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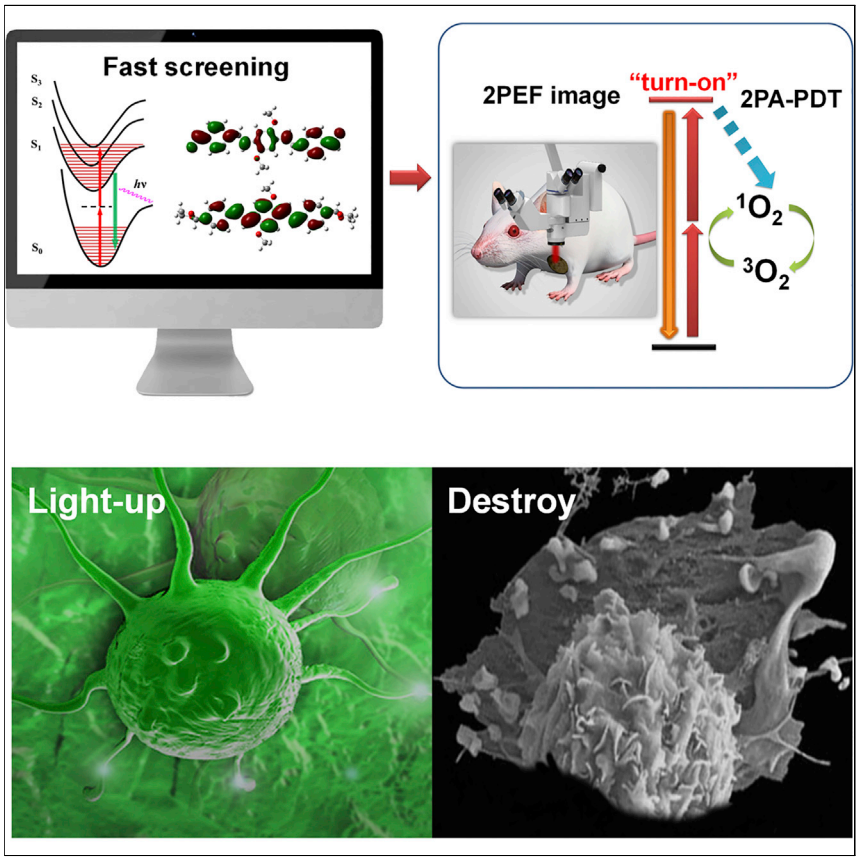

We report a cost-effective theory-assisted molecular screening method for the rational design of turn-on-type two-photon absorption (2PA) dyes. The obtained dyes exhibit high efficiency in two-photon excited fluorescence and singlet oxygen generation. The turn-on process can occur in mild and nontoxic conditions, even in the intracellular environment. With such a turn-on feature, two-photon laser confocal scanning microscopic imaging and two-photon excited photodynamic therapy can be performed in a well-controlled manner for MCF-7 cells and melanoma tumor.

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HIGHLIGHTS

A simulation-assisted strategy is used to design turn-on-type 2PA dyes

The 2PA dyes are suitable for imaging and photodynamic therapy of tumor

Phototoxicity to normal tissue can be reduced by turn-on in the tumor area only

This design strategy is useful for fast screening of new theranostic reagents

Sun et al., Chem 5, 1–17
March 14, 2019
https://doi.org/10.1016/j.chempr.2018.12.001

Please cite this article in press as: Sun et al., Rational Design of Organic Probes for Turn-On Two-Photon Excited Fluorescence Imaging and Photodynamic Therapy, Chem (2018), https://doi.org/10.1016/j.chempr.2018.12.001

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Rational Design of Organic Probes for Turn-On Two-Photon Excited Fluorescence Imaging and Photodynamic Therapy

Chun-Lin Sun,[1] Jun Li,[1,6] Xiao-Zhen Wang,[1] Rong Shen,[4] Sha Liu,[4] Jian-Qiao Jiang,[1] Ting Li,[5] Qi-Wei Song,[1] Qing Liao,[3] Hong-Bing Fu,[3,*] Jian-Nian Yao,[2,*] and Hao-Li Zhang[1,7,*]

SUMMARY

The rational design of two-photon absorption (2PA) organic dyes with 2PA cross-section (δ) values tunable by mild stimuli under physiological conditions remains a challenge. Using a simulation-assisted structure screening method, we have designed an acetal terminated distyrylbenzene derivative (Ace-DSB), which can be readily converted into aldehyde terminated molecules (Ald-DSB) to exhibit a significant increase in δ values at 760 nm. The conversion reaction, which dramatically turns on the 2PA absorption capability of the molecules, can occur in an intracellular environment. Furthermore, Ald-DSB exhibits 40 times higher efficiency of two-photon generation of singlet oxygen than Ace-DSB. These turn-on features allow enhanced two-photon laser confocal scanning microscopic imaging and two-photon excited photodynamic therapy (2PE-PDT) for MCF-7 cancer cells and melanoma tumors. This work opens a new avenue toward the design of turn-on-type two-photon absorption molecules for both *in vitro* and *in vivo* tumor imaging and 2PA-PDT.

INTRODUCTION

Organic molecules exhibiting two-photon absorption (2PA)[1,2] activity are of interest for many practical applications, including nonlinear optics,[3,4] two-photon microscopy,[5,6] and photodynamic therapy.[7,8] The two-photon excitation using near-infrared (NIR) light gives a better definition of the focal spot and resolution, lower phototoxicity, and deeper transmission in biological tissues compared with common one-photon excitation using UV and visible light.[9] To facilitate these applications, the design and discovery of 2PA molecules with large 2PA cross-section (δ) are essential. However, two-photon absorption and excitation are intrinsically much less efficient than the one-photon processes, and the occurrence probability of 2PA, reflected by the parameter δ, is generally small for most organic molecules. Therefore, developing an efficient method to design highly efficient 2PA dyes with ideal functions remains a challenge.

So-called "turn-on" fluorescence dyes are strongly desired in a variety of applications, such as bioimaging, biosensors, and photodynamic therapy.[9–11] An ideal turn-on dye is dark at the initial state but can be switched to brighter state after being triggered by certain stimuli. To date, most of the turn-on dyes belong to conventional one-photon absorption materials. Given the many advantages of 2PA, turn-on-type 2PA dyes are highly desirable for many biological applications. The turn-on feature of the two-photon excited fluorescence dyes can reduce the autofluorescence background and enhance imaging resolution.[12,13] Moreover,

The Bigger Picture

Photodynamic therapy (PDT) is a clinically accepted treatment for tumors, which relies on the photogeneration of singlet oxygen with specific photosensitizers. Organic dyes with two-photon absorption (2PA) features allow PDT to be carried out in deeper tissue than conventional one-photon absorption dyes. We report herein the rational design of turn-on-type two-photon absorption (2PA) dyes for fluorescent imaging and singlet oxygen generation by a simulation-assisted structure screening method. With such 2PA dyes, microscopic imaging and photodynamic therapy can be performed in a well-controlled manner according to the requirements. The partial turn-on state allows long-time imaging with reduced light damage to normal tissue, whereas the full turn-on state allows efficient photodynamic therapy to the selected tumor tissue. This simulation-assisted design strategy paves a new way for the rational design of 2PA photosensitizers for future clinical tumor therapy.

