

Article

A Near-Infrared Fluorescent and Photoacoustic Probe for Visualizing Biothiols Dynamics in Tumor and Liver

Weizhong Ding ^{1,2,†}, Shankun Yao ^{1,2,†}, Yuncong Chen ^{1,2,3,*} , Yanping Wu ^{1,2}, Yaheng Li ^{1,2}, Weijiang He ^{1,3,*} and Zijian Guo ^{1,2,3,*} 

¹ State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, China

² Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, Nanjing 210023, China

³ Nanchuang (Jiangsu) Institute of Chemistry and Health, Nanjing 210000, China

* Correspondence: chenyc@nju.edu.cn (Y.C.); hewei69@nju.edu.cn (W.H.); zguo@nju.edu.cn (Z.G.)

† These authors contributed equally to this work.

Abstract: Biothiols, including glutathione (GSH), homocysteine (Hcy) and cysteine (Cys), play crucial roles in various physiological processes. Though an array of fluorescent probes have been designed to visualize biothiols in living organisms, few one-for-all imaging agents for sensing biothiols with fluorescence and photoacoustic imaging capabilities have been reported, since instructions for synchronously enabling and balancing every optical imaging efficacy are deficient. Herein, a new near-infrared thioxanthene-hemicyanine dye (Cy-DNBS) has been constructed for fluorescence and photoacoustic imaging of biothiols in vitro and in vivo. Upon treatment with biothiols, the absorption peak of Cy-DNBS shifted from 592 nm to 726 nm, resulting in a strong NIR absorption as well as a subsequent turn-on PA signal. Meanwhile, the fluorescence intensity increased instantaneously at 762 nm. Then, Cy-DNBS was successfully utilized for imaging endogenous and exogenous biothiols in HepG2 cells and mice. In particular, Cy-DNBS was employed for tracking biothiols upregulation in the liver of mice triggered by S-adenosyl methionine by means of fluorescent and photoacoustic imaging methods. We expect that Cy-DNBS serves as an appealing candidate for deciphering biothiols-related physiological and pathological processes.

Keywords: fluorescence; photoacoustic imaging; near-infrared probe; biothiols; liver; tumor



Citation: Ding, W.; Yao, S.; Chen, Y.; Wu, Y.; Li, Y.; He, W.; Guo, Z. A Near-Infrared Fluorescent and Photoacoustic Probe for Visualizing Biothiols Dynamics in Tumor and Liver. *Molecules* **2023**, *28*, 2229. <https://doi.org/10.3390/molecules28052229>

Academic Editors: Li-Ya Niu and Xuewen He

Received: 3 January 2023

Revised: 19 February 2023

Accepted: 24 February 2023

Published: 27 February 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Sulfhydryl-containing amino acids, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) are bound up with many diseases [1–3]. For example, malfunction of Cys level has been associated with hair depigmentation, slowed growth rate and so on. In particular, the abnormal concentration of Cys in the tumor site is significantly higher than that in normal tissues, and it can even reflect different stages of tumor progression [4,5]. An anomalous level of Hcy gives rise to cardiovascular and Alzheimer's disease [6,7]. Alterations in GSH homeostasis have profound impacts on cell physiology, and it has been demonstrated that GSH deficiency is linked with cancer, liver damage and neurodegenerative diseases [8–10]. Additionally, targeting glutathione metabolic pathways has been extensively studied [2,11,12]. For instance, S-Adenosyl methionine (SAM), an intermediate metabolite of methionine, is used for the treatment of depression, liver disorders and osteoarthritis [13–17]. Moreover, the metabolites of SAM, such as Hcy, GSH and decarboxylated SAM, exert vital influences on the function of liver [18–21]. Unfortunately, the contents of SAM are significantly reduced in liver diseases. To maintain normal liver function, exogenous SAM must be supplemented to compensate for its deficiency, which is beneficial to various tissues, especially the liver [22–24]. Thus, tracking the levels of biothiols in the liver is highly conducive for investigating the liver-protective effects of drugs such as SAM.

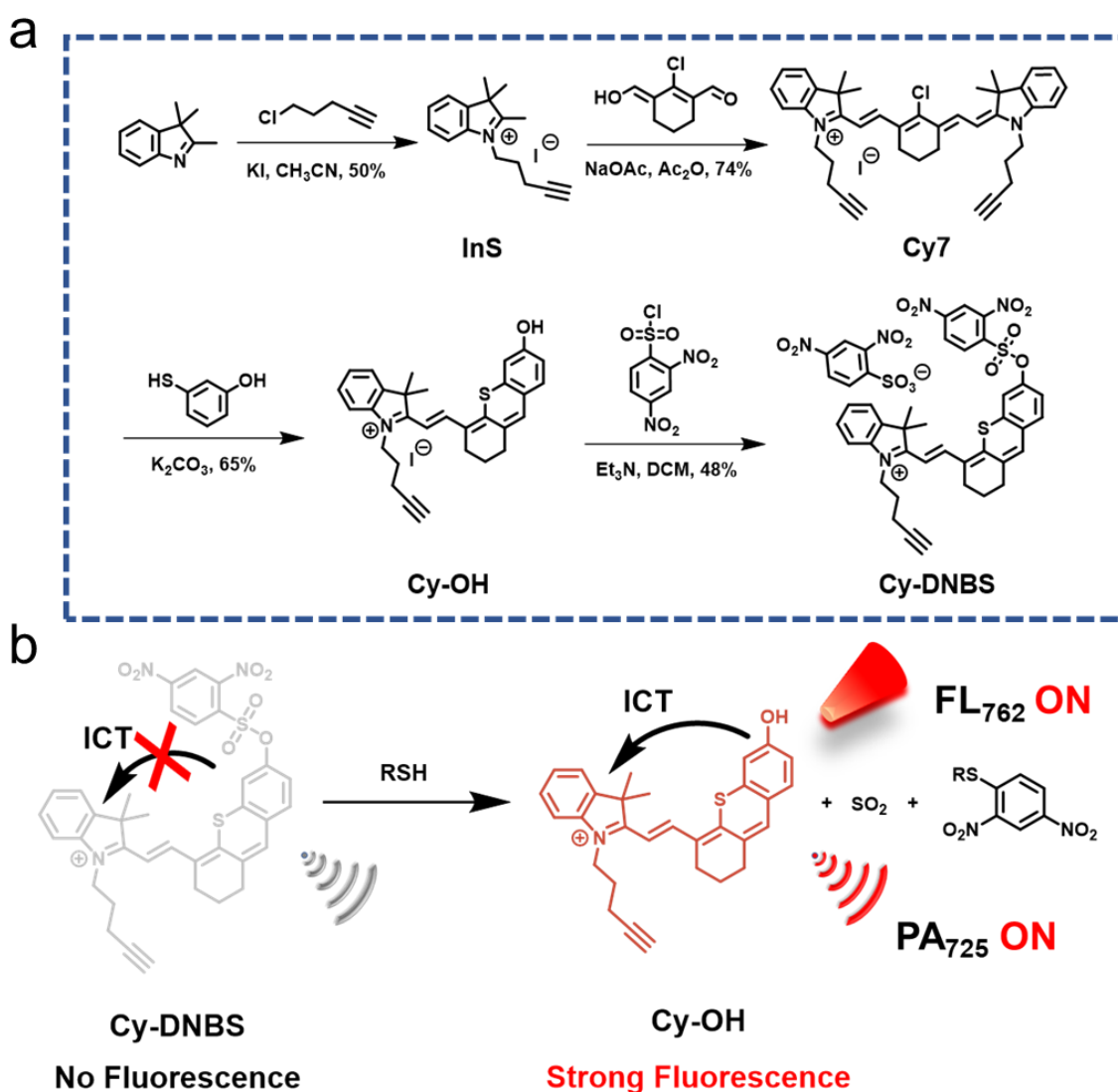
Owing to their significant roles, efforts to design efficient and handy methods for detecting biothiols with high sensitivity and selectivity are still advancing, which contributes to the understanding of cellular biothiols-related physiopathology [25–30]. Among these approaches, optical imaging, such as near-infrared fluorescence imaging (NIRFI), enables non-invasive and real-time visualization of disease-relevant biomarkers with high sensitivity and specificity, drawing great attention [31–37]. However, the penetration depth of NIRFI is usually limited, making it inaccessible for visualizing bioactive species in deep tissue. In contrast, photoacoustic imaging (PAI) is a burgeoning molecular imaging technique that utilizes the PA effect to convert absorbed light energy into ultrasound signals, which assists researchers to profile deeper tissues with high spatial resolution [38–40]. Hence, the drawbacks of NIRFI can be offset by PAI, bringing out the best in each other. Therefore, NIRFI/PAI with complementary advantages of fluorescence and photoacoustic imaging, is expected to perfect imaging output of biomarkers, which endows the ability to self-calibrate to avoid the false-positive signals and systematic errors [41–44]. Nevertheless, to date, the combination of NIRFI and PAI for multiscale analysis of biomarkers has not yet been fully exploited. Recently, thioxanthene-hemicyanine dye reported by Liang's group provided an energy balance strategy by transforming existing hemicyanine dyes into thioxanthene-hemicyanine scaffolds, permitting reliable dual modality NIRFI/PAI of biomolecules of interest [45]. We envisioned that an all-in-one imaging agent based on thioxanthene-hemicyanine scaffold for imaging biothiols is highly desirable.

