

# Evaluation of a dansyl-based amino acid DNSBA as an imaging probe for apoptosis detection

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**Abstract** Imaging agents that enable direct detection of apoptosis are highly desirable in the field of monitoring chemotherapeutic response as well as early diagnosis and disease monitoring. Previous work demonstrated that the dansylated amino acid DNSBA is used to specifically and selectively detect apoptotic cancer cells at the both early and late stages, but the mechanism remains unclear. In this work, we evaluated DNSBA as a tool for monitoring cell apoptosis in CNE1 tumor cell models both in vitro and ex vivo after its in vivo administration, which was confirmed by other assays. The ability of DNSBA to detect multiple pathways and different stages of apoptosis leading

to cell death may be advantageous in the evaluation of cancer treatment indicative of a positive therapeutic outcome. The uptake change of molecular probes DNSBA in CNE1 cells represented the changes of apoptotic rate in a caspase-dependent manner. However, the accumulation of DNSBA in apoptotic cells did not increase with the enhanced membrane permeability. Furthermore, ex vivo study demonstrated DNSBA has a similar pattern as the TUNEL-positive cells. In conclusion, DNSBA cellular imaging is useful for the early assessment of treatment-induced apoptosis, and thus may act as a substitute for Annexin V for assessing treatment response.

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## Introduction

Imaging of biological processes using specific molecular probes allows exploration of the mechanism of action and efficacy for new therapies. Molecular imaging can be used to explore many of the hallmarks of cancer biology, including angiogenesis, proliferation, tissue invasion, evasion of apoptosis, and self-sufficiency in growth signals [1]. As we all know, tumorigenesis is a complex biological process in which dysregulation of apoptosis leads to uncontrolled proliferation and high degree of malignancy. Apoptosis is a phenomenon of programmed cell death, which occurs in particular time and space, tightly regulated by cellular mechanism. Selective induction of tumor cells apoptosis has become one of the main strategies for the treatment of malignant tumors [2, 3]. In addition to a decline in cellular proliferation, many effective treatments also generate an early increase in cell death, particularly

apoptosis. Therefore, strategies that enable *in vivo* imaging of apoptotic process, which is independent of changes in glucose metabolism or cell proliferation, would be of enormous benefit for monitoring therapeutic response [4]. Not unexpectedly, imaging tools capable of monitoring apoptosis have been developed on the basis of a wide range of specific biochemical changes that occur in cell or tissue undergoing apoptosis. To date, several of these molecular steps have been evaluated, such as the detection of phosphatidylserine (PS) exposure at the extracellular face of the plasma membrane by Annexin V; the monitoring of caspase activation in the intracellular compartment through labeled enzyme substrates or inhibitors; and the evaluation of mitochondrial membrane potential collapse via reduced levels of phosphonium cations that normally accumulate in healthy mitochondria. Among them, one commonly used probe of apoptosis is Annexin-V, a protein with high affinity for PS flipped from the inner leaflet of the plasma membrane bilayer to the outer surface during the late stages of apoptosis. Based on this protein, a number of imaging probes for detecting apoptotic cells have been developed, including FITC Annexin-V, Cy5.5 Annexin-V and  $^{99m}\text{Tc}$ -labeled Annexin-V, which allow the detection of apoptosis *in vivo* by radionuclide and optical imaging techniques, respectively [5, 6]. Although the use of Annexin-V based probes offers several advantages, including high affinity to apoptotic cells, ready production by recombinant DNA technology, and its *in vivo* non-toxicity, its application in clinic has been limited unfortunately partly due to the non-specific biodistribution profile, low target/background contrast ratio, slow clearance as well as the cost. Therefore, there is an increasing need for the development of noninvasive molecular imaging strategies to confer quantitative, repetitive and dynamic measurement of detecting quantifying apoptosis status of diseases (especially for cancer) for diagnosis and monitoring treatment response. Accordingly, noninvasive imaging of apoptosis may be valuable in future clinical care for diagnosis of disease, monitoring of disease course, evaluation of treatment efficacy, or assistance in drug development. Since traditional biopsies and *ex vivo* assays from tumor sampling are generally time-consuming, expensive and could yield biased results, it is highly desirable to have non-invasive, *in vivo* imaging test which could bring optimal benefits to patients and further improves their management [7].

