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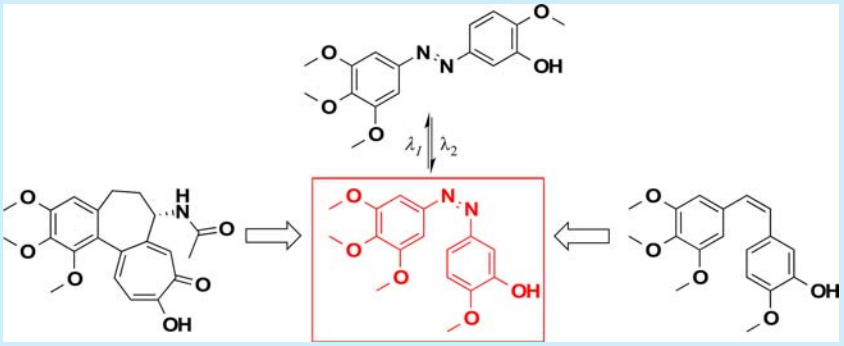
Synthesis, Characterization, and Bioactivity of the Photoisomerizable Tubulin Polymerization Inhibitor azo-Combretastatin A4

Ashton J. Engdahl, Edith A. Torres, Sarah E. Lock, Taylor B. Engdahl, Pamela S. Mertz, and Craig N. Streu*,†

Department of Chemistry & Biochemistry, St. Mary's College of Maryland, 18952 East Fisher Road, St. Mary's City, Maryland 20686, United States

Supporting Information



ABSTRACT: Combretastatin A4 is a stilbenoid tubulin binding mitotic inhibitor whose conformation greatly influences its potency, making it an excellent candidate for adaptation as a photoactivatable tool. Herein we report a novel synthesis, the facile isomerization with commercial grade equipment, and biological activity of azo-combretastatin A4 *in vitro* and in human cancer cells. Photoisomerized azo-combretestatin A4 is at least 200-fold more potent in cellular culture, making it a promising phototherapeutic and biomedical research tool.

In recent decades, improvements in drug delivery systems and drug activation have allowed greater targeting of therapies, maximizing effect and minimizing toxicity.[1] In cancer, tumor growth results from uncontrolled replication of mutated cells; therefore, many cancer therapeutics target the cell division cycle. However, interfering with cell division can lead to undesired chemotherapeutic side effects since healthy cells in the body also undergo cell division as a part of the normal cell cycle. As a result, cancer therapeutic methodologies that are able to target tumor cells in a very selective manner are highly desirable.[2−4]

Perhaps just as importantly, drug molecules, by virtue of their ability to manipulate cellular processes, are essential tools for understanding biological systems. However, the vast majority of drugs lack the ability to target cellular processes in a spatially and temporally selective way, making them less useful for answering questions about the precise timing and location of cellular processes. For this purpose, the ability to target drug molecules would be exceptionally advantageous.[1,5]

The photoisomerizable group gathering the most attention is the azo-stilbene.[1] Originally developed a century ago as dyes, azo-stilbenes are particularly notable for their facile and reversible trans/cis reversible photoisomerizability (Figure 1A). Typically, blue or near-UV light induces a trans → cis isomerization, which can be reversed quickly with longer wavelength light or slowly at room temperature in the absence of light.[1,11] Precisely because they are so robust, the photoisomerizability of azo-stilbenes has been exploited extensively throughout chemistry and biology.[12−14] Finally, although they are not utilized for their photoisomerization, there are several FDA-approved azo-stilbenes, such as the common food coloring Allura Red AC (Figure 1B).[6]

The wholesale structural and electronic changes that accompany photoisomerization between trans and cis forms would be predicted to create large differences in bioactivity between the isomers of a given azo-stilbene.[11] Fortunately, the azo-stilbene inhibitors that have been developed serve to illustrate their potential for photoactivation.