Hemicyanine dyes that display intramolecular charge transfer (ICT) characteristic are important building blocks for advanced optical probes. The ICT process depends on both donor and acceptor strength. When a strong electron-withdrawing group was introduced into the hydroxyl group of the hemicyanine fluorophore, the electron-donating ability of the donor group was weakened, which hampers the charge transfer in the fluorophore. Thus, the fluorescence of the probe could be quenched through blocking ICT process [46,47]. With all this in mind, we constructed a biothiols-activable probe Cy-DNBS with DNBS. Upon exposure to biothiols, the 2,4-dinitrophenylsulfonyl (DNBS) moiety was specifically cleaved and then Cy-DNBS was converted into Cy-OH, resulting in a strong NIR absorption at 726 nm accompanied by the enhanced fluorescence signal at 762 nm. Subsequently, Cy-DNBS was utilized for NIRFI/PAI of endogenous and exogenous biothiols in living cells and mice. Notably, Cy-DNBS have been employed successfully for real-time visualizing alterations in biothiols levels in liver induced by administration of SAM. Taken together, with complementary merits of fluorescence and photoacoustic imaging, Cy-DNBS was successfully applied for dynamically monitoring biothiols *in vitro* and *in vivo*, suggesting its enormous potential in imaging biothiols.

2. Results and Discussion

2.1. Design and Synthesis of Cy-DNBS

We first considered the leaving moiety of the probe since small biomolecules had a limited lifetime in biological system, which called for cleaved groups with excellent electron-withdrawing ability to translate biomarkers into optical signals rapidly. Given that, the first choice of masking group was 2,4-dinitrophenylsulfonyl group (DNBS), which has often been chosen as the responsive unit for biothiols [48–51]. Therefore, by modifying the hydroxyl group in the fluorophore, we envisioned that the fluorescence could be totally quenched through blocking intramolecular charge transfer (ICT) process, thus minimizing background fluorescence interference. Then, by covalently connecting DNBS to fluorophore, we designed and constructed Cy-DNBS. The reaction of biothiols and Cy-DNBS generated Cy-OH and thereby restored the strong absorption and emission at 726 nm, 762 nm, respectively. The detailed synthetic route and recognition mechanism were shown in Scheme 1. Furthermore, the structures of Cy7, Cy-OH and Cy-DNBS were fully characterized by ^1H NMR, ^{13}C NMR and high-resolution mass spectra (HRMS), with the details compiled in the supporting information (Figures S10–S19).



Scheme 1. (a) Synthetic route of Cy-DNBS and (b) proposed biothiols-sensing mechanism of Cy-DNBS.

2.2. Optical Properties of Cy-DNBS towards Biothiols

Before exploring the feasibility of Cy-DNBS for sensing biothiols *in vitro* and *in vivo*, the response behaviors of Cy-DNBS to biothiols were investigated. The absorption spectra of Cy-DNBS were first studied after three biothiols were added to PBS buffer (pH 7.4). As depicted in Figure 1a–c, free Cy-DNBS exhibited a maximum absorption peak at 592 nm ($\epsilon = 3.33 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and possessed excellent photostability (Figure S2). However, upon the addition of three biothiols, the absorption peak at 592 nm attenuated sharply, and a new strong absorption peak appeared at around 726 nm ($\epsilon = 3.42 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$), resulting from the departure of DNBS moiety and the restored ICT process. To further elaborate on the shift in maximum absorption peak and the sensing mechanism of Cy-DNBS towards biothiols, HRMS and high-performance liquid chromatography (HPLC) analysis were performed to elucidate the reaction processes. The peaks of m/z 452.20277 and m/z 682.16604 (Figure S1) were assigned to Cy-OH and Cy-DNBS, respectively, which was consistent with the mechanism proposed by Chakrapani et al. [52]. In addition, the HPLC analysis (Figure S3) also supported the results as stated above, and the retention time of Cy-OH and Cy-DNBS were 20 min and 21.5 min, respectively. Since the major absorption of Cy-DNBS was in the range of NIR, we validated the capability of Cy-DNBS for PAI. Similarly, to the absorption spectra, as shown in Figure 1d–f, an unspectacular PA signal was observed from free probe. In contrast, upon addition of 2 equivalents of Cys,

Hcy and GSH, augmented PA signals positioned at 725 nm occurred, and the PA intensity was 4.3-fold, 3.3-fold and 6.0-fold higher than that of the probe alone, respectively. Taken together, these results indicated that Cy-DNBS appeared to be a promising NIR PAI agent for imaging biothiols.

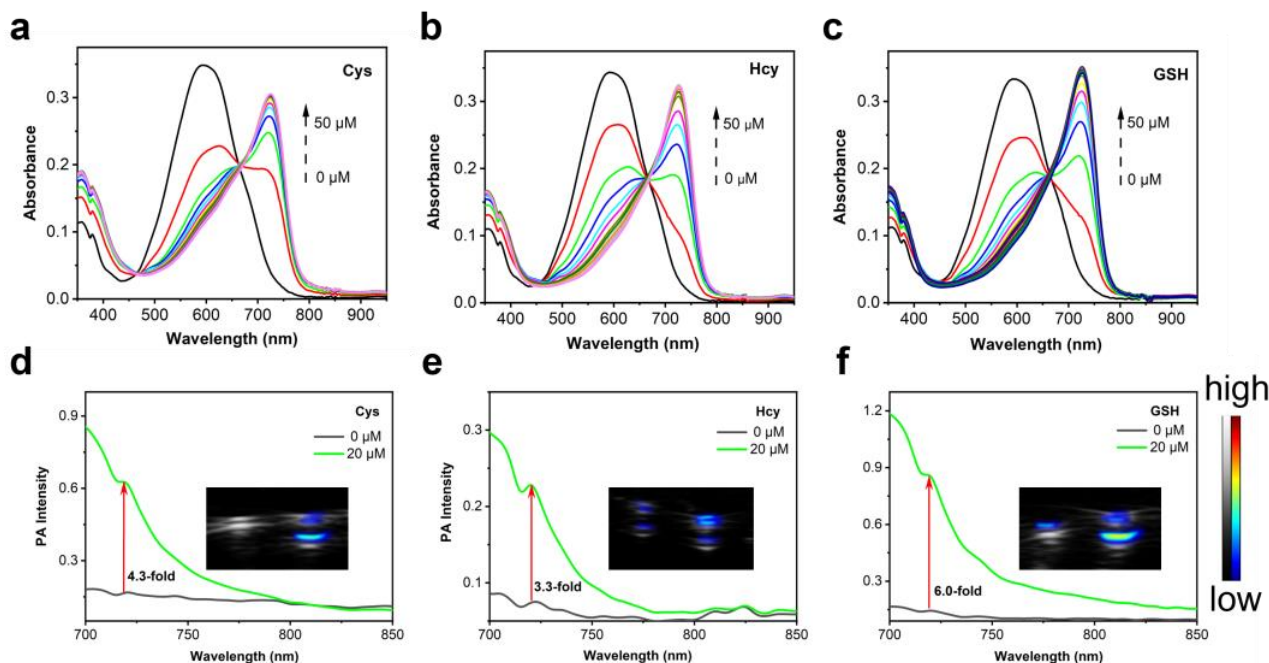


Figure 1. Absorption spectra of Cy-DNBS (10 μ M) in the presence of different concentrations (0–50 μ M) of (a) Cys, (b) Hcy, (c) GSH and PA spectra of Cy-DNBS (10 μ M) mixed with 20 μ M (d) Cys, (e) Hcy or (f) GSH in PBS solution. (Insets in (d–f): PA images of the Cy-DNBS solution with or without biothiols).

Then, the fluorescent responses of Cy-DNBS to a series of concentrations of biothiols were investigated. As illustrated in Figure 2a–d, the fluorescence spectra showed that Cy-DNBS displayed an augmented fluorescence peak centered at 762 nm and featured concentration-dependent behaviors. From the fluorescence titration curves and regression equation, the limit of detection of Cys, Hcy and GSH were measured to be as low as 0.25, 0.04 and 0.13 μ M, respectively (Figure S4). Selectivity was a key parameter for the probe, and Cy-DNBS responded specifically to biothiols over other bioactive species needed to be assessed. After incubating Cy-DNBS with other competing amino acids in PBS buffer, negligible fluorescence and PA signals were found from interfering species compared to three biothiols, hinting its outstanding selectivity (Figures 2e, S5 and S6).