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turn-on-type 2PA photodynamic therapy provides good control to the treatment process and reduces phototoxicity.[13,14] However, the development of 2PA dyes with tunable δ have met with limited success.[11,15] The main difficulty in the design of switchable 2PA emission dyes comes from the fact that the photophysical processes associated with two-photon excitation and subsequent light emission are much more complex than the conventional one-photon excitation process. Structural features that could dominate the δ values are not well understood, so finding an efficient mechanism to switch the 2PA efficiency on and off appears to be very difficult.

There are two key problems that need be solved for the design of 2PA dyes with tunable δ value. First, we need to identify a relatively small structure change that could significantly increase the δ in a given 2PA framework. Second, an easily operated chemical reaction needs to be identified, which can trigger the aforementioned structural change so that it gives rise to a significant change in δ value. Given that the structure-property correlation of 2PA dyes is not well understood, the first problem, i.e., identifying an appropriate structure change, is the more challenging to solve.

Obviously, experimental screening of all the structural modification possibilities among a large number of known 2PA frameworks is costly and impractical. Therefore, a simulation-assisted structure screening method must be used to assist the design of 2PA dyes. Unfortunately, 2PA process is a third-order nonlinear phenomenon and δ is related to the imaginary part of the third-order polarizability so that traditional theoretical calculations of δ are too time consuming for a practical screening of organic structures.

In this work we demonstrate, for the first time, the design of a turn-on 2PA probe by using a simple theoretical simulation method. We have chosen the distyrylbenzene (DSB) structure as the model 2PA framework. The theory-assisted screening of different DSB derivatives is based on a facile theoretical method analysis of the excited states.[16] Aided by the simulation, we found that through a simple conversion from ethylene glycol acetal terminal to aldehyde terminal DSB structure, a 26-fold enhancement of the δ values is achieved. The rationally designed DSB molecules were successfully applied in both turn-on *in vitro* and *in vivo* two-photon excited fluorescence (2PEF) imaging and turn-on two-photon excited photodynamic therapy (2PE-PDT).

RESULTS AND DISCUSSION

Simulation-Assisted Molecular Design

The DSB structure is selected as the model framework because many DSB derivatives are highly fluorescent and are widely used in various optoelectronic applications.[17–23] In our previous work, we have discussed how small modification of DSB frameworks could significantly affect their spectral properties.[24–27] Our basic idea here is to design carbonyl group modified DSB molecule as a turn-on-type 2PA imaging reagent, which is based on two considerations. First, an sp^2 hybrid carbon in carbonyl group connected directly to the DSB framework could extend the conjugation system. Previous studies have suggested that 2PA dyes extended π-conjugation with electron push-pull motifs is beneficial for high δ values.[10,28] Second, it is very important that most carbonyl groups are relatively reactive, which helps to find an appropriate trigger reaction for δ value tuning. Several DSB derivatives have been designed for initial screening, which is based on terminated substituted 1,4-dimethoxy-2,5-di((E)-styryl)benzene. The terminal substituent groups include

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https://doi.org/10.1016/j.chempr.2018.12.001

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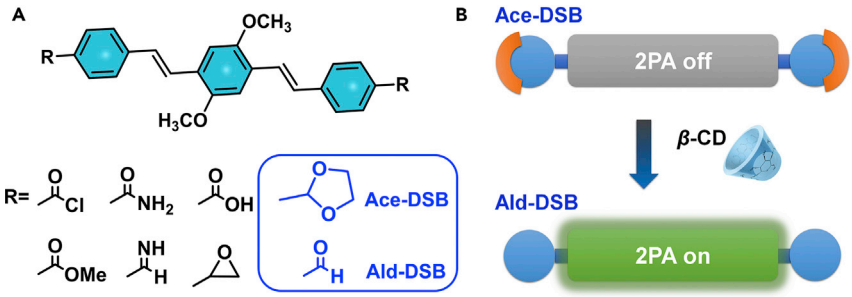

Figure 1. Structures of the DSB Compounds Studied in This Work

(A) The DSB derivatives designed for initial screening.

(B) "Turn-on" 2PA cross-section from Ace-DSB to Ald-DSB by mild and nontoxic reaction condition.

acyl chloride, amide, carboxylic acid, ester, methanimine, oxiane, acetal, and aldehyde with the molecular structures shown in Figure 1A. The possible transformation among DSB carbonyl derivatives is shown in Figure S8. The bottom-right panel of Figure 1A illustrates the two representative DSB molecules, including the aldehyde-containing molecule (Ald-DSB) and its ethylene glycol acetal (Ace-DSB). Ace-DSB hydrolysis yields Ald-DSB stoichiometrically under various mild conditions, i.e., β-CD in water solution and room temperature (Figure 1B).[29]

The next step is to assess the potential 2PA performance of the molecules and select a suitable structure for practical experimental measurement. Several theoretical methods have been developed for the calculation of δ values, the most well known of which is called the summary over states (SOS).[30] However, a full SOS calculation for DSBs, which contains 210 or more electrons, would be time consuming. In fact, a full *ab initio* calculation is unnecessary for initial structural screening. Instead we have used a simpler method for screening purposes.

In our recent work, we reported a facile theoretical method for the fast screening of small molecules as potential 2PA dyes. This method is based on analysis of the natural transition orbitals (NTOs) directly associated with the final state of 2PA transition.[16,31] As a highly delocalized final excited state is likely to give a large δ than a localized one, visualization of the NTOs not only can help to understand the excited state associated with the 2PA process but can also help to quickly identify candidate molecules with high δ. For centrosymmetric chromophores such as DSB derivatives studied in this work, the 2PA transition is from the ground state (S_0, 1Ag) to the lowest excited state with Ag symmetry (S_2 or/and S_3).[10,28] Therefore, we paid particular attention to the S_2 and S_3 states (for more details, see Figure S4).

We calculated the NTOs of the excited states (S_2 and S_3) of the DSB molecules shown in Figure 2A. To investigate the excited states, we conducted time-dependent density functional theory (TD-DFT) calculations using the Gaussian 09 package.[32] NTOs are used to study the related ground and excited states. More details of NTO calculation results and screening processes are provided in Figure S9. We have considered possible reactions including esterification, amidation, hydrolysis, chlorination, epoxidation, and deacetylation (conversion from acetal terminal to the aldehyde). NTO analysis suggests that most reactions on the carbonyl groups do not bring about obvious change to the S_2 states, and therefore are unlikely to enhance δ very much. However, the conversion from the acetal terminated structure (Ace-DSB) to the aldehyde terminated structure (Ald-DSB) is associated with a large change of the NTO orbitals involved.

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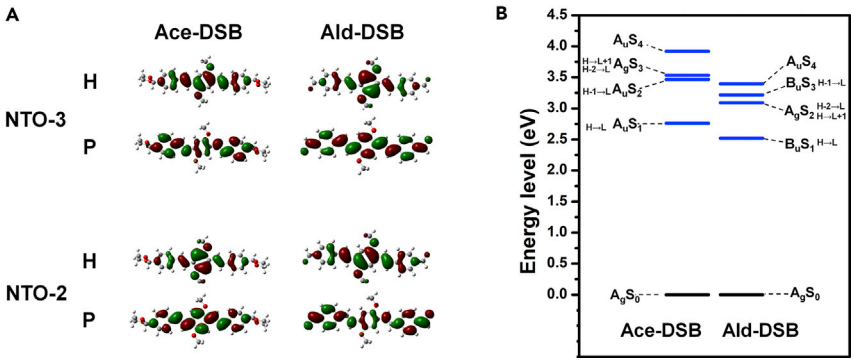

Figure 2. NTOs and Energy Levels for Ace-DSB and Ald-DSB

(A) NTOs of S_2 and S_3 for Ald-DSB and Ace-DSB related to the 2PA process (detailed NTOs and data shown in Figure S4), the H represents the hole and P represents the particles.