One of the most validated targets for chemotherapeutics, tubulin, is the building block of microtubules and plays a critical role in mitosis. Specifically, the vinca alkyloids and taxanes are common classes of natural products, which have been extensively mined for inhibitors of tubulin polymerization and depolymerization, respectively.[3] Another, more recent, tubulin polymerization inhibitor is combretastatin A4. Combretastatins are a class of phenolic natural products that have been examined extensively for biological activity. While combretastatin itself has limited chemotherapeutic activity, its trans-stilbenoid derivative, combretastatin A4 (CA4), was found to

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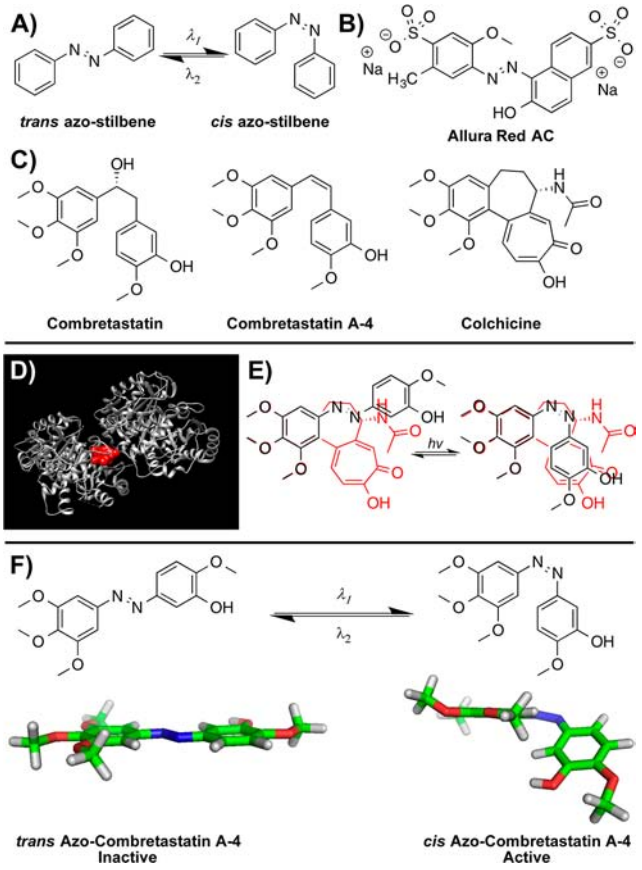

Figure 1. (A) Azo-stilbenes, which have a long history as dyes, undergo reversible light-induced trans/cis photoisomerization. (B) Allura Red AC, a common red food dye, is just one of several FDA-approved azo-stilbenoid compounds.[6] (C) Combretastatin and its more potent tubulin binding structural analogues combretastatin A4 (CA4) and colchicine. (D) Cocrystal structure of colchicine binding to β-tubulin.[7] (E) Two-dimensional overlay of azo-combretastatin in both conformations (black) over colchicine (red) helps visualize their similar β-tubulin binding modes. (F) Azo-combretastain A4 cis/trans photoisomerization and associated MM2 energy minimized conformational changes.

be far more potent (Figure 1C).[15] This lead structure has since been elaborated into a more bioavailable drug, known as ombrabulin, which is undergoing clinical trials for a variety of cancer subtypes. Specifically, combretatstatin A4 and ombrabulin have been found to be effective antiangiogenesis agents and are therefore being examined in numerous combination therapies for solid tumors.[16]

The antiangiogenic activity of combretastatin A4 has been reported to result largely from binding the same site in β-tubulin as colchicine (Figures 1D/E).[7,17] Interestingly, the trans-stilbenoid isomer of combretastatin A4 has been found to be far less active than the cis isomer.[15] Additionally, slow isomerization of the central cis double bond to its trans form limits the efficacy of the drug, demonstrating that the biological activity of combretastatin A4 indeed relies upon the stereochemical configuration of its central double bond. To this end, my group has synthesized azo-combretastatin A4 (Figure 1F), examined its photodynamic properties, measured its light-activated inhibition of tubulin polymerization, and demonstrated its light-induced bioactivity in whole cell experiments.