Compared with protein probes, small molecules have advantageous clinical translation potentials, including amenable structural optimization and favorable pharmacokinetic properties. To date, a number of small molecules based probes have been developed as an alternative to Annexin-V [8–11]. For example, Ziv et al. reported that dansyl compounds NST-732 can be used for imaging apoptosis process in three experimental, clinically relevant

animal models of: renal ischemia/reperfusion, cerebral stroke, and irradiation treated lymphoma. The NST-732 showed high sensitivity and specificity in targeting apoptotic cells *in vivo* in all three models used. Uptake of NST-732 in apoptotic cells was higher than in the non-apoptotic ones, and the specificity of NST-732 targeting was demonstrated by its localization in regions of apoptotic/necrotic cell death, detected morphologically and by TUNEL staining. Similarly, based on dansyl groups, we designed and synthesized a series of small organic molecular probes in the previous work [12–14]. By means of the structural modification and transformation of the dansyl groups, these molecular probes were found to have good fluorescence properties, selectivity and specificity for the identification and monitoring of apoptosis in breast cancer cell line MCF-7. Recently, we found a novel dansyl-based molecular probe, 4-(5-dimethylamino-naphthalene-1-sulfonamido)-3-(4-iodophenyl)butanoic acid (DNSBA), which possesses the characteristics of targeting apoptotic cells at both early and late stage of apoptosis [14]. The binding of DNSBA with apoptotic cells induced by paclitaxel showed an increasing ratio compared to those without treatment, confirmed by flow cytometric analysis. However, the mechanism of DNSBA for detecting apoptotic cells still remains unclear. In this work, different agents are used for cells apoptosis and three classical apoptotic pathways are established to determine whether DNSBA has been involved in any kind of apoptotic pathways in CNE1 tumor cell models. The favorable results demonstrate the DNSBA has potentiality for disease diagnosis and treatment monitoring, especially in the cases of cancer.

## Materials and methods

### Cell culture and animals

CNE1 cells (human nasopharyngeal carcinoma cells) were cultured in RPMI 1640 medium supplement with 10 % newborn calf serum (NBS) with 5.0 %  $\text{CO}_2$  at 37 °C in a humidified atmosphere of incubator. For cell imaging, cells ( $2 \times 10^4$ ) were plated in 0.5 mL of complete cell growth medium on a Millicell EZ slide and incubated for 24 h. RPMI 1640, NBS were purchased from Gibco (Grand Island, USA).

*Balb/C* mice (8–10 week old, weight 20–25 g) were injected subcutaneously with  $8 \times 10^6$  CNE1 cells per site (1 injection sites/animal) in RPMI 1640. Tumors were allowed to grow for 14 days until established, and animals were then randomly allocated to each treatment group. Tumor appearance was detected and/or growth measured by vernier caliper. The mice were immobilized and subject to deep anesthesia with ketamine (100 mg/kg), then

imaged in animal in vivo fluorescence imager (M-MSI-500-FL, CRIG). All animal studies were performed according to the Guiding Principles for Research Involving Animals, and were approved by the local Animal Care Committee.

#### Induction of cell apoptosis

A series of experiments was performed to evaluate the efficacy of DNSBA in detecting apoptosis in CNE1 cells. The apoptosis of CNE1 cells was induced by exposing the cells to 150 nM paclitaxel, or 200 ng/mL recombinant human TNF- $\alpha$ , or 200  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at 37 °C for 16 h, or 2  $\mu$ M thapsigargin at 37 °C for 24 h. To inhibit apoptosis, CNE1 cells were incubated with 10  $\mu$ M of the pan-caspase inhibitor Z-Vad-fmk at 37 °C for 1 h. Paclitaxel, thapsigargin and Z-Vad-fmk were purchased from Sigma (St. Louis, MO, USA). Recombinant human TNF- $\alpha$  was purchased from R&D Systems (Minneapolis, MN, USA). H<sub>2</sub>O<sub>2</sub> was purchased from Amresco (Solon, OH, USA).