To further explore the response of Cy-DNBS to biothiols following the change in time, the fluorescence spectra were immediately acquired upon Cy-DNBS mixed with three biothiols. As illustrated in Figure 2f, fluorescence intensity of Cy-DNBS increased rapidly and reached a plateau within 100 s. The results indicated that Cy-DNBS could fulfill the demand of rapid identification of biothiols, which was beneficial to capture the variation of biothiols quickly. In addition, it was worth noting that the fluorescent quantum yield of Cy-OH was 0.04 in DMSO by using ICG ($\Phi = 0.13$ in DMSO) as a reference. Fortunately, the low-fluorescence quantum yield promoted the outputs of photoacoustic signals, ensuring a balanced platform for bimodal imaging. These impressive properties encouraged us to explore applications of Cy-DNBS in cells.

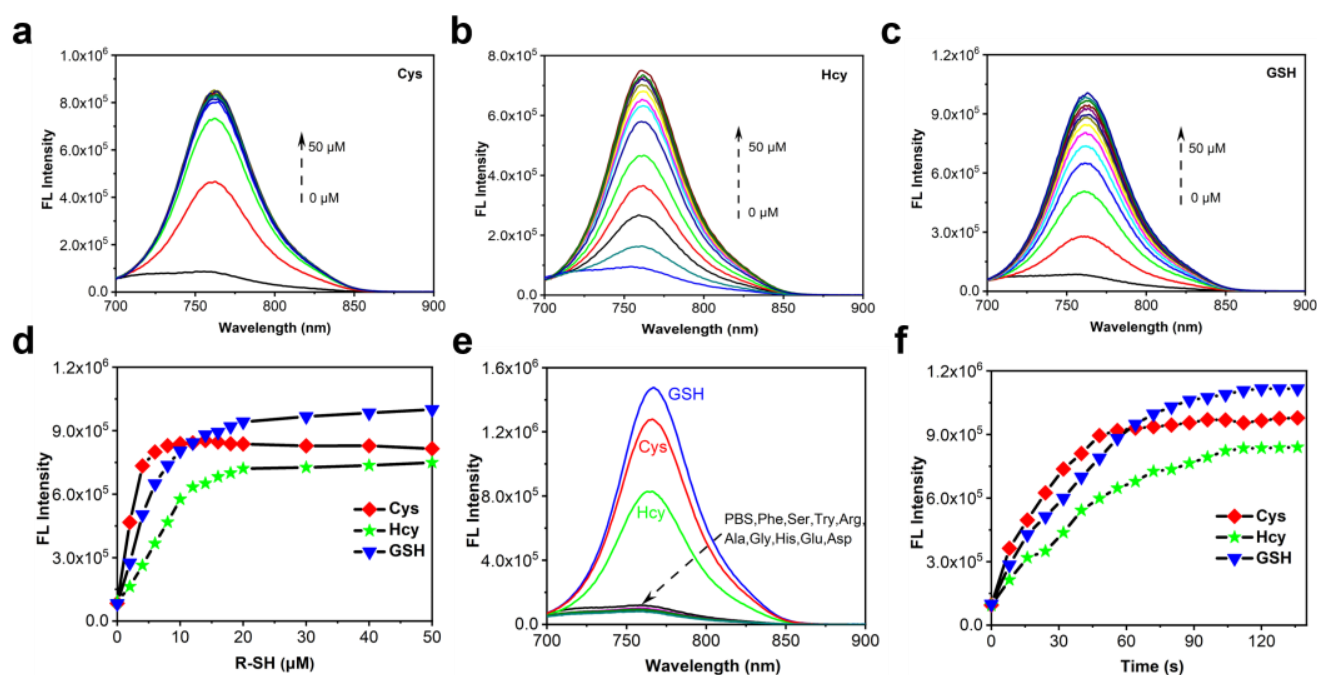


Figure 2. Fluorescence spectra of Cy-DNBS (10 μ M) towards different concentrations of (a) Cys, (b) Hcy and (c) GSH. (d) The plot of fluorescence intensity against concentration in PBS solution determined from (a–c). (e) Selectivity of Cy-DNBS (10 μ M) towards 20 μ M biothiols and other competing species. (f) Time-dependent behaviors of Cy-DNBS towards biothiols. $\lambda_{\text{ex}} = 680$ nm, $\lambda_{\text{em}} = 700$ –900 nm. Slits, 5 nm/5 nm.

2.3. Intracellular Biothiols Imaging with Cy-DNBS

Prior to imaging biothiols in cells, we evaluated the biocompatibility of Cy-DNBS in HepG2 cells through conventional MTT assay. It can be seen from Figure S7 that no obvious cytotoxicity was observed within the tested concentration range. Following on from this, we enquired the locating tendency of Cy-DNBS in organelles. After Cy-DNBS were co-incubated with three commercial organelle dyes (Mito-, Lyso-Tracker and DAPI) for 30 min, the confocal images were acquired. The results (Figure S8) illustrated that Cy-DNBS were successfully uptaken by cells and prone to remain in the mitochondria and lysosome, with Pearson correlation coefficients (PCC) of 0.74, 0.76 and 0.29 for mitochondria, lysosome and nuclei, respectively. The results were exciting because biothiols are abundant in the two organelles and remarkable indicators of organelles' function [53,54]. We next examined whether Cy-DNBS could be used to image intracellular biothiols. Firstly, N-Ethylmaleimide (NEM), a thiol-specific scavenger, was used to reduce intracellular biothiols prior to incubation of cells with Cy-DNBS [55]. As displayed in Figure 3a, the cells treated with NEM exhibited much weaker fluorescence than untreated cells. Then, exogenous Cys, Hcy and GSH were added to improve intracellular biothiols content. As expected, fluorescence intensity of cells significantly enhanced compared to Cy-DNBS alone (Figures 3b and S9). All the results showed that Cy-DNBS was suitable for real-time visualization of endogenous biothiols fluctuations in living cells and could be used for a follow-up survey of biothiols in vivo.

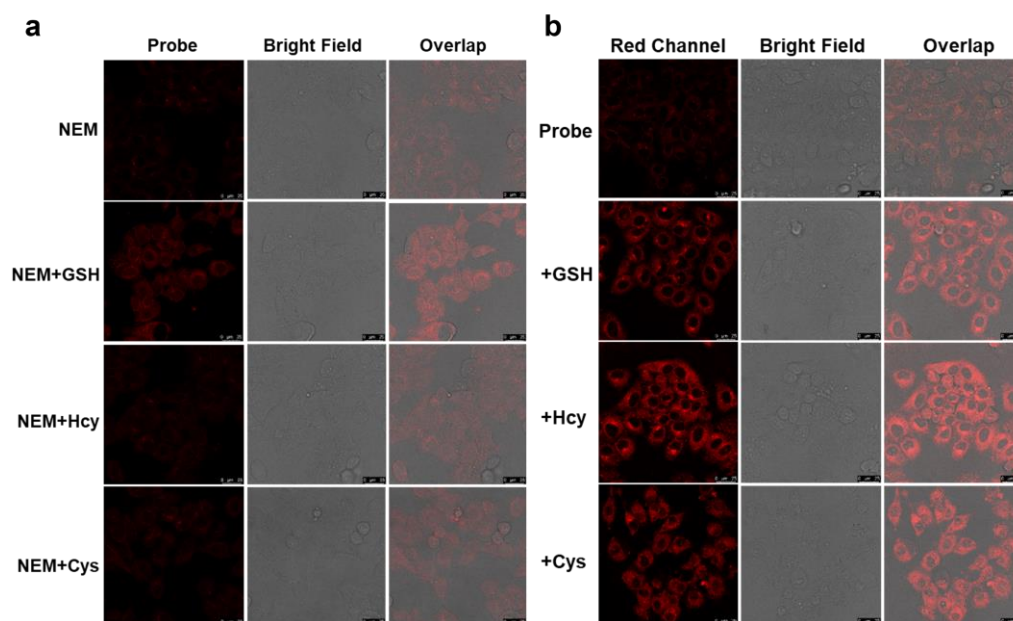


Figure 3. (a) HepG2 cells were pretreated with NEM (4 μ M) for 30 min, followed by biothiols (4 μ M) incubation for another 30 min, then images were obtained after incubation with Cy-DNBS (4 μ M) for 30 min. (b) Fluorescent images of HepG2 cells pretreated with different biothiols (4 μ M) for 30 min followed by Cy-DNBS (4 μ M) incubation for another 30 min. λ_{ex} = 633 nm, λ_{em} = 680–800 nm. Scale bars: 25 μ m.

2.4. Dynamically Tracking Biothiols in Tumor by NIRFI and PAI

Given the probe's excellent performance in biothiols detection in HepG2 cells, we further studied the applications of Cy-DNBS for tracking biothiols-related dynamic processes in the tumor of a mouse model. By using an excitation wavelength of 740 nm and emission wavelength of 790 ± 20 nm, the interference of spontaneous fluorescence was effectively avoided. After Cy-DNBS (100 μ L, 50 μ M) was injected into the tumor (tumor group) and normal tissue (control group), respectively, images were recorded immediately. Fluorescence signals increased significantly in both groups within one minute and fluorescence intensity in tumor tissues was nearly four times that of normal tissue (Figure 4a,b). The result could be explained by the reported truth that cancer cells possess higher biothiols content than normal tissues [11,56]. In addition, there were no obvious changes in fluorescence intensity of tumor and normal tissue after 2 min, indicating that Cy-DNBS has reached equilibrium with endogenous biothiols. These results demonstrated that Cy-DNBS could respond quickly to biothiols and differentiate tumor from normal tissues and therefore provided guidance for early diagnosis of tumor progression.