(B) Energy-level diagram and symmetry for the singlet state of Ald-DSB and Ace-DSB, where H represents the highest occupied molecular orbital and L represents the lowest unoccupied molecular orbital.

Figure 2B illustrates the calculated orbital energy level and symmetry for the singlet states (to S_4) of the Ald-DSB and Ace-DSB. The symmetry of ground state is A_g for both molecules. The S_2 state of the Ace-DSB has A_u symmetry, suggesting that the 2PA transition from S_0 to S_2 is forbidden. For the Ald-DSB symmetry of S_2 state is A_g, which allows 2PA transition to occur. It is noted that this symmetry analysis to the different excited states does not consider possible vibronic coupling. Meanwhile, it is noted that the S_3 state of Ace-DSB has A_g symmetry and lies very close to its S_2 state. Therefore, it is likely that the 2PA process of Ace-DSB may involve transition from S_0 to S_3. Nevertheless, the information from the orbital symmetry analysis further verifies that the Ald-DSB is likely to have much a larger δ than Ace-DSB.

As illustrated above, the TD-DFT based NTOs and symmetry analysis to the excited states help us to quickly identify the Ace-DSB molecules as the promising candidate for turn-on 2PA probe. It is noted that although NTO analysis provides only qualitative information, it helps us to quickly screen out most of the candidates and focus on only Ace-DSB and Ald-DSB molecules. More quantitative information can be obtained from a significantly more expensive calculation method. Here we have verified the above calculation results regarding 2PA properties of the two molecules using the Dalton program, which is based on coupled cluster response theory.[33,34] Details of such calculations are shown in Figure S5. In the 690- to 850-nm range, the calculation predicts that the maximum δ values for the Ace-DSB and Ald-DSB are 10 and 3,500 GM, respectively, which confirms that the prediction based on our NTO analysis is correct. The result of simulation of 2PA cross-section based on a simplified sum-over-states model is consistent with Dalton calculations (details shown in Figure S6). It is worth mentioning that the 2PA spectra calculation using the Dalton program cost about 1,800 CPU hr, whereas the NTO calculation cost only about 20 CPU hr. Given the simplicity of the NTO analysis, it is much easier to be used in practice for fast molecule screening.

Spectroscopic Characterization

We then verified the above theoretical prediction by experimental measurements. The synthesis and structural characterization data of the Ald-DSB and Ace-DSB are provided in the Supplemental Information. The experimental one-photon absorption and emission spectra of the two molecules in diluted tetrahydrofuran (THF)

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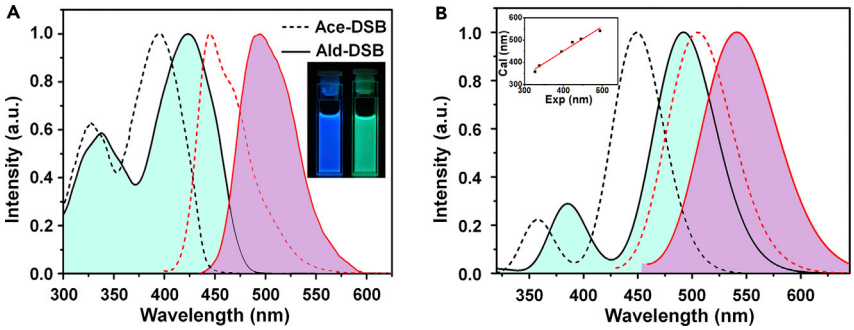

Figure 3. Steady-State One-Photon Absorption and Emission Spectra

(A) Steady-state one-photon absorption and fluorescence of Ald-DSB (solid line) and Ace-DSB (dashed line) in THF.

(B) Simulated absorption and fluorescence spectra. Inset shows the linear fitting to the calculated and experimental energies including the two absorption peaks and the emission peak energy.

solution are shown in Figure 3A, with the key parameters summarized in Table 1. The absorption spectra of the Ace-DSB show two prominent absorptions in the range of 300–500 nm. The lowest electronic transition (S_0-S_1) gives the maximum absorption band around 395 nm, and the (S_0-S_2) transition gives the slightly weaker band at around 327 nm. The emission wavelength is in the range of 400–600 nm with a peak at 445 nm. The absorption and fluorescent emission bands of Ald-DSB show bathochromic shifts compared with Ace-DSB. The first and second absorption peaks are around 423 and 338 nm, respectively. The emission peak of Ald-DSB also shows a bathochromic shift of about 40 nm compared with the Ace-DSB. The inset of Figure 3A shows that the emission of Ace-DSB is blue and Ald-DSB is green. The fluorescence quantum yields (Φ) of Ace-DSB and Ald-DSB were measured as 0.72 and 0.57, respectively.

We simulated the one-photon absorption spectra and fluorescence emission spectra by TD-DFT methods (Figure 3B). The linear fitting to the calculated and experimental transition energy shows good linearity with a small systematic error of 0.3 eV, indicating that the simulations were reliable (Figure 3B, inset).[26] The experiment and simulation results of the one-photon properties, i.e., bathochromic shifts for Ald-DSB compared with Ace-DSB, offer preliminary evidence that the electron orbital delocalization of Ald-DSB is indeed higher than that of Ace-DSB.

The 2PA spectra of the Ald-DSB and Ace-DSB were measured by the 2PEF method. Figure 4 shows the 2PA spectra between 730 and 840 nm. Both molecules show the maximum 2PA absorption around 760 nm, corresponding well with the two-photon absorption for the S_0-S_2 and S_0-S_3 transition. Meanwhile, the Ace-DSB and Ald-DSB showed no obvious degradation upon the femtosecond laser irradiation for 2 hr (120 fs, 1,000 Hz, 80 mW, Figure S10), indicating high photostability.

As shown in Figure 4, Ald-DSB exhibits very high δ in the measurement range, the largest δ being about 2,421 GM. In contrast, the 2PA for Ace-DSB is much smaller, with maximum δ of only about 93 GM, similar to that of unmodified distyrylbenzene. These 2PA measurement results are in good agreement with our theoretical prediction, confirming that our NTO analysis method is also applicable to the styryl-benzene-like molecules. Moreover, these results indicate that if we are able to convert Ace-DSB to Ald-DSB, the δ will enhance 26-fold, which is ideal for turn-on applications. Although the Φ for Ald-DSB is slightly lower than that

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Table 1. Spectroscopic Parameters of Ald-DSB and Ace-DSB

	λ^{Abs}_{exp}	λ^{em}_{exp}	Φ	τ	δ	Φ_Δ	$\delta\Phi_\Delta$	k_r	k_{nr}
Ace-DSB	395	445	0.72	1.58	93	0.18	17 (14)	0.47	0.17
Ald-DSB	423	484	0.57	1.77	2421	0.28	678 (600)	0.32	0.24

λ^{Abs} (nm) is the vertical transition wavelength of the lowest excited states, λ^{em} (nm) is the energy of the peak of the fluorescence band, Φ is the fluorescence quantum yields, τ (ns) is the lifetime of fluorescence, δ (GM) is the 2PA cross-section, Φ_Δ is the singlet oxygen quantum yield, and $\delta\Phi_\Delta$ presents the 2PA singlet oxygen generation capability. The data in parentheses show the test value with tetraphenylporphyrin as a reference. The last two columns provide the rate constant for radiative deactivation S_1 to S_0, k_r (ns^{-1}) and the rate constant for nonradiative deactivation, k_{nr} (ns^{-1}).

of Ace-DSB, 2PA measurement shows that the Ald-DSB is significantly brighter than Ace-DSB under the two-photon excitation fluorescence condition (inset of Figure 4A). Figure 4B shows that the 2PEF emission from the Ace-DSB solution was enhanced obviously upon the addition of β-CD, indicating the transformation from Ace-DSB to Ald-DSB.