#### Western blotting analysis

Cells ( $1 \times 10^6$ ) were disrupted with cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 2 % SDS, 5 mM DTT, 10 mM PMSF) followed by 30 s ultrasonic dispersion, 10 min boiling water bath for protein denaturation, and 5 min of centrifugation at 13,000 $\times g$  for cell fragment clearance. Proteins (100  $\mu$ g) were separated by SDS-PAGE, and electro-transferred to nitrocellulose membranes. Primary antibody for cleaved caspase-3, caspase-8, caspase-9 and poly ADP ribose polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA, USA).  $\beta$ -actin was purchased from Sigma (St. Louis, MO, USA). After incubation with primary and second antibodies, the processed proteins were stained with a chemiluminescent substrate (Supersignal West Pico Chemiluminescent Substrate, Thermo) according to manufacturer's instruction. Positive bands were exposed and their luminescent intensities normalized to  $\beta$ -actin as an internal loading control.

#### Evaluation of DNSBA binding to apoptotic cells in vitro

For assessment of apoptosis, translocation of PS was detected using the Annexin V-FITC apoptosis detection kit (BD Biosciences, Heidelberg, Germany) and flow cytometry (FCM) analysis (Moflo XDP, Beckman). Apoptotic CNE1 cells were detected by FCM after double staining with Annexin V-FITC and propidium iodide (PI) or DNSBA, respectively. Cells were harvested by centrifugation and followed washed twice in ice-cold PBS. The

cell density was adjusted to  $1 \times 10^6$  cells/mL using PBS and those cells were then resuspended in  $1 \times$  binding buffer. After addition of 5  $\mu$ L of Annexin V solution and 5  $\mu$ L of PI solution or 50  $\mu$ M of DNSBA solution per 100  $\mu$ L cells, cells were incubated for 15 min at room temperature in the dark. Thereafter, 400  $\mu$ L of  $1 \times$  binding buffer were added, and 10,000 cells of each sample were analyzed by FCM. Unstained cells, cells stained with Annexin V alone, and cells stained with PI alone were used as controls to adjust channel compensation.

#### Ultrasound treatment

For ultrasound exposure experiments, cells were harvested from logarithmically growing suspension cultures, pelleted, and resuspended in fresh RPMI1640 10 % NBS. Cells were then mixed in sterile 60 mm dish with an equal volume of RPMI1640 and 10 % NBS in order to control CNE1 Cell concentrations roughly  $5 \times 10^5$  cell/mL. Final calcein acetoxymethyl (calcein-AM) concentration was adjusted to 0.5  $\mu$ M, and dishes were incubated at 37 °C for 30 min and protected from light before ultrasound irradiation. The dishes containing the cells were fixed at the center of a sonication bath (Sonicator Q700, Misonix) for each ultrasound irradiation (20 kHz). The ultrasound bath was  $10 \times 10$  cm in size with 5 cm water depth from the acoustic generating source at the bottom of the container to the sample tube near the water surface. The coupling water was degassed before ultrasound treatment and temperature was maintained at 37 °C during irradiation. Cells were subsequently centrifuged, washed with PBS, resuspended in 500  $\mu$ L cold PBS, then 5  $\mu$ L of DNSBA probe solution was added and cells were gently blending and incubated for 5–10 min at room temperature in the dark. The cell membrane permeability (stained with Calcein-AM) was evaluated by flow cytometric under ultrasound with acoustic intensity of 1.88 W/cm<sup>2</sup> for 2 and 3 min. The rate of cells with increased cell membrane permeability as function of acoustic intensity (1.88 W/cm<sup>2</sup>) and exposure duration (30 s, 1, 2 and 3 min) was evaluated by FCM.

#### Immunofluorescence staining, TUNEL assay and fluorescence microscopy

For microscopic analysis and fluorescence imaging, 5  $\mu$ m-thick sections of paraffin-embedded transplanted tumor tissue were evaluated either by fluorescence microscopy or by light microscopy analysis with hematoxylin and eosin (HE) staining for apoptotic and/or necrotic lesion distribution. Furthermore, the presence of apoptotic cell nuclei was confirmed by In Situ Cell Death Detection Kit staining, using the TUNEL method (terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end

labeling). The prolonged gold antifade mounting solution containing 4',6-diamidino-2-phenylindole (DAPI) was added to tissue sections prior to coverslips mounting. All assays were performed according to the manufacturer instructions. Sections were counterstained with HE staining. In Situ Cell Death Detection Kit was purchased from Roche (Basel, Switzerland). Prolong gold antifade reagent was purchased from Invitrogen (Grand Island, NY, USA).

### Statistical analysis

Group variations were shown as mean  $\pm$  standard deviation (SD). Statistical analyses were performed to compare the uptake values of DNSBA at different treatments (Z-Vad vs. paclitaxel) in the CNE1 cell. Mean uptake values were compared using 1-way ANOVA. *p* value less than or equal to 0.05 was considered statistically significant.