Then, we employed Cy-DNBS for PAI of biothiols in a tumor-bearing mouse model. As depicted in Figure 4c,d, upon injection with Cy-DNBS in two different groups (tumor, normal tissue), respectively, almost no obvious PA signals were observed in normal tissue over time, while strong PA signals were found in tumor, and the intensity was about four times as strong as that of normal tissue. Interestingly, both the fluorescence and photoacoustic intensity of tumor tissue were four times that of normal tissue, and the two were mutually compatible, indicating the excellent authenticity of the imaging outputs.

PAI possessed the benefits of high resolution and deep imaging depth, which enabled us not only to detect biothiols in tumor, but also to precisely assess the content of biothiols in different tumor regions through capturing numerous two-dimensional tomographic images. It can be seen from Figure 4e that PA intensity was very weak on the tumor surface and increased sharply as the tumor depth deepened, suggesting that there was a highly heterogeneous distribution of biothiols in solid tumors. By contrast, in normal tissues, PA signals hardly change with tissue depth, indicating that normal tissues had

no characteristics of distribution differences. These results suggested that PAI provided us with a toolkit for accurately locating and mapping the distribution and abundance of bioactive species at different depths of tumor.

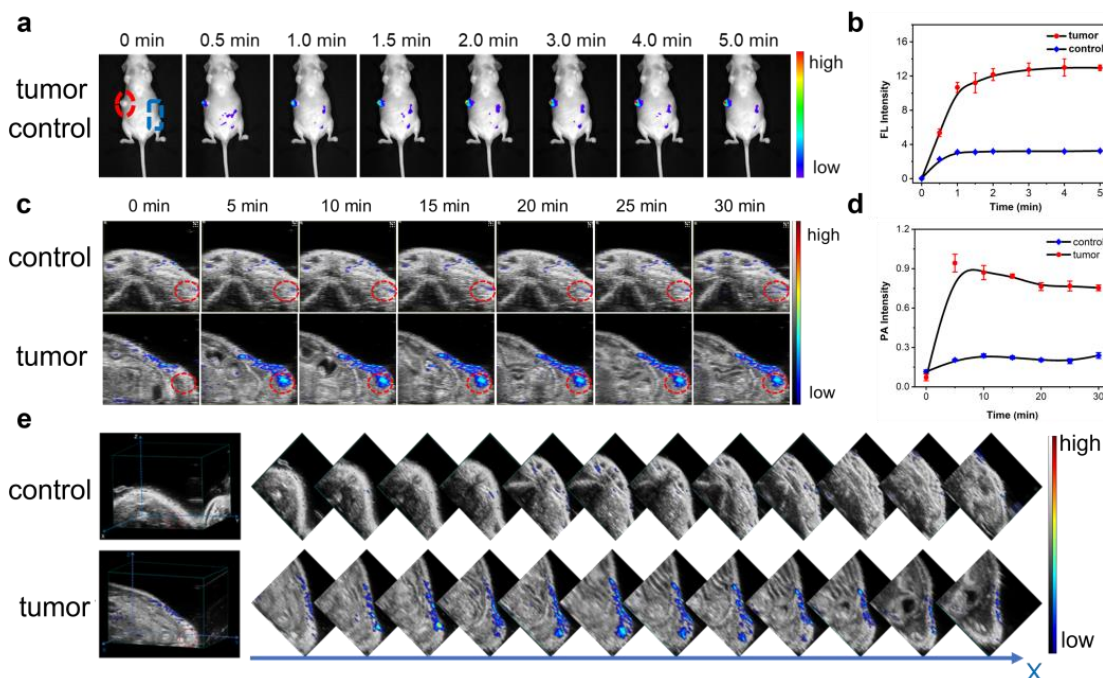


Figure 4. In vivo optical imaging of HepG2 tumor-bearing mice. (a) Fluorescence images of mice subjected to intratumoral injection of Cy-DNBS (50 μ M, 200 μ L; $\lambda_{\text{ex}} = 740$ nm, $\lambda_{\text{em}} = 790 \pm 20$ nm). Tumor and normal tissues are indicated by the red dotted circles and blue dotted rectangles, respectively. (b) Fluorescence intensity of tumor and the normal tissues determined over time. (c) PA signals of tumor and normal tissues collected at 725 nm by tumor injection of Cy-DNBS (50 μ M, 200 μ L). (d) PA₇₂₅ signals plotted as a function of time. (e) Representative two-dimensional images of the whole tumor region and the normal tissues at 10 min post injection of the Cy-DNBS (50 μ M, 200 μ L).

2.5. Real-Time Visualizing Biothiols in Liver of Drug-Treated Mouse Model

Liver is vital for digesting food and getting rid of toxic substances, and its dysfunction can lead to a series of diseases. It is well confirmed that the treatment of SAM could be effective against liver disease; hence, we are determined to select SAM as a model drug for visualizing biothiols kinetics in opposing liver diseases [18,20,21]. To prove the feasibility of Cy-DNBS for detecting excess biothiols caused by SAM treatment, we established an SAM-treated mouse model and subcutaneously injected SAM (200 μ L, 100 mg/kg) every other day for two weeks. After post injection of Cy-DNBS via tail vein, the fluorescence signals of PBS and SAM group were monitored. As shown in Figure 5a,b, it is clear that mouse administrated with SAM exhibited significantly brighter fluorescence in the liver than that of PBS-treated mouse. Meanwhile, more biothiols were directly found in the liver of the SAM group based on the fluorescence intensities of main organs resected from the mice at 10 min post injection (Figure 5c,d). To further investigate the biothiols fluctuations induced by administration of SAM, the PAI was carried out. The intensity sharply enhanced over time and reached its plateau at 5 min post injection (Figure 5e,f). Noticeably, only when mice were incubated with SAM could we observe apparent augmented signals from the acquired PA images. These results implied that Cy-DNBS could be used for tracking biothiols dynamics in the liver during drug therapy and thus provided feedback on the effects of medicine.