In Vitro Application

To examine Ald-DSB's capability as 2PEF probe, we imaged Ald-DSB with MCF-7 cells by using two-photon laser confocal scanning microscopy (TPLCSM). The fluorescence emission excited by the 760-nm laser is strong enough for the high-resolution microscopic imaging, as shown in Figure 5A.

z-stack confocal images (depth interval 0.5 μm) were taken continuously from the bottom to the top via TPLCSM. The image stacks were processed by ImageJ software to obtain the 3D reconstruction movie of MCF-7 cell (Video S1). Intense intracellular luminescence was observed with femtosecond laser pulses at 760 nm, under which no background fluorescence from the MCF-7 cells would interfere. Benefiting from the high δ of the Ald-DSB molecule, high signal strength can be obtained. The large δ and high photostability of Ald-DSB make it a good candidate for *in vivo* TPLCSM imaging.

The Ace-DSB was also incubated with the MCF-7 cell, and the TPLCSM image is shown in Figure 5B. Compared with the cells stained by Ald-DSB, those stained by Ace-DSB are much darker. Ace-DSB can be transformed to Ald-DSB by treating with β-CD in solution.[29] This reaction was established in cell-culture conditions and monitored by thin-layer chromatography and mass spectra (shown in Figure S3). The β-CD is a Food and Drug Administration-approved biological safe material. The mild and nontoxic reaction condition is suitable for intracellular translation. We treated the Ace-DSB-stained cells with the β-CD solution, and the TPLCSM shows significantly enhanced intensity (Figures 5B and 5C). These results confirm that the turn-on 2PA *in vitro* has been achieved.

We measured the singlet oxygen quantum yields (Φ_Δ) of the DSBs by the singlet oxygen luminescent method, using rose bengal as a reference. The singlet oxygen $^1\Delta_g$ emission spectra are shown in Figure 6 and the determined Φ_Δ is displayed in Table 1. The Φ_Δ of Ald-DSB is about 0.28, which is twice that of the Ace-DSB, i.e., 0.14. However, the efficiency of singlet oxygen generated by the two-photon excitation is defined not only by Φ_Δ but also by δ. In fact, the factor $\delta\Phi_\Delta$ is a useful figure with which to estimate the two-photon excited photodynamic therapy (2PE-PDT) efficiency. The $\delta\Phi_\Delta$ values are 678 for Ald-DSB and 17 for Ace-DSB, indicating that Ald-DSB is 40 times more efficient than Ace-DSB in singlet oxygen production. In Figure 6, two-photon excited luminescence of singlet oxygen sensitized with Ace-DSB or Ald-DSB was used to directly evaluate their $\delta\Phi_\Delta$, which gave $\delta\Phi_\Delta$ values of

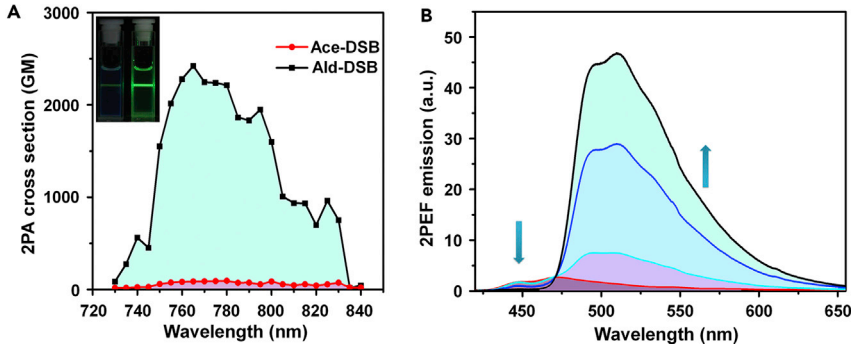

Figure 4. 2PA Spectra of Ald-DSB and Ace-DSB in Solution

(A) 2PA cross-section of Ald-DSB and Ace-DSB in THF solutions. The inset shows the corresponding 2PA fluorescence photographs of the Ace-DSB (left) solution and the Ald-DSB (right) solution under 760-nm illumination.

(B) 2PEF emission changes upon the addition of β-CD.

14 and 600 for Ace-DSB and Ald-DSB, respectively. Figure S10 shows the turn-on singlet oxygen process through transformation from Ace-DSB to Ald-DSB.

Next, the 2PE-PDT of the two compounds were studied. The MCF-7 cancer cells were incubated with different concentrations of Ace-DSB and Ald-DSB for 24 hr and then divided into two equal series. One series was illuminated with 760 nm for 20 min. Another series was kept in the dark as the control group. After illumination, all cells were incubated in darkness for 24 hr before the viability test. The cell viability of control-group data exhibits low cytotoxicity for both Ald-DSB and Ace-DSB in darkness. The cell viabilities were higher than 90% even when the concentration was as high as 32 μg/mL (about 80 μM). After the irradiation (control experiments confirmed that the laser does not damage the cells), the viabilities were very different between the cell samples containing Ald-DSB and Ace-DSB.

There is no obvious viability change in the cell containing Ace-DSB, indicating weak 2PE-PDT effect. In contrast, Ald-DSB resulted in a viability lower than 20%, suggesting a good 2PE-PDT effect (Figure 7). The lower 2PE-PDT efficiency of Ace-DSB can be attributed to its lower singlet oxygen production efficiency.

Finally, we tested the capability of Ace-DSB as 2PE-PDT turn-on reagent. The cellular phototoxicity of Ace-DSB in the presence of β-CD was carried out on MCF-7 cells under NIR illumination. Figure 8 shows the 2PE-PDT results during the turn-on process. In this experiment, the MCF-7 cells incubated with Ace-DSB (8 μg/mL, 20 μM) were divided into two batches. One batch was incubated in the standard cell-culture medium and the other was incubated in the cell-culture medium containing β-CD (16 μg/mL, 28 μM) for 24 hr. Half of each batch was then illuminated with 760-nm light (10 mW cm^{-2}, 120 fs, 1,000 Hz) for 20 min (others remained in darkness). After the illumination, all cells were incubated in the dark for another 24 hr. The cell viabilities were monitored by using microscopy, as shown in Figure 8.