## Results

### Detection of apoptosis using DNSBA

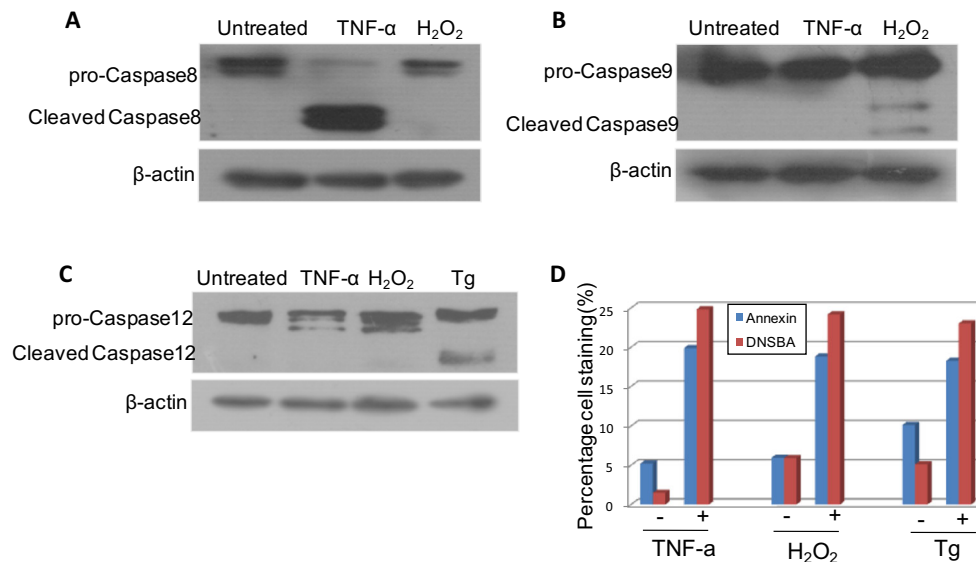
Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs or radiotherapy kill tumor cells. The mitochondrial (intrinsic) pathway, the death receptor (extrinsic) pathways and the endoplasmic reticulum (ER) stress pathways are three of the common cell apoptotic pathways [15, 16]. Most signaling pathways activated by induce agents ultimately result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death. Furthermore, different agents were used for cell apoptosis-inducing and three classical apoptotic pathways were established to determine whether DNSBA had been involved in selectivity of apoptotic pathways in tumor cells. The detection of the extrinsic and the intrinsic apoptotic pathways are determined through the levels or activity of caspase-8 and caspase-9, respectively. TNF- $\alpha$  and H<sub>2</sub>O<sub>2</sub> have been chosen as the extrinsic and the intrinsic apoptotic pathways induce agents, respectively. Then we therefore treated CNE1 cells with thapsigargin (Tg), a selective inhibitor of SERCA (seroplasmic endoplasmic reticulum calcium ATPase) and analyzed the levels of caspase-12 proteins only by western blotting. Interestingly, an increased cleavage in caspase-12 protein level is not exposure to the TNF- $\alpha$  or H<sub>2</sub>O<sub>2</sub> group, only upon the treatment of thapsigargin (Fig. 1 a–c). Annexin V/PI double staining and DNSBA were used to detect apoptosis in CNE1 cells of three classical apoptotic pathways (Fig. 1d). The percentage of apoptotic cells was changed with the corresponding concentration of apoptosis inducers.

### Molecular probe DNSBA labeled cells and xenograft tissue display apoptosis

The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment [16]. Annexin V-FITC staining precedes the loss of membrane integrity which accompanies the latest stage of cell death resulting from either apoptotic or necrotic processes [17]. However, one major disadvantage of Annexin V conjugations is its low specificity to apoptotic cells, which was attributed to natural PS turnover observed in non-apoptotic cells.