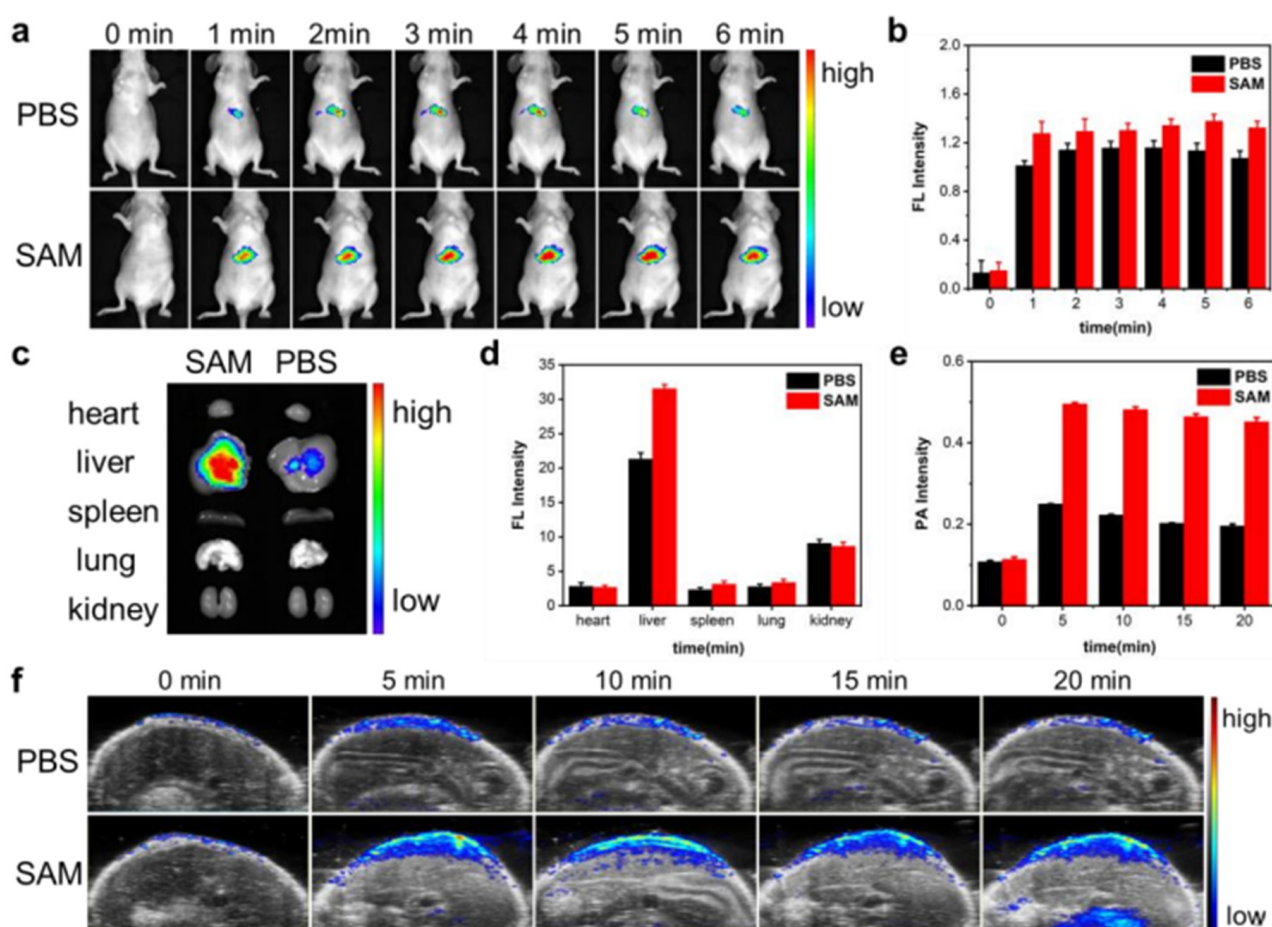


Figure 5. In vivo optical imaging of biothiols in liver of SAM/PBS-treated mouse model. (a) Both of SAM/PBS-treated mice were injected with Cy-DNBS (50 μ M in PBS, 200 μ L) via tail vein, and fluorescent images were acquired immediately at one-minute intervals. $\lambda_{\text{ex}} = 740$ nm, $\lambda_{\text{em}} = 790 \pm 20$ nm. (b) Changes in fluorescence intensity in the liver over time measured in part a. (c) Fluorescent images of representative organs of mice injected tail intravenously with Cy-DNBS (50 μ M in PBS, 200 μ L). $\lambda_{\text{ex}} = 740$ nm, $\lambda_{\text{em}} = 790 \pm 20$ nm. (d) Relative fluorescence intensity of different organs in (c). (e) Temporal profile of PA₇₂₅ signals determined in (f). (f) PA₇₂₅ signals of lung of mice injected tail intravenously with Cy-DNBS (50 μ M in PBS, 200 μ L) were recorded at five-minutes intervals.

3. Conclusions

In summary, a near-infrared fluorescent and photoacoustic probe Cy-DNBS was designed and synthesized by covalently linking thioxanthene-hemicyanine fluorophore with a biothiols-responsive unit (DNBS). Cy-DNBS could selectively respond to biothiols and realized sensitive detection of endogenous and exogenous biothiols in HepG2 cells. In addition, Cy-DNBS could fulfill visualization of biothiols in tumor and differentiate tumor region from normal tissues with high confidence. Furthermore, with the complementary advantages of NIRFI and PAI, Cy-DNBS was successfully applied to precisely monitor the fluctuations of biothiols in the liver triggered by administration of SAM. Accordingly, the dual-modality imaging probe can report the treatment efficacy of drugs that work by regulating the content of biothiols, which may offer a molecular tool for drug screening.

4. Materials and Methods

All chemicals used in the synthesis of Cy-DNBS were purchased commercially and used directly without other purification. The intermediates and probe were purified by column chromatography. Silica gel was shipped from Qingdao Haiyang Chemical Co., Ltd. (China), and the size of silica gel particle was around 300–400 mesh. All solvents used

for column chromatography were distillation grade. HPLC was carried out on Thermo Scientific Dionex Ultimate 3000 with acetonitrile/H₂O as eluents. All ¹H NMR and ¹³C NMR spectra were acquired at 298 K in CDCl₃ or CD₃OD on Bruker DRX-400, using TMS as internal reference. The ESI-MS spectra were obtained through Thermo Fisher Scientific LCQ Fleet electron-spray mass spectrometer with positive or negative mode. The HR-MS spectra were presented in positive mode unless otherwise noted. Absorption and fluorescence spectra were recorded using Perkin-Elmer Lambda 35 spectrophotometer and FluoroMax-4 spectrofluorometer, respectively. All the spectroscopic experiments were performed in 3 mL PBS buffer (pH 7.40) by applying a 1 cm optical path standard quartz cuvette as a container. Additionally, the spectral data were processed by Origin 2019 software. The melting point was measured by SGW[®]X-4 melting point apparatus. In vivo imaging of mice was carried out on IVIS Lumina K Series III instrument (PerkinElmer), and isoflurane for anesthetizing mice was purchased from RWD Life Science. The acquired raw images of mice were studied with Living Image 4.5. Confocal imaging was performed with Leica SP8 STED 3X and Zeiss LSM710 microscope. The PCC was determined by one-to-one-pixel matching method in ImageJ software.

4.1. Synthesis and Characterization

4.1.1. Synthesis of Indolenine Salt (InS)

To a solution of 5-chloro-1-pentyne (2.3 mL, 14.2 mmol, 1 eq) in 25 mL acetonitrile, was added potassium iodide (4.71 g, 28.4 mmol, 2 eq). After stirring at 50 °C for 30 min, 2,3,3-trimethylindoles (2.26 g, 14.2 mmol, 1 eq) were added dropwise to the mixture, and then the mixture was heated to reflux. After the reaction completed, the flask was cooled to room temperature, and the inorganic salt was removed by filtration, washed by DCM for several times and used in the next step without further purification (2.51 g, 50% yield. Mp: 189–191 °C, *R*_f = 0.74 (dichloromethane/methanol = 20:1)). ESI-MS calculated 226.15902 for C₁₆H₂₀N⁺, [M-I]⁺; found *m/z*: 226.15829.

4.1.2. Synthesis of Cy7

Compound InS (2.51 g, 7.11 mmol, 2 eq), trans-2-chloro-3-(hydroxymethyl) cyclohexyl-1-ene-1-formaldehyde (3.68 g, 3.56 mmol, 1 eq) and sodium acetate (0.59 g, 7.11 mmol, 2 eq) in 100 mL acetic anhydride were heated to reflux under nitrogen atmosphere for 3 h. After the flask was cooled to room temperature, the mixture was poured into 300 mL of cold diethyl ether. The precipitated yellow-green solid was washed with cold diethyl ether, and dried to afford light green powder (1.9 g, 74% yield. Mp: 231–234 °C, *R*_f = 0.48 (dichloromethane/methanol = 20:1)). ¹H NMR (400 MHz, CDCl₃) δ 8.37 (d, *J* = 14.1 Hz, 2H), 7.47–7.39 (m, 6H), 7.33 (s, 2H), 6.35 (d, *J* = 14.1 Hz, 2H), 4.37 (t, *J* = 7.3 Hz, 4H), 2.77 (t, *J* = 6.1 Hz, 4H), 2.47 (td, *J* = 6.6, 2.5 Hz, 4H), 2.19–2.02 (m, 6H), 2.01–1.92 (m, 2H), 1.76 (d, *J* = 21.4 Hz, 12H); ¹³C NMR (101 MHz, CDCl₃) δ 172.5, 150.7, 144.6, 142.1, 140.9, 128.9, 127.8, 125.4, 122.2, 111.1, 101.5, 82.9, 70.2, 49.3, 43.4, 28.2, 26.9, 26.0, 20.6, 16.2. ESI-MS calculated 587.31875 for C₄₀H₄₄ClN₂⁺, [M-I]⁺; found *m/z*: 587.31747.

4.1.3. Synthesis of Cy-OH

3-Hydroxythiophenol (0.27 g, 2.14 mmol, 1.5 eq) and potassium carbonate (0.29 g, 2.10 mmol, 1.5 eq) were dissolved in 15 mL acetonitrile, and the mixture was stirred at room temperature for 20 min. Then, 10 mL Cy7 (1 g, 1.4 mmol, 1 eq) in acetonitrile was added dropwise into the reaction solution, which was heated to 50 °C. After the reaction completed, the solvent was removed by rotary distillation under reduced pressure, and the resulting crude product was purified by column chromatography (CH₂Cl₂/CH₃OH = 50:1, *v/v*) to give a blue solid of 0.53 g, with a yield of 65%. Mp: 290–293 °C, *R*_f = 0.63 (dichloromethane/methanol = 20:1). ¹H NMR (400 MHz, CD₃OD) δ 8.19 (d, *J* = 13.5 Hz, 1H), 7.58–7.48 (m, 3H), 7.45–7.38 (m, 1H), 7.33 (d, *J* = 7.9 Hz, 1H), 7.30–7.24 (m, 1H), 6.96–6.90 (m, 1H), 6.85 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.31 (d, *J* = 13.5 Hz, 1H), 4.25 (t, *J* = 7.4 Hz, 2H), 2.82–2.75 (m, 2H), 2.70 (t, *J* = 6.1 Hz, 2H), 2.51 (t, *J* = 2.6 Hz, 1H), 2.38 (td, *J* = 6.5, 2.6 Hz, 2H), 2.03

(dd, $J = 14.1, 6.8$ Hz, 2H), 1.93 (dd, $J = 11.9, 6.0$ Hz, 2H), 1.76 (s, 6H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.4, 171.4, 159.9, 157.1, 143.6, 142.3, 141.4, 141.1, 135.5, 131.2, 129.8, 126.2, 123.8, 123.5, 122.0, 114.6, 113.2, 111.7, 101.0, 83.9, 71.3, 50.4, 43.8, 33.2, 29.0, 27.6, 27.0, 22.0, 16.5. ESI-MS calculated 452.20236 for $\text{C}_{30}\text{H}_{30}\text{NOS}^+$, $[\text{M-I}]^+$; found m/z : 452.20426.