Until recently, azo-combretastatin A4 (azo-CA4) had never before been reported.[18] Most likely, this is because the single-step synthesis between its component aniline and phenol gives the undesired regioisomer, requiring a longer synthesis that is less accessible with high throughput methods. We therefore synthesized the molecule in high yield in three steps from allyl-protected catechol (Figure 2). Unlike silyl protecting groups,

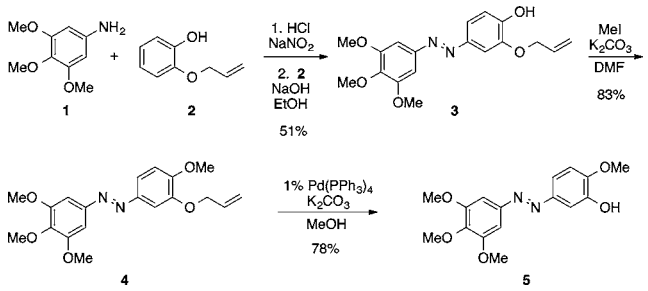

Figure 2. Synthesis of azo-combretastatin A4.

which may migrate, this protecting group was found to be particularly amenable to azo-bond forming reactions, which require both harshly acidic and harshly basic conditions.

Interestingly, azo-combretastatin A4 demonstrates quite favorable photodynamic properties as demonstrated by ^{1}H NMR and UV/vis isomerization assays. At 10 mM, the compound shows ~90% trans → cis isomerization in minutes by NMR using simply an $8 consumer-grade 400 nm LED flashlight (Figure 3A). Chemical shifts for the cis-azo-combretastatin A4 are substantially upfield of those for trans-azo-combretastatin A4, indicating that the cis-ring stacked conformation is the predominant form of the irradiated compound in solution, since ring currents in the stacked form result in an upfield shift of protons above or below the plane of aromatic rings. (It is worth noting at this point that cis-azostilbenes like azo-CA4 have helical chirality, which has implications for their target binding, off-target effects, and potency since only one stereoisomer would be predicted to bind tubulin.)

At lower, more biologically relevant concentrations (30 μM), photoisomerization was tracked by changes in the UV/vis spectrum, which are pronounced for the trans/cis photoisomerization of azo-combretastatin A4. Under these conditions, photoisomerization reached a maximum level after less than 10 s of irradiation with an identical light source (Figure 3). Longer wavelengths of light were able to induce cis → trans reversion. Strikingly, a simple hand-held white light flashlight is able to induce approximately ~25% reversion in seconds and >50% reversion in just minutes (Figure 3).

Having established appropriate conditions to induce trans → cis photoisomerization, the light-activated inhibition of tubulin polymerization was confirmed *in vitro* by turbidity assay (Figure 4). Strikingly, azo-combretastatin A4 shows essentially no inhibition of tubulin polymerization in the absence of isomerizing light. However, this changes markedly in the presence of an appropriate light source. Under such conditions, azo-combretastatin A4 shows similar polymerization inhibition to that of CA4, albeit at slightly higher concentrations. Although qualitatively useful, such assays require large concentrations of tubulin to achieve acceptable signal-to-noise ratios and are therefore stoichiometrically unable to determine the lower IC_{50} or EC_{50} values. Therefore, further investigation of the cytotoxicity of the azo-combretastain A4 compound was conducted using human cervical cancer (HeLa) cells.