To explore the apoptotic mechanism of DNSBA, the subcellular localization in apoptotic cells of probe DNSBA was investigated by confocal microscopy. For comparison, Annexin V-FITC labeling was used as it is the gold standard for apoptotic detection. CNE1 cells were incubated with paclitaxel (150 nM) for 16 h then Annexin V-FITC staining for 20 min. The staining pattern was evaluated under a fluorescence microscope equipped with relevant filters. Fluorescence images of the sections were acquired using a Leica 3000B epifluorescence microscope with the appropriate excitation (340/80 and 450/90 nM) and emission (435/85 and 500/50 nM). CNE1 cells were stained with Annexin V-FITC (green color in Fig. 2a) as a positive control. Simultaneously, the cells were stained with PI (red color in Fig. 2a) to display the nuclei area. The fluorescent images were visualized with a laser scanning confocal microscope. When three images were merged, blue signals indicated the localization of probe DNSBA was in the nucleus and cytoplasm. The cytoplasm of apoptotic CNE1 cells was induced by paclitaxel and an accumulation of DNSBA in nucleus was observed (Fig. 2a). DNSBA marker-positive and -negative cells were collected by FCM sorting after 150 nM paclitaxel treatment. The caspase-3 protein expression levels were shown in Fig. 2b, it was observed that sorting-out probe labeled negative cells could not detect the cleaved caspase-3. Additionally, DNA ladder assay showed DNA breakage occurred in the sorting DNSBA labeled positive cell (Fig. 2c).

In consideration of the spectrum of probe DNSBA in visible UV region, the *in vivo* studies can only be carried out in tissue sections level to evaluate the efficacy of probe DNSBA. The xenograft bearing nude mice were employed to evaluate the apoptosis detection of DNSBA in tumor tissue *in vivo*. The uptake of molecular probe DNSBA was significantly increased in paclitaxel treated tumor tissues



**Fig. 1** Selectivity analysis of DNSBA for different targeting tumor cells apoptosis pathways **a, b, c** CNE1 cells were treated as indicated and later were harvested in lysis buffer as described in experimental procedures. Analysis for expression of caspases in CNE1 cells treated with apoptosis inducers. Changes in cleaved caspase-8/9/12 levels

were measured by western blot analysis.  $\beta$ -actin was used as loading control. CNE1 cells were treated for 24 h with either 200 ng/mL TNF- $\alpha$  or 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 2  $\mu$ M thapsigargin (Tg) or untreated. **d** Annexin V-FITC or DNSBA labeling assay in CNE1 cells treated with apoptosis inducers by FCM

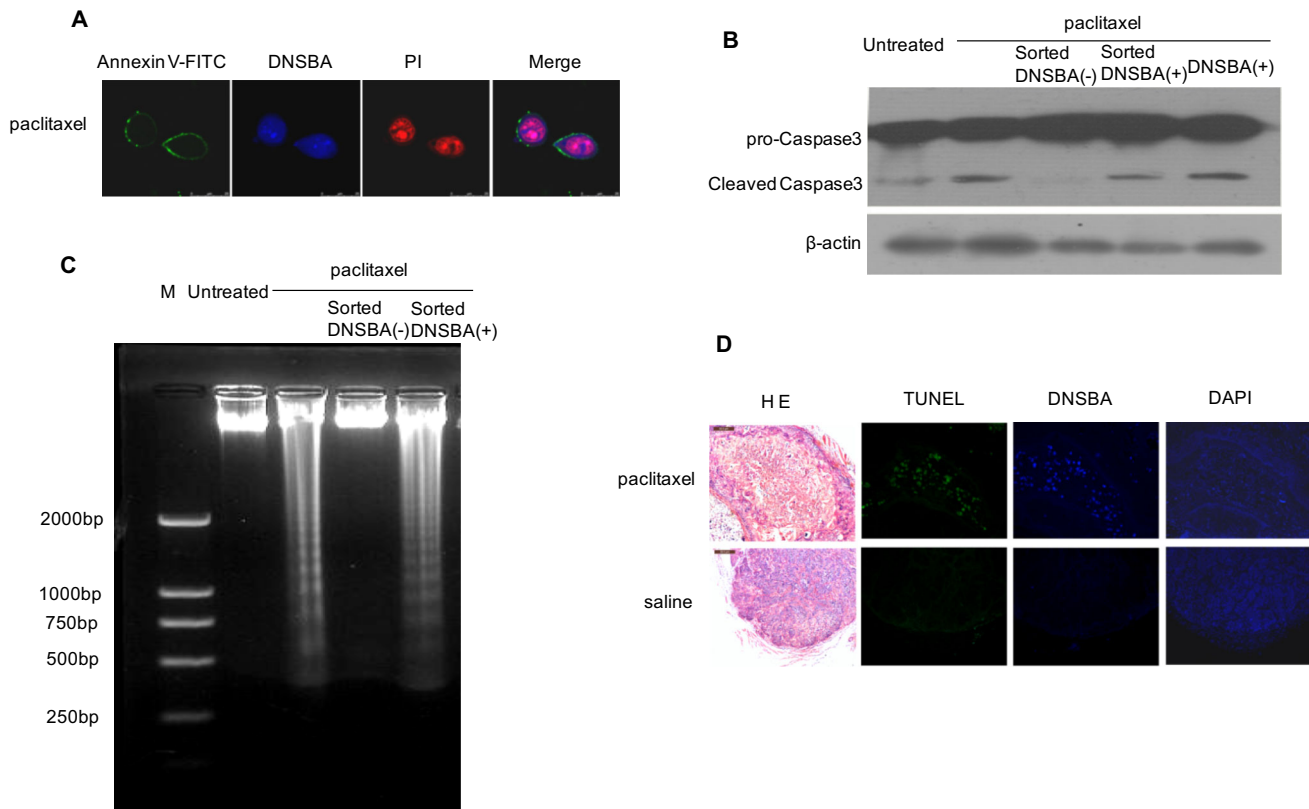
when compared with the normal saline-treated group. The result is similar to the positive position of TUNEL assay detection. DNSBA targeting apoptotic and dying cells within the xenograft bearing nude mice was demonstrated by fluorescent histological analyses of excised tumor tissue. Each tumor tissue was paraffin embedding and sliced into 5  $\mu$ m thick sections, and stained with TUNEL and DAPI. Tumor tissue sections from mice that were treated with paclitaxel showed a much stronger DNA strand break signal than that from non-treated mice, indicating the increased amounts of cell death (Fig. 2d). Furthermore, the evaluation of the same tissues by fluorescence microscopy showed that the regions solely stained by DNSBA displayed high TUNEL signal. The number of TUNEL-positive cells dramatically increased in the tissue section of paclitaxel-treated mice in comparison of that in the normal saline-treated mice. Therefore, DNSBA demonstrated a similar pattern for the TUNEL-positive cells and these two signals were colocalized (Fig. 2d).