4.1.4. Synthesis of Cy-DNBS

To a stirred solution of intermediate compound Cy-OH (0.53 g, 0.91 mmol, 1 eq) and triethylamine (0.09 g, 0.91 mmol, 1 eq) in 10 mL CH_2Cl_2 under argon atmosphere, 2,4-dinitrobenzenesulfonyl chloride (0.36 g, 1.37 mmol, 1.5 eq) was added quickly. After the reaction finished, the mixture was diluted with CH_2Cl_2 and then washed with saturated brine, the organic layer was dried over sodium sulfate anhydrous and concentrated under reduced pressure. The residue was separated by column chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH} = 30:1$, v/v) to obtain a blue solid of 0.41 g, yield 48%. Mp: 96–99 °C, $R_f = 0.33$ (dichloromethane/methanol = 20:1). ^1H NMR (400 MHz, CD_3OD) δ 8.91 (d, $J = 2.2$ Hz, 1H), 8.62 (dd, $J = 8.7, 2.3$ Hz, 1H), 8.50 (d, $J = 2.2$ Hz, 1H), 8.47–8.40 (m, 2H), 8.32 (d, $J = 8.7$ Hz, 1H), 8.26 (d, $J = 8.6$ Hz, 1H), 7.77–7.69 (m, 2H), 7.64–7.50 (m, 4H), 7.20 (dd, $J = 8.5, 2.4$ Hz, 1H), 7.15 (s, 1H), 6.92 (d, $J = 14.8$ Hz, 1H), 4.60 (t, $J = 7.4$ Hz, 2H), 2.84–2.78 (m, 2H), 2.73 (t, $J = 6.1$ Hz, 2H), 2.56 (t, $J = 2.6$ Hz, 1H), 2.47–2.39 (m, 2H), 2.18–2.09 (m, 2H), 1.98–1.92 (m, 2H), 1.83 (s, 6H). ^{13}C NMR (101 MHz, CD_3OD) δ 181.2, 153.1, 151.2, 150.1, 147.7, 144.3, 142.5, 137.1, 135.5, 135.1, 133.5, 133.2, 133.2, 132.2, 130.6, 130.5, 129.8, 129.7, 128.0, 126.8, 124.0, 122.6, 122.1, 120.0, 119.7, 115.0, 109.7, 83.5, 71.9, 52.9, 45.9, 33.4, 28.1, 27.9, 27.9, 21.3, 16.4. ESI-MS calculated 682.16762 and 246.96664 for $\text{C}_{36}\text{H}_{32}\text{N}_3\text{O}_7\text{S}_2^+$ and $\text{C}_6\text{H}_3\text{N}_2\text{O}_7\text{S}^-$, found m/z : 682.16803 and 246.96629, respectively.

4.2. Detection Methods

4.2.1. Optical Study

Cy-DNBS was dissolved in 1.5 mL dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions. The stock solution of three biothiols were prepared by dissolving Cys, Hcy and GSH in PBS buffer, respectively. Then, the stock solution was stored in the refrigerator at -20 °C for use. Fluorescence spectra were measured with a 5 nm slit for both excitation and emission.

4.2.2. Quantum Yield Calculation

The quantum yield was measured by using ICG ($\Phi = 0.13$ in DMSO) as a standard. The following equation was used to calculate quantum yield of Cy-DNBS Φ_x :

$$\Phi_x = \Phi_s (A_s F_x / A_x F_s) (n_x/n_s)^2$$

where Φ_s is the quantum yield of ICG, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used, respectively.

4.2.3. MTT Assay

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and glutamine (2 mM) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cellular cytotoxicity of Cy-DNBS towards HepG2 cells were evaluated using the classical MTT assay. An amount of 100 μL of DMEM medium containing 1000–10,000 HepG2 cells was seeded into each well of 96-well plate. Then, Cy-DNBS was diluted to different concentrations with culture medium and gradually added to each well sequentially according to the concentration gradient. The cells were incubated at 37 °C, 5% CO_2 , for 24 h. Next, 40 μL of MTT solution (2.5 mg/mL) was added to each well, and the cells were incubated for another 4 h. After the original medium were removed, 150 μL of DMSO was added to each well and placed on a shaker for 10 min to allow the crystals to dissolve effectively. Finally, the absorbance value of each well at 490 nm was measured in a microplate reader and repeated three times to calculate cell viability.

4.2.4. Cell Imaging

Firstly, to improve the biothiols content of cells, DMEM medium containing exogenous Cys (2 mL, 4 μ M) was incubated with HepG2 cells in the confocal dishes for 30 min. Then, the medium was replaced with Cy-DNBS (2 mL, 4 μ M) in DMEM medium. Meanwhile, the cells were incubated with three commercial organelle dyes (Mito-Tracker, Lyso-Tracker and DAPI, 1.5 mL, 1 μ M) for 30 min, respectively. At last, the cells were washed with 2 mL PBS buffer 3 times before imaging on laser confocal microscope. Green channel ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 500$ –620 nm), Red channel ($\lambda_{\text{ex}} = 633$ nm, $\lambda_{\text{em}} = 680$ –800 nm), Blue channel ($\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 420$ –550 nm).

4.2.5. Imaging of Cy-DNBS towards Endogenous and Exogenous Biothiols

The HepG2 cells were divided into two groups, which were incubated with DMEM medium in the presence and absence of NEM (2 mL, 1 mM) for 30 min. Then, the medium was replaced with DMEM medium containing Cys, Hcy and GSH solution (2 mL, 1 mM), respectively. After culturing the cells for 30 min, the medium was replaced with DMEM medium containing Cy-DNBS (2 mL, 4 μ M) and incubated for another 30 min. At last, the cells were washed with PBS buffer 3 times and placed under confocal fluorescence microscope for direct imaging.

4.2.6. In Vivo Fluorescence Imaging

For in vivo fluorescence imaging of biothiols in tumor, mice were anaesthetized using isoflurane. Cy-DNBS (100 μ L, 50 μ M) was injected into the subcutaneous tumor and normal tissues (control group) of the mice, respectively, and then mice were immediately imaged. As for imaging biothiols in the liver, the mice were divided into two groups, including SAM and PBS Group. Both groups received SAM/PBS (200 μ L, 100 mg/kg) intraperitoneally every two days for two weeks. Then, Cy-DNBS (100 μ L, 50 μ M) was injected by tail vein. After imaging was completed, the mice were sacrificed, and the main organs (heart, liver, spleen, lung and kidney) were harvested and subjected to ex vivo imaging. The fluorescence imaging was performed with a band path of 770–810 nm upon excitation at 740 nm.

4.2.7. In Vivo Photoacoustic Imaging

For in vivo photoacoustic imaging of biothiols in tumor, isoflurane was used to anaesthetize mice during imaging. Cy-DNBS (100 μ L, 50 μ M) was injected into the subcutaneous tumor and normal tissues (control group) of the mice, respectively. Then, mice were immediately imaged. Other operating conditions of PA imaging in the liver are similar to that of fluorescence imaging. The excitation wavelength at 725 nm was selected according to the UV-Vis spectra.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28052229/s1>, Figure S1: Mass spectrum acquired by mixed Cy-DNBS with GSH; Figure S2: The photostability of Cy-DNBS determined by UV-Vis spectrometer; Figure S3: HPLC analysis of the reaction between Cy-DNBS and GSH; Figure S4: linear relationship between fluorescence intensity and concentration of biothiols; Figure S5: histogram of the fluorescence intensity of Cy-DNBS towards various amino acids; Figure S6: histogram of the PA intensity of Cy-DNBS towards various amino acids; Figure S7: The cell viability of HepG2 cells after incubating with Cy-DNBS; Figure S8: the distribution of probes in organelles determined by confocal imaging system; Figure S9: fluorescence intensity of probes was calculated from Figure 3. Figures S10–S19: ^1H NMR, ^{13}C NMR and HRMS of Cy7, Cy-OH and Cy-DNBS.

Author Contributions: W.D.: synthesis, characterization, measurements, data analysis and writing of the manuscript; S.Y.: project design, experiment guide, data analysis and interpretation of results; Y.W. and Y.L.: discussed the results and commented on the manuscript; Y.C.: supervised the project and funding; W.H. and Z.G.: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Natural Science Foundation of China (22122701, 21731004, 21907050, 91953201, 21907044), the Natural Science Foundation of Jiangsu Province (BK20202004), and the Excellent Research Program of Nanjing University (ZYJH004).

Institutional Review Board Statement: The animal experiments in this study were approved by the Institutional Animal Care and Use Committees of GemPharmatech, approval No.: SYXK (Jiangsu) 2018-0027.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the reported results can be found within the article and its Supplementary Materials.

Acknowledgments: We are grateful to the Natural Science Foundation of China (22122701, 22293050, 91953201, 92153303, 21907044), the Natural Science Foundation of Jiangsu Province (BK20202004), and the Excellent Research Program of Nanjing University (ZYJH004).