Figures 8A and 8B show that there is no obvious cell death to the Ace-DSB stained cells after the illumination, indicating that Ace-DSB has low 2PE-PDT efficiency. In contrast, in the cell batch containing β-CD distinct cell death was observed within the irradiation area, as shown in Figure 8D. As discussed above, the Ald-DSB has

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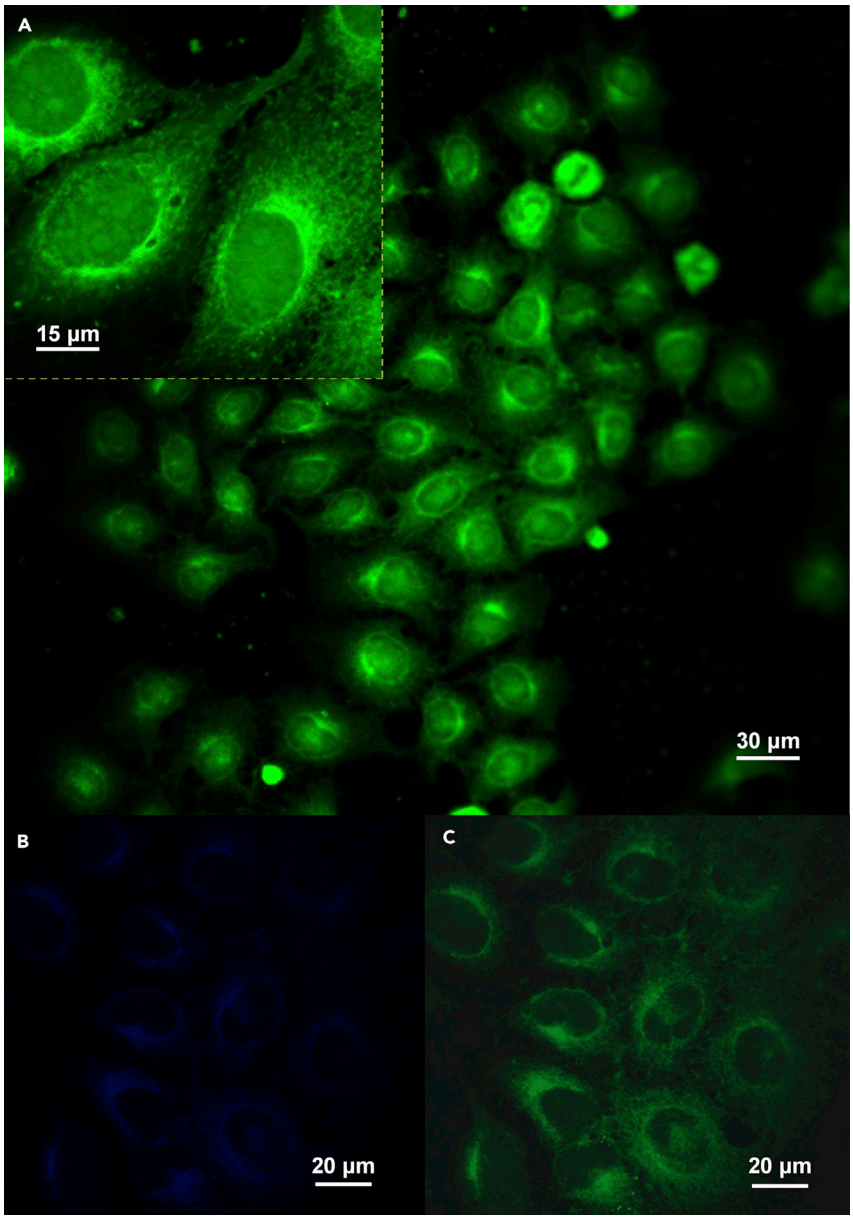

Figure 5. *In Vitro* Bioimage Application

(A) TPLCSM image of MCF-7 incubated with Ald-DSB upon excitation at 760 nm; the inset shows the high-resolution image.

(B and C) Before (B) and after (C) treatment with β-CD in same exposure time.

much higher $\delta\Phi\Delta$ than its precursor Ace-DSB. Therefore, the Ald-DSB generated a significant amount of singlet oxygen upon two-photon excitation, which resulted in the death of almost all cancer cells.

The above results demonstrate that the low 2PE-PDT active precursor Ace-DSB can be converted into Ald-DSB under cell-culture conditions when treated by a low concentration of β-CD. Due to the high 2PA fluorescence intensity of the Ald-DSB, the treated area can be readily imaged by TPLCSM. Furthermore, the turn-on regions can be selectively illuminated by NIR light to produce singlet oxygen, which is

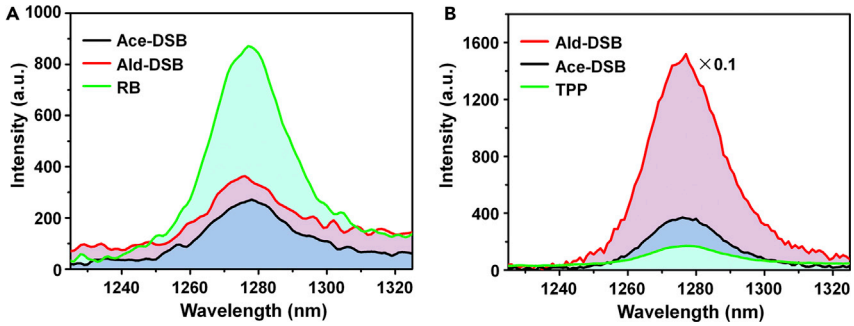

Figure 6. Singlet Oxygen Quantum Yields

(A) Photoexcited luminescence of singlet oxygen sensitized with Ace-DSB, Ald-DSB, and rose bengal (RB) as a reference.

(B) Photoexcited luminescence of singlet oxygen sensitized with Ace-DSB, Ald-DSB, and tetraphenylporphyrin (TPP) as a reference.

used in 2PE-PDT. Such a strategy may be applied in the treatment of various cancers or disease, such as skin cancer and fundus lesions, with higher selectivity and fewer side effects.

In Vivo Application

The mice tumor model (the building process of the model is described in the Experimental Procedures) was used for evaluating the turn-on TPLCSM *in vivo* image (Figure 9A). The mice were injected with Ace-DSB at the tumor location. The TPLCSM in Figure 9B shows only very low signal and most of the emission can be attributed to the background luminescence. After β-CD injection as the turn-on stimulant, TPLCSM (Figure 9C) shows the details of *in vivo* tumor cells. Compared with Figure 9B, the tumor cell signal is very easily distinguished from the background luminescence.

The mice tumor model was then used for evaluating the turn-on 2PA-PDT therapeutic effect, as shown in Figure 10A, and details are given in the Experimental Procedures. Melanoma is the most dangerous form of skin and eye cancer and is the most suitable cancer for PDT treatment.[14] When light treatment of 80 mW/cm^2 was combined with Ace-DSB (turn-on by β-CD) for 15 days, the tumors in the treated group were significantly smaller than in the control group and the Ace-DSB group (Figure 10B). From histopathological analysis using H&E staining (Figures 10C, 10D, and S14), we observed that the tumor tissue from the control group displayed compact tumor cells with an intact structure. No significant difference between Ace-DSB-injected and control groups was detected, which suggests that the tumor tissue is not affected by Ace-DSB/β-CD injection (Figure S14). In the Ace-DSB/β-CD and irradiation groups (Figure 10D), the tumor tissue was no longer structurally integrated, and there were many sections and numerous nuclear fragments. With the help of TPLCSM imaging of the tumor tissue section, the details can be observed. Before 2PA-PDT the morphology of tumor cells was normal, whereas cavity and cell disintegration were observed on the tumor image after the 2PA-PDT treatment (Figures 10E and 10F).