#### Analysis of molecular probe DNSBA label rate and uptake

Generally, the target-background ratio is dependent on the signal strength of molecular probe binding to the targets. Considering the experiments results obtained from FCM, it is observed that the average fluorescence intensity of DNSBA uptake changed with different apoptosis level. First, after staining by Annexin V-FITC, paclitaxel

induced cells were sorted to positive and negative cells by FCM. Second, the parallel groups were labeled with DNSBA and assayed by FCM (Fig. 3a). There is a marked fluorescence enhancement in sorted Annexin V-FITC labelled positive cells (apoptotic cells, dark blue line). An apparent fluorescence decrease was observed in the sorted negative cells of Annexin V-FITC labeled, and its fluorescence intensity restored to the status of the control cells. Then, the positive rate in the corresponding two groups was compared. In CNE1 cells untreated group and paclitaxel induced molecular probe DNSBA markers positive rate were 1.07 and 33.85 % respectively. Paclitaxel induced cell apoptosis, Annexin V-FITC labeled apoptotic cells by FCM sorting, the molecular probe DNSBA markers positive rate were 0.14 and 84.25 % respectively, in Annexin marked negative and positive cells. According to our data, the uptake change of molecular probes DNSBA in CNE1 cells represented the changes of apoptotic rate, which provided strong experimental evidence for the application of the molecular probe DNSBA. As seen in Fig. 3b, the uptake of DNSBA in the paclitaxel-treated CNE1 cell was significantly higher than that untreated CNE1 cell ( $p = 0.0111$ ), and the highest amount of DNSBA accumulation has been observed in the paclitaxel-treated associated with Annexin V-FITC stained positive cells. Uptake of DNSBA was also higher than the paclitaxel-treated Annexin V-FITC stained negative cells, which is attributed to DNSBA targeting the cell death in CNE1 cell.





