaged microscopically. Fig. 3 shows the red fluorescence of dead CHO-K1 cells and green fluorescence of healthy Jurkat cells. As expected there was no cell death observed with control cells that were not laser illuminated or treated with **1** (Fig. S6, ESI†). These results support the idea that photothermal inactivation of dye labeled cells does not also inactivate nearby unlabeled cells. An alternative explanation is the Jurkat cells are simply a more thermoresistant cell-line.[33] Indeed, a series of comparative photothermal experiments showed that Jurkat cells labeled with **1** are more resistant to laser-induced inactivation than labeled CHO-K1 cells.‡ As shown in Fig. S7 (ESI†), complete laser-induced inactivation of Jurkat cells required a much higher loading of **1** and a longer 10 minute laser irradiation period. With this new insight, we conducted a reverse cell labeling and laser heating experiment. That is, the more heat-resistant Jurkat cells were labeled with a high loading of **1** (50 μM) and the CHO-K1 cells were left unlabeled. The two populations of cells were mixed

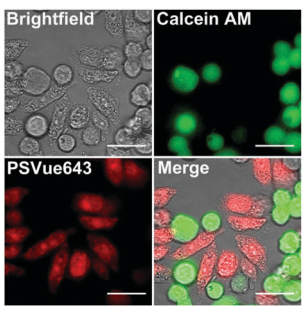


Fig. 3 Selective photothermal inactivation of CHO-K1 cells. A mixture comprised of adherent CHO-K1 cells (labeled by incubating with 5 μM of **1**) and non-adherent Jurkat cells (unlabeled) was laser irradiated (2 W cm^{-2}) for 125 seconds which raised the microwell temperature from 37 °C to 41.5 °C. After irradiation, the cells were incubated overnight in fresh media at 37 °C, then treated with a mixture of red fluorescent live-cell stain Calcein AM (3 μg mL^{-1}) and red fluorescent dead-cell stain PSVue643 (10 μM) and imaged microscopically. Scale bar = 10 μm.

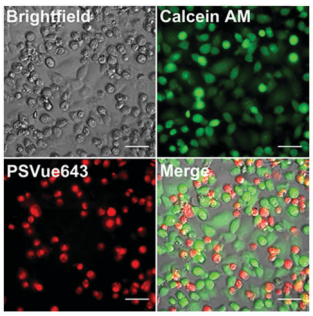


Fig. 4 Selective photothermal inactivation of Jurkat cells. A mixture comprised of adherent CHO-K1 cells (unlabeled) and non-adherent Jurkat cells (labeled by incubating with 50 μM of **1**) was laser irradiated (2 W cm^{-2}) for 10 minutes which raised the microwell temperature from 37 °C to a steady state of 45 °C. After irradiation, the cells were incubated overnight in fresh media at 37 °C, then treated with a mixture of red fluorescent live-cell stain Calcein AM (3 μg mL^{-1}) and red fluorescent dead-cell stain PSVue643 (10 μM) and imaged microscopically. Scale bar = 25 μm.

together in a microwell and irradiated with the 808 nm laser (2 W cm^{-2}) for 10 minutes, which raised the well temperature to a steady state of 45 °C (Fig. S8, ESI†). After replacing the media and incubating overnight, the cells were treated with the binary mixture of fluorescent live and dead cell stains and imaged microscopically. The micrographs revealed that about half of the labeled Jurkat cells were killed by the photothermal treatment (Fig. 4 with control experiments in Fig. S9, ESI†) whereas all of the neighboring unlabeled CHO-K1 cells remained healthy. The same outcome was observed when the experiment was repeated using the same photothermal conditions, but employed propidium iodide as a red fluorescent dead-cell stain that identifies necrotic cells (Fig. S10, ESI†). It is notable that the unlabeled CHO-K1 cells remained viable even when the temperature of the solution surrounding the cell mixture was maintained by laser heating to be around 45 °C for 10 minutes.§ This is in contrast to the high levels of cell death observed when labeled CHO-K1 cells were laser heated for only 2 minutes and the temperature of the surrounding solution never exceeded 42 °C.

In summary, we find that photothermal inactivation of cells caused by laser heating of near-infrared croconaine dye molecules is more effective when the dye molecules are located inside the cell. Furthermore, cell inactivation is spatially confined and does not harm adjacent cells, which enables selective laser-induced cell destruction with micron resolution. Future research efforts will move towards practical applications that aim to selectively destroy pathological cells with high spatial precision. This will require development of new molecular dye conjugates that target the cells and accumulate in specific intracellular locations that induce high sensitivity to photothermal-induced cell death.[34]

This work was supported by the NSF (CHE-1401783) and a Walther Cancer Foundation Advancing Basic Cancer Research Grant administered by the Harper Cancer Research Institute.

Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ The study focused on mild laser heating conditions designed to trigger cell apoptosis due to thermal stress. Thus, each cell inactivation experiment employed an overnight incubation after laser heating to ensure that the cell apoptosis process was complete. One possible reason why Jurkat cells are more resistant than CHO-K1 cells to laser-induced inactivation is they express higher levels of heat shock protein 70 which is known to inhibit apoptosis. For additional details, see Section S3 in the ESI.†

§ Not surprisingly, harsher laser heating conditions (long periods >50 °C) inactivates both labeled and unlabeled cells.

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