The trends of tumor size and percentage survival are reported in Figures 10G and 10H. The final relative tumor volume of the control group was about 6.5 (650 mm^3). The Ace-DSB group showed very similar tumor-growing trend with the control group, suggesting no inhibitory effect on the tumor. In the turn-on group, the relative tumor volume was reduced obviously compared with the control group,

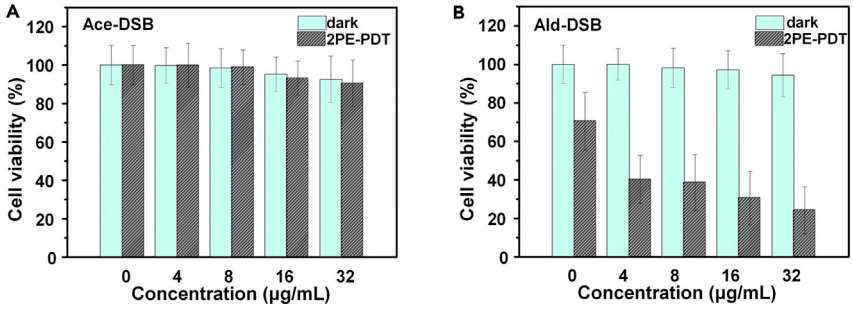

Figure 7. Cytotoxicity of Ace-DSB and Ald-DSB

Viability of MCF-7 cells incubated with (A) Ace-DSB and (B) Ald-DSB for 24 hr and then illuminated by a 760-nm laser (10 mW cm^{-2}, 1,000 Hz, 120 fs) for 20 min (2PE-PDT series) or no light (dark series). The error bars denote the SD of three replicates.

indicating significant inhabitation to the tumor tissue due to 2PE-PDT. The mice survival percentages after the 2PE-PDT are shown in Figure 10H. The survival percentage of the control and Ace-DSB-only group fell rapidly after 40 days, and all the mice died after 60 days. In contrast, the survival percentage of the Ace-DSB/β-CD group remained 40% after 70 days Figure 10H indicates that the 2PA-PDT using Ace-DSB/β-CD significantly prolonged the survival of the tumor-bearing mice and inhibited tumor growth. The above results demonstrate that we have successfully achieved both turn-on 2PEF tumor cell imaging and turn-on 2PA-PDT on melanoma tumor cells. It is noted that the current experiments were performed on melanoma cells, which are exposed at the skin surface. Further molecular optimization and more sophisticated drug-delivery systems may be needed to take advantage of the unique turn-on feature of the dyes and perform 2PA-PDT treatments for other tumors.

In summary, we have demonstrated that by using NTO analysis of the excited states, fast screening of the potential 2PA dyes can be achieved. We have used this method to quickly evaluate a series of carbonyl functionalized distyrylbenzene derivatives for their potential as turn-on 2PA dyes without the need of actually synthesizing all the molecules. Under consideration of both the 2PA activity and chemical reactivity, we have identified the acetal terminated molecule Ace-DSB as a preferable structure for the 2PA probes. We then conducted more careful theoretical and spectroscopic characterization focusing only on this structure. The measurements confirmed that the conversion of the Ace-DSB into Ald-DSB under mild conditions gave rise to a 26-fold increase of the δ values at 760 nm, along with a 40-fold increase in efficiency of two-photon generation of singlet oxygen. The conversion reaction, which dramatically turns on the 2PA absorption capability of the molecules, can occur under mild and physiological conditions. This turn-on feature of the Ace-DSB molecule allows enhanced imaging of cancer cells using TPLCSM. Importantly, by controlled conversion of Ace-DSB into Ald-DSB, we have successfully achieved turn-on two-photon excited photodynamic therapy for MCF-7 cancer cells. These probes were also successfully applied in *in vivo* 2PA photodynamic therapy on melanoma tumor using a mouse model. This work highlights the importance of theory-assisted molecular screening methods for the cost-effective rational design of novel 2PA-based theranostic reagents.

EXPERIMENTAL PROCEDURES

Calculation

All the calculations were performed with the Gaussian 09 software package.[32] The ground states of these four molecules were optimized using DFT with the B3LYP

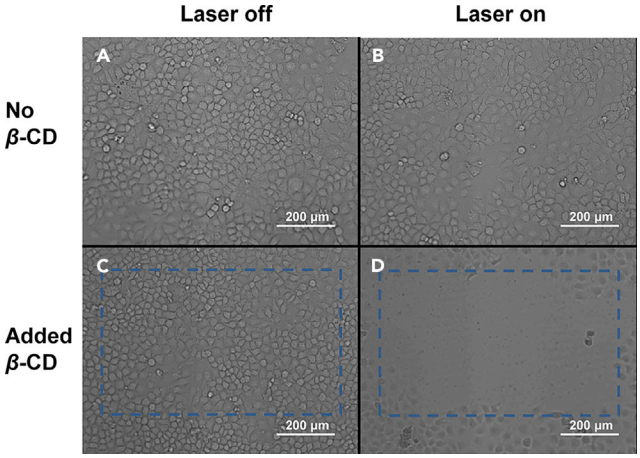

Figure 8. Cellular Phototoxicity in Response to 2PE-PDT by Turn-On Process

(A and B) Viability of MCF-7 cells incubated with Ace-DSB and no β-CD (A) in darkness and (B) illuminated by the 760-nm laser.

(C and D) Viability of MCF-7 cells incubated with Ace-DSB and added β-CD, when deprotection happens, (C) in darkness and (D) illuminated by the 760-nm laser; the box shows the illuminated area.

functional (DFT/B3LYP/6-31G++(d, p)), and the excitation energies were calculated using the TD-DFT at the B3LYP functional (DFT/B3LYP/6-311G++(2d,2p)). Adiabatic TD-DFT in the Kohn-Sham form was used for calculating the excited-state structures.[35,36] The S_1 excited states were then optimized using the restricted configuration interaction (singlet) (DFT/6-31G(++d,p)) approach, and the S_1-S_0 electronic transitions from the relaxed excited states were obtained from TD-DFT calculations using the optimized excited states as inputs with the B3LYP functional (DFT/B3LYP/6-311G++(2d,2p)). Natural transition orbitals (NTO)[37] calculations were performed after TD-DFT to describe the physical meanings of the orbitals of the hole and electron on the excitation states. Inhomogeneous line-broadening parameter for all absorption spectra calculations has been fixed to Γ = 0.17 eV for all chromospheres based on a typical line width of respective experimental spectra.[38] The transition dipole moments between the excited states were calculated by the Multiwfn wavefunction analyzer software with the Gaussian fchk and out file as input.[39]

Synthesis

The synthesis and structural characterization of the DSB molecules are shown in Figures S1 and S2.

Absorption and Fluorescence Spectra

Steady-state absorption spectra were recorded using a T6 UV-vis spectrometer (Purkinje General, China). Fluorescence measurements were performed on an LS55 fluorescence spectrometer (PerkinElmer, USA). The solutions were bubbled with N_2 for 10 min before fluorescence measurement. Absolute fluorescence quantum yield (Φ) of Ald-DSB (0.01 mM in THF) was measured by an FLS920 fluorescence spectrometer using an integrating sphere (Edinburgh Instruments, UK). Relative fluorescence quantum yield was measured in THF solution with Ace-DSB in THF as a reference, using the relative methodology based on the following equation: $\Phi_x/\Phi_r = (A_r/A_x)\,(D_x/D_r)\,(n_x/n_r)^2$, where A is the absorbance at the excitation wavelength, n the refractive index, and D the integrated luminescence intensity. r and x stand for reference and sample.