**Fig. 2** Cells and xenograft tissue display apoptosis labeled with probe DNSBA molecular probe DNSBA accumulate nuclear and cytoplasm of apoptotic CNE1 cells induced by paclitaxel. CNE1 cells were stained with annexin V-FITC (*green*) as positive control. Simultaneously, the cells were stained with propidium iodide (*red*) to display the nuclei. The fluorescent images were visualized with a laser scanning confocal microscope. *Scale bar* = 25  $\mu\text{m}$ . **b** Western blot analysis of caspase-3 cleavage in sorting DNSBA labeled CNE1 cells.  $\beta$ -actin is shown as a loading control. **c** Photograph of a 2 %

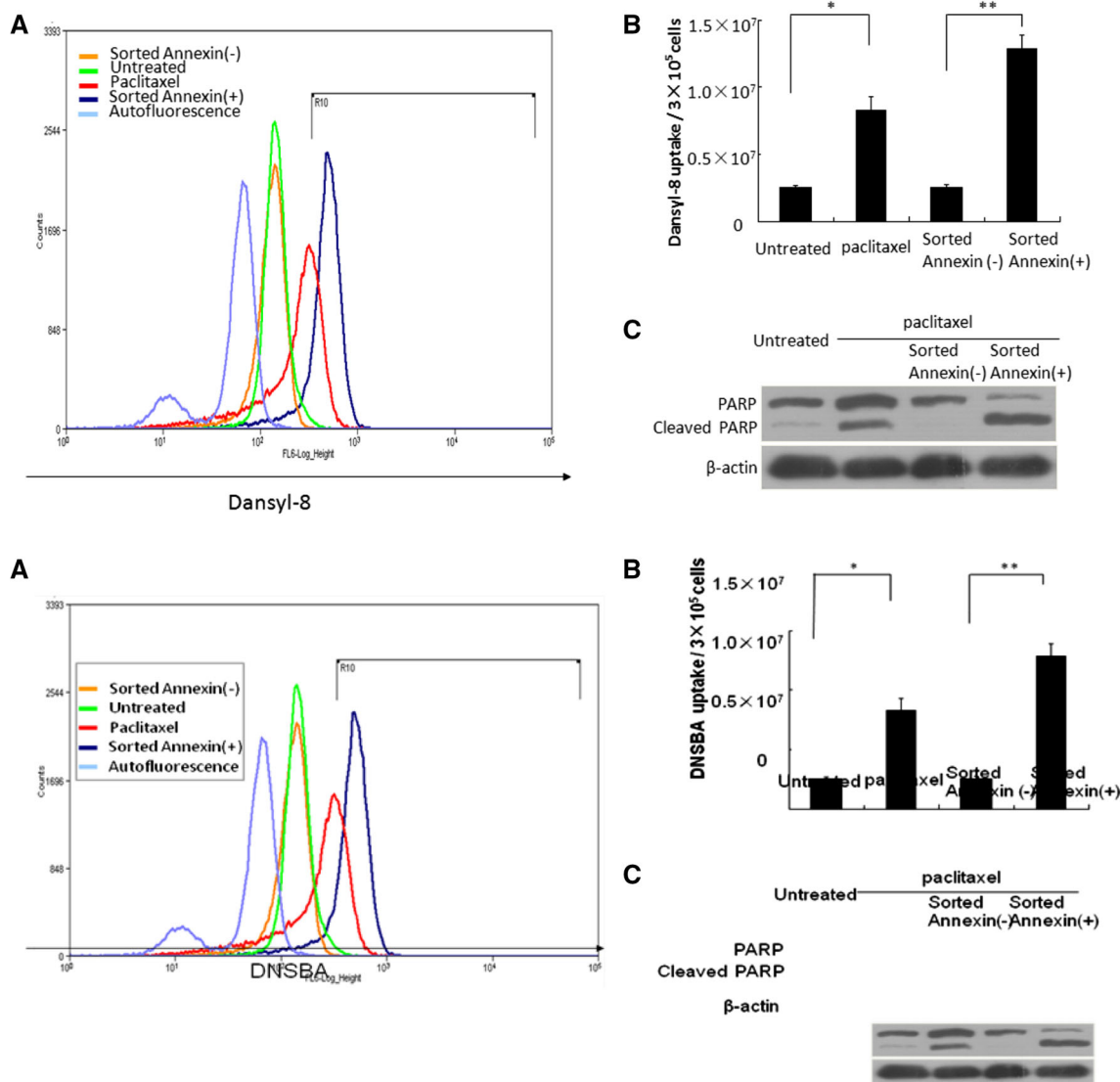
agarose gel electrophoresis of DNA extracted from CNE1 cells. CNE1 cells were pretreated with paclitaxel (150 nM) then sorted by FCM. **d** Tumor tissues processed for histological analysis and stained for TUNEL assay and molecular probe DNSBA detection. Mice were treated with a dose of either paclitaxel (30 mg/kg) or saline delivered at 24 h before sacrificed. The 5  $\mu\text{m}$  thick xenograft histological sections were counterstained with DAPI and TUNEL. Other serial sections were stained with DNSBA. *Scale bar* = 50  $\mu\text{m}$