Conflicts of Interest: The authors declare no conflict of interest.

References

- Balendiran, G.K.; Dabur, R.; Fraser, D. The role of glutathione in cancer. *Cell Biochem. Funct.* **2004**, *22*, 343–352. [\[CrossRef\]](#)
- Kennedy, L.; Sandhu, J.K.; Harper, M.E.; Cuperlovic-Culf, M. Role of Glutathione in Cancer: From Mechanisms to Therapies. *Biomolecules* **2020**, *10*, 1429. [\[CrossRef\]](#)
- Zhang, S.; Ong, C.N.; Shen, H.M. Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells. *Cancer Lett.* **2004**, *208*, 143–153. [\[CrossRef\]](#)
- Ball, R.O.; Courtney-Martin, G.; Pencharz, P.B. The in vivo sparing of methionine by cysteine in sulfur amino acid requirements in animal models and adult humans. *J. Nutr.* **2006**, *136*, 1682–1693. [\[CrossRef\]](#)
- Jouandin, P.; Marelja, Z.; Shih, Y.H.; Parkhitko, A.A.; Dambowsky, M.; Asara, J.M.; Nemazany, I.; Dibble, C.C.; Simons, M.; Perrimon, N. Lysosomal cystine mobilization shapes the response of TORC1 and tissue growth to fasting. *Science* **2022**, *375*, eabc4203. [\[CrossRef\]](#)
- Ozkan, Y.; Ozkan, E.; Simsek, B. Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. *Int. J. Cardiol.* **2002**, *82*, 269–277. [\[CrossRef\]](#)
- Seshadri, S.; Beiser, A.; Selhub, J.; Jacques, P.F.; Rosenberg, I.H.; D’Agostino, R.B.; Wilson, P.W.; Wolf, P.A. Plasma homocysteine as a risk factor for dementia and Alzheimer’s disease. *N. Engl. J. Med.* **2002**, *346*, 476–483. [\[CrossRef\]](#)
- Franco, R.; Schoneveld, O.J.; Pappa, A.; Panayiotidis, M.I. The central role of glutathione in the pathophysiology of human diseases. *Arch. Physiol. Biochem.* **2007**, *113*, 234–258. [\[CrossRef\]](#)
- Hwang, C.; Sinsky, A.J.; Lodish, H.F. Oxidized Redox State of Glutathione in the Endoplasmic Reticulum. *Science* **1992**, *257*, 1496–1502. [\[CrossRef\]](#)
- Armstrong, J.S.; Steinauer, K.K.; Hornung, B.; Irish, J.M.; Lecane, P.; Birrell, G.W.; Peehl, D.M.; Knox, S.J. Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line. *Cell Death Differ.* **2002**, *9*, 252–263. [\[CrossRef\]](#)
- Desideri, E.; Ciccarone, F.; Ciriolo, M.R. Targeting Glutathione Metabolism: Partner in Crime in Anticancer Therapy. *Nutrients* **2019**, *11*, 1926. [\[CrossRef\]](#)
- Ogiwara, H.; Takahashi, K.; Sasaki, M.; Kuroda, T.; Yoshida, H.; Watanabe, R.; Maruyama, A.; Makinoshima, H.; Chiwaki, F.; Sasaki, H.; et al. Targeting the Vulnerability of Glutathione Metabolism in ARID1A-Deficient Cancers. *Cancer Cell* **2019**, *35*, 177–190. [\[CrossRef\]](#)
- Sarris, J.; Murphy, J.; Stough, C.; Mischoulon, D.; Bousman, C.; MacDonald, P.; Adams, L.; Nazareth, S.; Oliver, G.; Cribb, L.; et al. S-Adenosylmethionine (S-AMe) monotherapy for depression: An 8-week double-blind, randomised, controlled trial. *Psychopharmacology* **2020**, *237*, 209–218. [\[CrossRef\]](#)
- Williams, A.L.; Girard, C.; Jui, D.; Sabina, A.; Katz, D.L. S-adenosylmethionine (S-AMe) as treatment for depression: A systematic review. *Clin. Investig. Med.* **2005**, *28*, 132–139.
- Sharma, A.; Gerbarg, P.; Bottiglieri, T.; Massoumi, L.; Carpenter, L.L.; Lavretsky, H.; Muskin, P.R.; Brown, R.P.; Mischoulon, D. S-Adenosylmethionine (S-AMe) for Neuropsychiatric Disorders: A Clinician-Oriented Review of Research. *J. Clin. Psychiatry* **2017**, *78*, e656–e667. [\[CrossRef\]](#)
- Di Padova, C. S-adenosylmethionine in the treatment of osteoarthritis. Review of the clinical studies. *Am. J. Med.* **1987**, *83*, 60–65. [\[CrossRef\]](#)
- Hosea Blewett, H.J. Exploring the mechanisms behind S-adenosylmethionine (S-AMe) in the treatment of osteoarthritis. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 458–463. [\[CrossRef\]](#)
- Lu, S.C.; Mato, J.M. S-adenosylmethionine in liver health, injury, and cancer. *Physiol. Rev.* **2012**, *92*, 1515–1542. [\[CrossRef\]](#)
- Mato, J.M.; Lu, S.C. Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology* **2007**, *45*, 1306–1312. [\[CrossRef\]](#)