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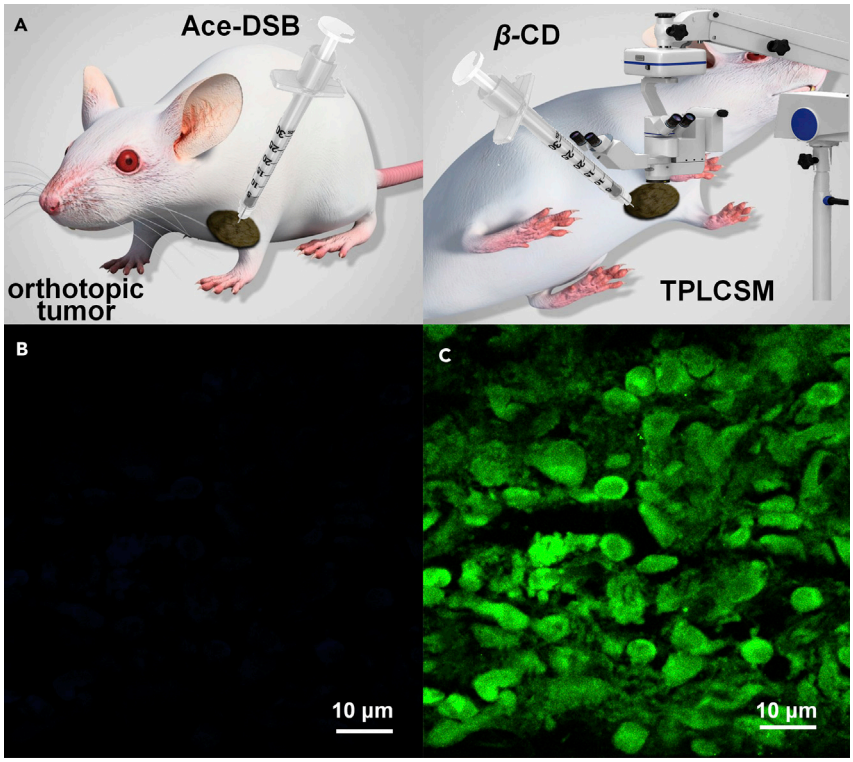

Figure 9. *In Vivo* Bioimaging Application
(A) Diagram of *in vivo* turn-on TPLCSM image of tumor stained by the Ace-DSB/β-CD.
(B and C) Before (B) and after (C) treatment with β-CD in same exposure time.

For measurements of the fluorescence time profiles, the time-correlated single-photon counting method using a nanosecond pulsed LED sources (376 nm, full width at half-maximum ca. 800 ps) with 40-MHz repetition rate was employed. A photomultiplier tube and a counting board (PicoQuanta, PicoHarp 300, Germany) were used for signal detection (Figure S7).

Singlet Oxygen Quantum Yields

The singlet oxygen quantum yield Φ_Δ was estimated by the singlet oxygen luminescent method. Singlet oxygen phosphorescence (near 1,270 nm) was recorded with a liquid nitrogen-cooled, solid indium-gallium-arsenic detector (Edinburgh Instruments), and Φ_Δ values were deduced from an analogous methodology similar to that for fluorescence quantum yields (see above) with rose bengal as a reference (Φ_Δ = 0.76 in methanol).[40,41] We measured the two-photon excited $^1O_2$ generation ability ($\delta\Phi_\Delta$) by using tetraphenylporphyrin as a reference (λ_{2PA} = 790 nm, δ = 12 GM, Φ_Δ = 0.6, $\delta\Phi_\Delta$ = 7.2).[42] The InGaAs detector (NIRvana-640, Princeton Instruments) was used to detect $^1O_2$ emission.

2PA Cross-Section

2PA cross-sections were determined via a comparative method that measured the 2PEF by using rhodamine B as a reference. The fundamental of a mode-locked Ti:sapphire laser (690–850 nm, Tsunami) was focused into a quartz cuvette with an optical geometry and detected with a liquid-nitrogen-cooled charge-coupled device (SPEC-10-400 B/LbN, Roper Scientific) attached to a polychromator (Spectro-pro-550i, Acton).

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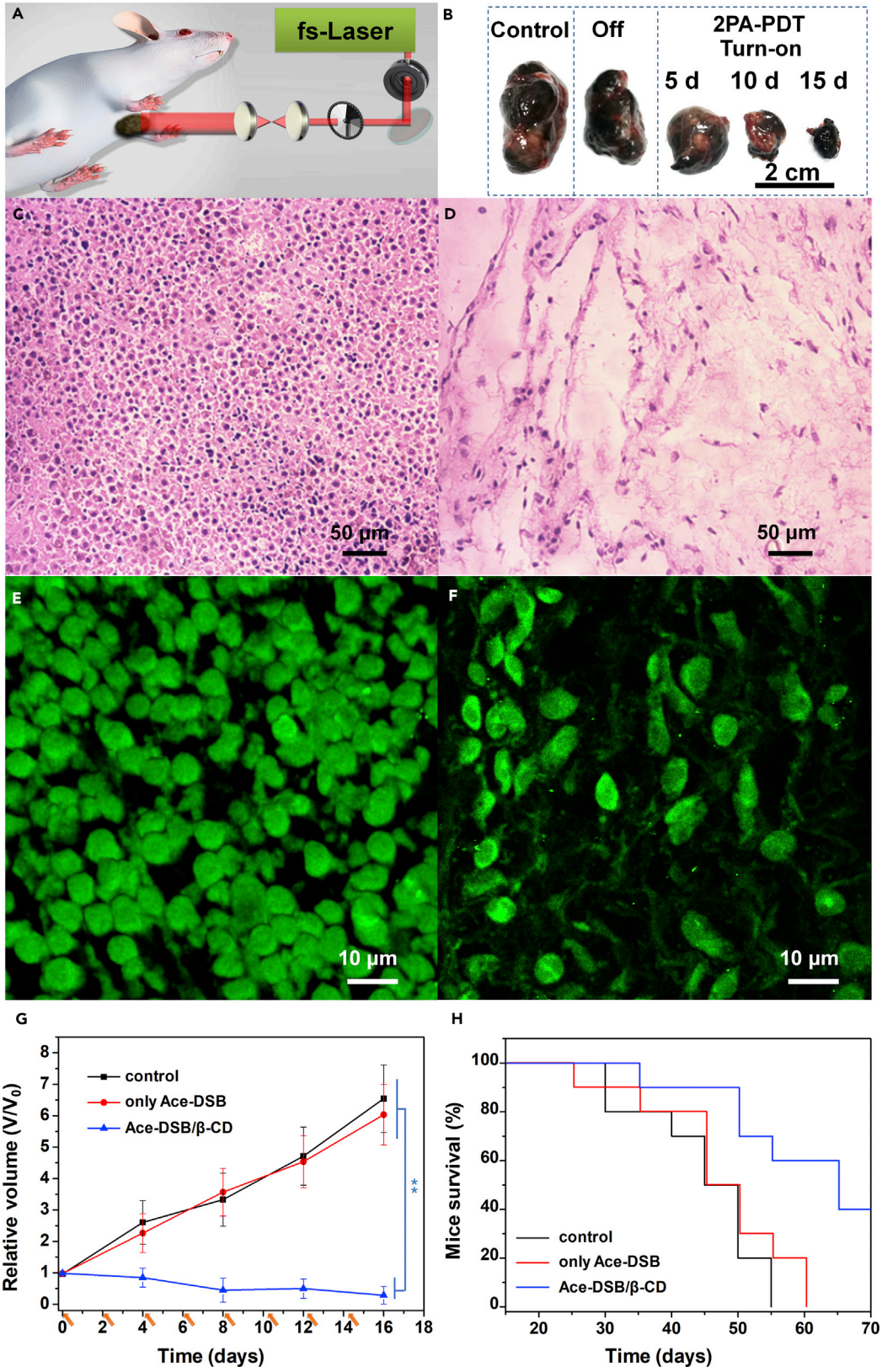

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Figure 10. *In Vivo* 2PA-PDT Application

(A) *In vivo* studies of Ace-DSB/β-CD as cancer turn-on 2PA-PDT agent, whereby an extending femtosecond laser pulse was used to irradiate the tumor.