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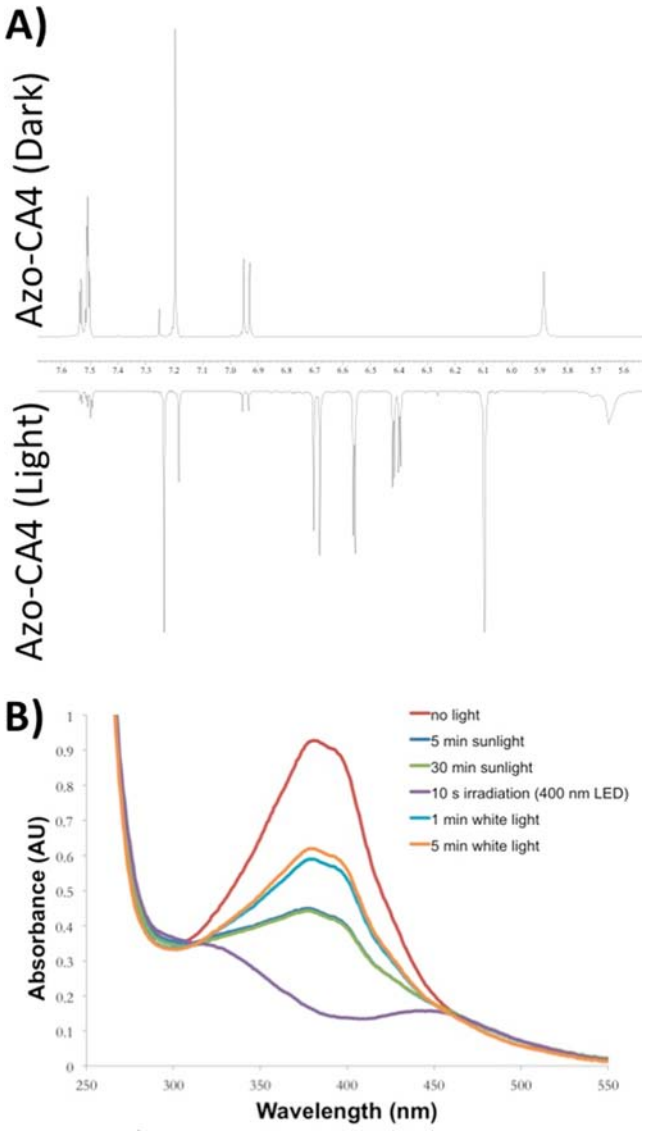

Figure 3. (A) ^{1}H NMR analysis of azo-CA4 in the dark (top) and after 10 min isomerization with 400 nm LED light source (inverted). (B) UV/vis analysis of 30 μM azo-combretastatin A4 *trans* → *cis* photoisomerization with 400 nm LED.

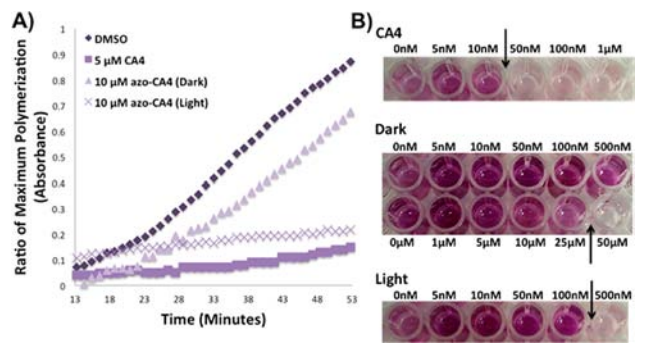

Figure 4. (A) *In vitro* inhibition of tubulin polymerization by azo-CA4 (10 μM) is significantly increased in the presence of isomerizing light. (B) MTT assays of CA4 (top), azo-CA4 in the dark (middle), and azo-CA4 in the presence of 400 nm isomerizing light (bottom) show increased cytotoxicity of azo-CA4 in the presence of light.

In these experiments, HeLa cells were grown in culture for 5 days with exposure to the azo-combretastatin A4 in both the presence of light and the absence of light. These five-day experiments in a CO_2 incubator required the development of an automated light switch system coded on an Arduino board (see Supporting Information). The entire setup (Arduino board, led flashlight, power source) was produced for less than $40 with consumer grade materials.