20. Mato, J.M.; Corrales, F.J.; Lu, S.C.; Avila, M.A. S-Adenosylmethionine: A control switch that regulates liver function. *FASEB J.* **2002**, *16*, 15–26. [\[CrossRef\]](#)
21. Avila, M.A.; Garcia-Trevijano, E.R.; Martinez-Chantar, M.L.; Latasa, M.U.; Perez-Mato, I.; Martinez-Cruz, L.A.; del Pino, M.M.; Corrales, F.J.; Mato, J.M. S-Adenosylmethionine revisited: Its essential role in the regulation of liver function. *Alcohol* **2002**, *27*, 163–167. [\[CrossRef\]](#)
22. Yan, Y.; Yan, Q.; Qian, L.; Jiang, Y.; Chen, X.; Zeng, S.; Xu, Z.; Gong, Z. S-adenosylmethionine administration inhibits levodopa-induced vascular endothelial growth factor-A expression. *Aging* **2020**, *12*, 21290–21307. [\[CrossRef\]](#)
23. Mora, S.I.; Garcia-Roman, J.; Gomez-Nanez, I.; Garcia-Roman, R. Chronic liver diseases and the potential use of S-adenosyl-L-methionine as a hepatoprotector. *Eur. J. Gastroen. Hepat.* **2018**, *30*, 893–900. [\[CrossRef\]](#)
24. Czarnecka, A.; Milewski, K.; Jaźwiec, R.; Zielińska, M. Intracerebral Administration of S-Adenosylhomocysteine or S-Adenosylmethionine Attenuates the Increases in the Cortical Extracellular Levels of Dimethylarginines Without Affecting cGMP Level in Rats with Acute Liver Failure. *Neurotox. Res.* **2017**, *31*, 99–108. [\[CrossRef\]](#)
25. Zeng, Q.; Guo, Q.; Yuan, Y.; Wang, B.; Sui, M.; Lou, X.; Bouchard, L.S.; Zhou, X. Ultrasensitive molecular building block for biotriol NMR detection at picomolar concentrations. *iScience* **2021**, *24*, 103515–103531. [\[CrossRef\]](#)
26. Dega, N.K.; Ganganboina, A.B.; Tran, H.L.; Kuncoro, E.P.; Doong, R.A. BSA-stabilized manganese phosphate nanoflower with enhanced nanozyme activity for highly sensitive and rapid detection of glutathione. *Talanta* **2022**, *237*, 122957–122966. [\[CrossRef\]](#)
27. Li, Y.L.; Jiang, L.; Zou, Y.Q.; Song, Z.B.; Jin, S.Z. Highly reproducible SERS sensor based on self-assembled Au nanocubic monolayer film for sensitive and quantitative detection of glutathione. *Appl. Surf. Sci.* **2021**, *540*, 148381–148388. [\[CrossRef\]](#)
28. Liu, T.; Chen, S.; Ruan, K.; Zhang, S.; He, K.; Li, J.; Chen, M.; Yin, J.; Sun, M.; Wang, X.; et al. A handheld multifunctional smartphone platform integrated with 3D printing portable device: On-site evaluation for glutathione and azodicarbonamide with machine learning. *J. Hazard. Mater.* **2022**, *426*, 128091–128102. [\[CrossRef\]](#)
29. Qian, Y.; Zhang, L.; Tian, Y. Highly Stable Electrochemical Probe with Bidentate Thiols for Ratiometric Monitoring of Endogenous Polysulfide in Living Mouse Brains. *Anal. Chem.* **2022**, *94*, 1447–1455. [\[CrossRef\]](#)
30. Yan, D.; Liu, L.; Liu, X.; Liu, Q.; Hou, P.; Wang, H.; Xia, C.; Li, G.; Ma, C.; Chen, S. Simultaneous Discrimination of Cys/Hcy and GSH With Simple Fluorescent Probe Under a Single-Wavelength Excitation and its Application in Living Cells, Tumor Tissues, and Zebrafish. *Front. Chem.* **2022**, *10*, 856994–857004. [\[CrossRef\]](#)
31. Umezawa, K.; Yoshida, M.; Kamiya, M.; Yamasoba, T.; Urano, Y. Rational design of reversible fluorescent probes for live-cell imaging and quantification of fast glutathione dynamics. *Nat. Chem.* **2017**, *9*, 279–286. [\[CrossRef\]](#)
32. Fang, H.; Chen, Y.; Wang, Y.; Geng, S.; Yao, S.; Song, D.; He, W.; Guo, Z. A dual-modal probe for NIR fluorogenic and ratiometric photoacoustic imaging of Cys/Hcy in vivo. *Sci. China Chem.* **2020**, *63*, 699–706. [\[CrossRef\]](#)
33. Guo, R.; Huang, F.; Zhang, B.; Yan, Y.; Che, J.; Jin, Y.; Zhuang, Y.; Dong, R.; Li, Y.; Tan, B.; et al. GSH Activated Biotin-tagged Near-Infrared Probe for Efficient Cancer Imaging. *Theranostics* **2019**, *9*, 3515–3525. [\[CrossRef\]](#)
34. Lim, S.Y.; Hong, K.H.; Kim, D.I.; Kwon, H.; Kim, H.J. Tunable heptamethine-azo dye conjugate as an NIR fluorescent probe for the selective detection of mitochondrial glutathione over cysteine and homocysteine. *J. Am. Chem. Soc.* **2014**, *136*, 7018–7025. [\[CrossRef\]](#)
35. Morozumi, A.; Kamiya, M.; Uno, S.N.; Umezawa, K.; Kojima, R.; Yoshihara, T.; Tobita, S.; Urano, Y. Spontaneously Blinking Fluorophores Based on Nucleophilic Addition/Dissociation of Intracellular Glutathione for Live-Cell Super-resolution Imaging. *J. Am. Chem. Soc.* **2020**, *142*, 9625–9633. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Tang, L.; Yu, F.; Tang, B.; Yang, Z.; Fan, W.; Zhang, M.; Wang, Z.; Jacobson, O.; Zhou, Z.; Li, L.; et al. Tumor Microenvironment-Activated Ultrasensitive Nanoprobes for Specific Detection of Intratumoral Glutathione by Ratiometric Photoacoustic Imaging. *ACS Appl. Mater. Interfaces* **2019**, *11*, 27558–27567. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Yang, Q.Q.; Ji, N.; Zhan, Y.; Tian, Q.Q.; Cai, Z.D.; Lu, X.L.; He, W. Rational design of a new near-infrared fluorophore and apply to the detection and imaging study of cysteine and thiophenol. *Anal. Chim. Acta* **2021**, *1186*, 339116–339126. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Huang, J.; Pu, K. Activatable Molecular Probes for Second Near-Infrared Fluorescence, Chemiluminescence, and Photoacoustic Imaging. *Angew. Chem. Int. Ed.* **2020**, *59*, 11717–11731. [\[CrossRef\]](#)
39. Liu, C.; Zheng, X.; Dai, T.; Wang, H.; Chen, X.; Chen, B.; Sun, T.; Wang, F.; Chu, S.; Rao, J. Reversibly Photoswitching Upconversion Nanoparticles for Super-Sensitive Photoacoustic Molecular Imaging. *Angew. Chem. Int. Ed.* **2022**, *61*, e202116802.
40. Wang, C.; Du, W.; Wu, C.; Dan, S.; Sun, M.; Zhang, T.; Wang, B.; Yuan, Y.; Liang, G. Cathepsin B-Initiated Cypate Nanoparticle Formation for Tumor Photoacoustic Imaging. *Angew. Chem. Int. Ed.* **2022**, *61*, e202114766.
41. Qi, J.; Li, J.; Liu, R.; Li, Q.; Zhang, H.; Lam, J.W.Y.; Kwok, R.T.K.; Liu, D.; Ding, D.; Tang, B.Z. Boosting Fluorescence-Photoacoustic-Raman Properties in One Fluorophore for Precise Cancer Surgery. *Chem* **2019**, *5*, 2657–2677. [\[CrossRef\]](#)
42. Ong, S.Y.; Zhang, C.; Dong, X.; Yao, S.Q. Recent Advances in Polymeric Nanoparticles for Enhanced Fluorescence and Photoacoustic Imaging. *Angew. Chem. Int. Ed.* **2021**, *60*, 17797–17809. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Zhu, Y.; Lai, H.; Guo, H.; Peng, D.; Han, L.; Gu, Y.; Wei, Z.; Zhao, D.; Zheng, N.; Hu, D.; et al. Side-Chain-Tuned Molecular Packing Allows Concurrently Boosted Photoacoustic Imaging and NIR-II Fluorescence. *Angew. Chem. Int. Ed.* **2022**, *61*, e202117433.
44. Zhao, Z.; Swartzchick, C.B.; Chan, J. Targeted contrast agents and activatable probes for photoacoustic imaging of cancer. *Chem. Soc. Rev.* **2022**, *51*, 829–868. [\[CrossRef\]](#) [\[PubMed\]](#)

45. Zhang, S.; Chen, H.; Wang, L.; Qin, X.; Jiang, B.P.; Ji, S.C.; Shen, X.C.; Liang, H. A General Approach to Design Dual Ratiometric Fluorescent and Photoacoustic Probes for Quantitatively Visualizing Tumor Hypoxia Levels In Vivo. *Angew. Chem. Int. Ed.* **2022**, *61*, e202107076.
46. Li, H.; Kim, H.; Xu, F.; Han, J.; Yao, Q.; Wang, J.; Pu, K.; Peng, X.; Yoon, J. Activity-based NIR fluorescent probes based on the versatile hemicyanine scaffold: Design strategy, biomedical applications, and outlook. *Chem. Soc. Rev.* **2022**, *51*, 1795–1835. [[CrossRef](#)] [[PubMed](#)]
47. Zeng, Z.; Liew, S.; Wei, X.; Pu, K. Hemicyanine-Based Near-Infrared Activatable Probes for Imaging and Diagnosis of Diseases. *Angew. Chem. Int. Ed.* **2021**, *60*, 26454–26475. [[CrossRef](#)]
48. Qin, Z.; Ren, T.B.; Zhou, H.; Zhang, X.; He, L.; Li, Z.; Zhang, X.B.; Yuan, L. NIRII-HDs: A Versatile Platform for Developing Activatable NIR-II Fluorogenic Probes for Reliable In Vivo Analyte Sensing. *Angew. Chem. Int. Ed.* **2022**, *61*, e202201541. [[CrossRef](#)]
49. Chen, D.; Long, Z.; Sun, Y.; Luo, Z.; Lou, X. A red-emission probe for intracellular biothiols imaging with a large Stokes shift. *J. Photochem. Photobiol. A Chem.* **2019**, *368*, 90–96. [[CrossRef](#)]
50. Li, B.; Zhang, D.; An, R.; Zhu, Y. A 7-Hydroxybenzoxazinone-Containing Fluorescence Turn-On Probe for Biothiols and Its Bioimaging Applications. *Molecules* **2019**, *24*, 3102. [[CrossRef](#)]
51. Lin, X.; Hu, Y.; Yang, D.; Chen, B. Cyanine-coumarin composite NIR dye based instantaneous -response probe for biothiols detection and oxidative stress assessment of mitochondria. *Dye. Pigment.* **2020**, *174*, 107956–107963. [[CrossRef](#)]
52. Maeda, H.; Matsuno, H.; Ushida, M.; Katayama, K.; Saeki, K.; Itoh, N. 2,4-Dinitrobenzenesulfonyl fluoresceins as fluorescent alternatives to Ellman's reagent in thiol-quantification enzyme assays. *Angew. Chem. Int. Ed.* **2005**, *44*, 2922–2925. [[CrossRef](#)]
53. Calabrese, G.; Morgan, B.; Riemer, J. Mitochondrial Glutathione: Regulation and Functions. *Antioxid. Redox Signal.* **2017**, *27*, 1162–1177. [[CrossRef](#)]
54. Hastings, K.T.; Cresswell, P. Disulfide reduction in the endocytic pathway: Immunological functions of gamma-interferon-inducible lysosomal thiol reductase. *Antioxid. Redox Signal.* **2011**, *15*, 657–668. [[CrossRef](#)] [[PubMed](#)]
55. Chen, Z.; Mu, X.; Han, Z.; Yang, S.; Zhang, C.; Guo, Z.; Bai, Y.; He, W. An Optical/Photoacoustic Dual-Modality Probe: Ratiometric in/ex Vivo Imaging for Stimulated H₂S Upregulation in Mice. *J. Am. Chem. Soc.* **2019**, *141*, 17973–17977. [[CrossRef](#)] [[PubMed](#)]
56. Bansal, A.; Simon, M.C. Glutathione metabolism in cancer progression and treatment resistance. *J. Cell Biol.* **2018**, *217*, 2291–2298. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.