(B) *In vivo* tumor inhibition assays of Ace-DSB/β-CD. Experimental conditions are described in the text; H&E-stained paraffin section image was used as control.

(C–F) Before 2PA-PDT (C) and after 2PA-PDT (D); the TPLCSM image of histological sections of tumor stained with Ace-DSB/β-CD for (E) control and (F) after 2PA-PDT.

(G) Relative tumor volume (compared with the same tumor on day 0, V_0) with 2PE-PDT; the arrows show the 2PE-PDT treatments (t test, $^{**}p < 0.01$).

(H) Percentage of survival after 2PE-PDT.

Cell Culture

MCF-7 human breast cancer cells were cultured in DMEM (Gibco) containing 10% bovine calf serum and 1% penicillin and streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2. Cells were removed with a solution of 0.05% trypsin in 0.53 mM EDTA, resuspended in serum-free medium (10^5 cells/mL) for cell seeding, and allowed to attach to the surface for 2 hr before the addition of serum-containing media. For passage, cells were resuspended in the same 10 mL of the medium that they were growing in, and 3 mL was transferred to 7 mL of fresh medium in a new flask. Cells were seeded into a Petri dish, 12-well plate, or confocal dish for different experiments.

Cell Staining and Two-Photon Fluorescence Microscopy Imaging

Cells were cultured on 12-well glass slides or confocal dish under normal culture conditions. After a 48 hr incubation period, the medium was removed and cells were incubated with 50 μM Ace-DSB or Ald-DSB in PBS (containing 0.5% DMSO) for 10 min, followed by two washes with PBS. The cell was visualized with 20×, 40×, and 63× objectives. The cell images were captured sequentially on an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus) with 760-nm excited light. Stacks of 18 optical sections (1,024 × 1,024 pixel arrays) were collected at 0.55-μm intervals in the z dimension. The 3D microscopy construction of MCF-7 cell images is shown in Video S1.

In Vitro 2PE-PDT

MCF-7 human breast cancer cells were seeded into a confocal dish (100,000 cells/mL) and cultured for 24 hr. The cells were then cultured for 4 hr with or without 8 μg/mL (about 20 μM) Ald-DSB or Ace-DSB in fresh culture medium containing 0.5% DMSO. After incubation with the DSB molecules, cells were washed twice, maintained in fresh culture medium, and submitted (or not) to laser irradiation. The β-CD added to the culture medium for the turn-on stimuli was 16 μg/mL (about 28 μM). The entire area of the well was irradiated at 760 nm (10 mW/cm^2 120 fs, 1,000 Hz) for 20 min.

Cytotoxicity

To assess the cytotoxicity of the Ald-DSB or Ace-DSB with (without) laser irradiation, we observed the viability after staining by using a LIVE/DEAD kit. Cells were cultured in a 24-well microplate (approximately 100,000 cells per well). After culture for 24 hr, a calcein and ethidium homodimer-1 PBS solution was added and cultured for 20 min in normal culture environment. We determined the cell viability by using a confocal microscope (Carl Zeiss LSM710) whereby we calculated viability by counting live (green) and dead (red) cells, comparing those numbers with that of the control well, and expressing them as percentages.

In Vivo Imaging and 2PA-PDT

BALB/c nude mice (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and all experimental procedures and protocols in the

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study were approved by the Ethics Committee of Lanzhou University, China. According to the requirements for Animal Ethics, all experiments were made to minimize the number of mice (20 mice per group) used and their suffering. Each 6-week-old female BALB/c nude mouse was inoculated subcutaneously with B16-F10 melanoma in mouse cells suspended in 0.2 mL of sterile saline solution. When the tumors grew to about 100 mm^3, the mice were distributed randomly into groups for the analysis of tumor development with PBS and Ace-DSB/β-CD treatments. One milligram of Ace-DSB was dissolved in 2 mL of 20% fat emulsion (Intralipid) by ultrasonication for 10 min (concentration: 1.25 mM); 4.5 mg β-CD was dissolved in 2 mL PBS to form a solution whose concentration was 2 mM (Figure S13). We have measured the δ spectra in different solutions (Figure S12), which show negligible solvent effect.

The dose of 50 μL of PBS (control) or Ace-DSB was injected into the tumors per mouse. The turn-on 2PEF was achieved by injection of 100 μL of β-CD solution. The *in vivo* tumor images were captured sequentially on an Olympus FluoView FV1000MPE confocal laser scanning microscope (Olympus). The 2PA PDT was performed by the home-made instrument shown in Figure 10A. The average power density of laser (760 nm, 120 fs, 1,000 Hz) delivered to the tumor was measured with a thermoelectric optical energy meter and was 80 mW/cm^2. Each 2PE-PDT process was performed for 10 min three times.

The initial tumor volumes were all pre-designed to be approximately 100 mm^3 before 2PE-PDT. To reduce individual initial tumor size differences, we introduced the relative tumor volume, which we calculated by using the following formula: relative tumor volume = V_x/V_0, where V_x is the absolute tumor volume of the respective tumor on day x, and V_0 is the absolute tumor volume of same tumor on day 0, when the treatment started.

In the *in vivo* theranostic process, the turn-on degree of the dyes can be controlled according to the experimental requirements (Figure S15). Partial turn-on state allows imaging with reduced light damage to normal tissue, and the full turn-on state allows efficient photodynamic therapy to the selected tumor tissue. For example, the dyes can be partially turned on by adding a 10% dose of β-CD solution, and enabled high-quality TPLCSM imaging with a low level of light toxicity. The full dose of β-CD was then imposed to fully turn on the photosensitizers for 2PE-PDT (Figure S15).

H&E Staining

Tumors and major organs were fixed with 10% formalin for at least 24 hr. Samples were then embedded in paraffin, sectioned, and stained with H&E. Histopathological changes were observed with a light microscope (DMI4000B; Leica, Germany). The TPLCSM images of histological sections of tumor stained with Ace-DSB/β-CD were captured on a Leica SP8 DIVE.

DATA AND SOFTWARE AVAILABILITY

X-ray crystallographic data can be obtained free of charge from the Cambridge Crystallographic Data Center (www.ccdc.cam.ac.uk/data_request/cif) under accession number CCDC: 934402.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, 15 figures, 1 video, and 1 data file and can be found with this article online at https://doi.org/10.1016/j.chempr.2018.12.001.

ACKNOWLEDGMENTS

This work is supported by National Natural Science Foundation of China (21702085 21602176, 51525303, 21233001, 21190034, 21573251, and 21673144), the Fundamental Research Funds for the Central Universities (lzujbky-2017-11), the Beijing Natural Science Foundation of China (2162011), and High-Level Teachers in Beijing Municipal Universities in the Period of 13th Five-Year Plan (CIT&TCD20180331).

AUTHOR CONTRIBUTIONS

Conceptualization, C.-L.S. and J.L. equally; Investigation, C.-L.S., J.L., X.-Z.W., R.S., S.L., J.-Q.J., T.L., Q.-W.S., and Q.L.; Writing – Original Draft, C.-L.S. and J.L.; Writing – Review & Editing, H.-L.Z.; Supervision, H.-B.F., J.-N.Y., and H.-L.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 16, 2018
Revised: July 12, 2018
Accepted: November 30, 2018
Published: January 10, 2019

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