An MTT assay was then used to measure the viability of the remaining cells. In the absence of light, azo-combretastatin A4 shows little to no toxicity to HeLa in concentrations as high as 100 μM. However, in the presence of isomerizing light, azo-combretastatin A4 resulted in complete cell death at concentrations as low as 500 nM, a greater than 200-fold increase in bioactivity. Similar results were obtained using white light microscopy with 500 μM azo-CA4 after just 12 h (Figure 5). It is worth noting that controls to measure the toxicity of

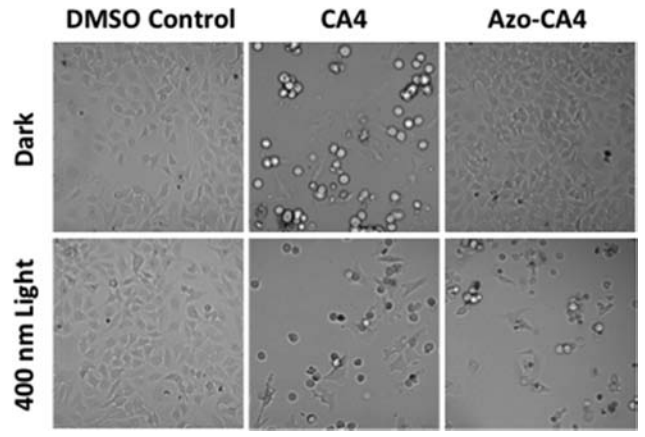

Figure 5. White light microscopy of HeLa cells incubated 12 h with DMSO, CA4 (50 nM), and azo-CA4 (500 nM) in the dark (top), and in the presence of 400 nm isomerizing LED light (bottom) clearly demonstrates increased cytotoxicity of azo-CA4 in the presence of light.

the light source (10 s of irradiation every 30 min with a 400 nm LED flashlight) showed no adverse effects on HeLa cell viability. Additionally, CA4 potency was not significantly impacted by the irradiation conditions.

During the preparation of this manuscript, Borowiak et al. published the synthesis of azo-CA4 and extensively characterized its biological activity.[18] Our simultaneous work confirms and complements their findings both qualitatively and quantitatively regarding the vast potential of this compound to work intracellularly.[19] However, since other close combretastatin A4 analogues have shown nontubulin specific activity,[20] we have characterized azo-combretastatin A4's ability to act directly as a light-activated tubulin polymerization inhibitor by a purified tubulin polymerization assay. Additionally, we described an efficient alternate synthesis of azo-combretastatin A4, which is not susceptible to silyl migration and makes use of an exceptionally mild and catalytic deprotection. Finally, and perhaps most importantly, we have demonstrated that photoisomerization from an inactive to an active inhibitor can be achieved with a simple consumer grade $8 LED flashlight and brief light pulse sequences that may even be produced manually. This finding has substantial implications for translating this work to therapeutic applications in the human body and research applications on tissues and cells since

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application of this robust technology does not require expensive equipment.

In conclusion, azo-CA4 is an analogue of CA4 that can be reversibly light activated. This photoisomerization can be initiated by relatively weak, inexpensive, light sources. Isomerization and reversion can be induced with medically useful wavelengths and short irradiation times that will limit light toxicity. As a result of this photoisomerization, azo-CA4 also shows a greater than 200-fold increase in cytotoxicity in human cancer cells following photoisomerization. These properties make azo-CA4 a robust, versatile, spatially and temporally targetable chemical tool for biomedical angiogenesis research or possibly even chemotherapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02262.

Experimental details, spectral data, Arduino programs (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: cstreu@albion.edu.

Present Address

†Albion College, Department of Chemistry, 611 E. Porter St., Albion, MI 49224.

Notes

The authors declare no competing financial interest.

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