### Molecular probe DNSBA uptake is dependent on the activation of caspase

One of the key factors in apoptosis is a family of cysteine-dependent aspartate-directed proteases (caspase). In the western blot experiments, Z-Vad-fmk treatment can inhibit the occurrence of apoptosis, and caspase-3 and PARP spliceosome components obviously reduced (Fig. 4a). In CNE1 cells, the occurrence of paclitaxel-induced apoptosis and cells uptake of molecular probe DNSBA was significantly increased (count  $3 \times 10^5$  cell fluorescence value), compared with the untreated group with remarkable differences ( $p < 0.05$ ). The addition of Z-Vad-fmk, a pan-caspase inhibitor, led to a significant decrease in uptake of DNSBA, compared with paclitaxel treatment group with significant differences ( $p < 0.05$ ) (Fig. 4b).

### Effect on molecular probe DNSBA label in cell membrane permeability enhanced model

Calcein AM can be utilized to detect the changes in cytoplasmic membrane permeability that occur in response to low intensity ultrasound. We then examined the status of cell membrane permeability by monitoring the population of calcein labeled cells with ultrasound treatment at different times (Fig. 5a) and by detecting the effect of DNSBA probe using flow cytometric analysis (Fig. 5b–c). The R2/R3 represented the percentage of calcein fluorescent (cell membrane permeability) cells and geometric mean of fluorescence intensity of membrane-permeable cells. The R4 was marked to show the percentage of DNSBA fluorescent cells. It was observed when ultrasonic irradiates, calcein fluorescent through the cell membrane increased, and the



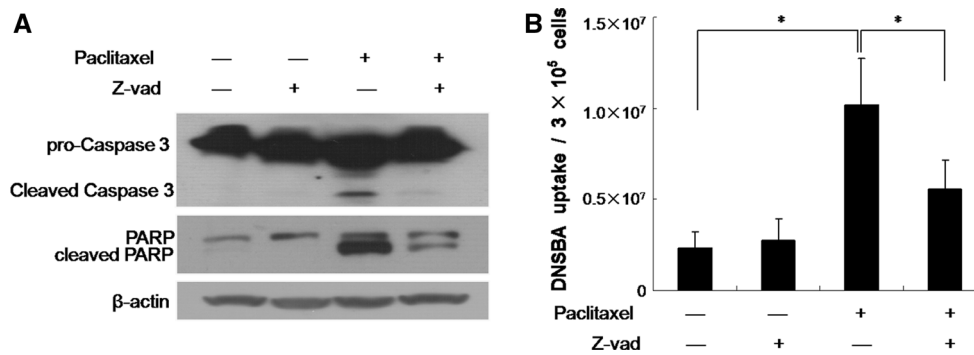
**Fig. 3** Analysis of sorting Annexin V-FITC labeled cells changes in DNSBA label rate and uptake **a** The histogram depicts the number of cells (*Y* axis) versus DNSBA fluorescence intensity (*X* axis). **b** The uptake fluorescence intensity of sorted annexin labeled CNE1 cells treated with paclitaxel. Molecular probe DNSBA of cells uptake increased significantly after paclitaxel treated ( $p = 0.011103$ ).

percentage of cells with fluorescence signals changed in a time-dependent increase manner. However, DNSBA labeled cells had no change significantly, which suggested that the cellular uptake of DNSBA did not improve with the increase of membrane permeability. Probe DNSBA showed a promising membrane permeable property, which could be attributed to its small molecule structure. To further get insight into the relationship between the changing of the permeability of cell membranes and the accumulation of DNSBA in apoptotic cells, a permeability enhancement mode of cell membrane was established and used for evaluating probe uptake. However, the cellular uptake of DNSBA has no

significant increase when the membrane permeability enhanced. Moreover, we also observed that DNSBA could label cells at a greater rate in the presence of apoptosis-inducing agent; this phenomenon further demonstrated that the molecular probe DNSBA shows great specificity to detect the apoptotic cells.

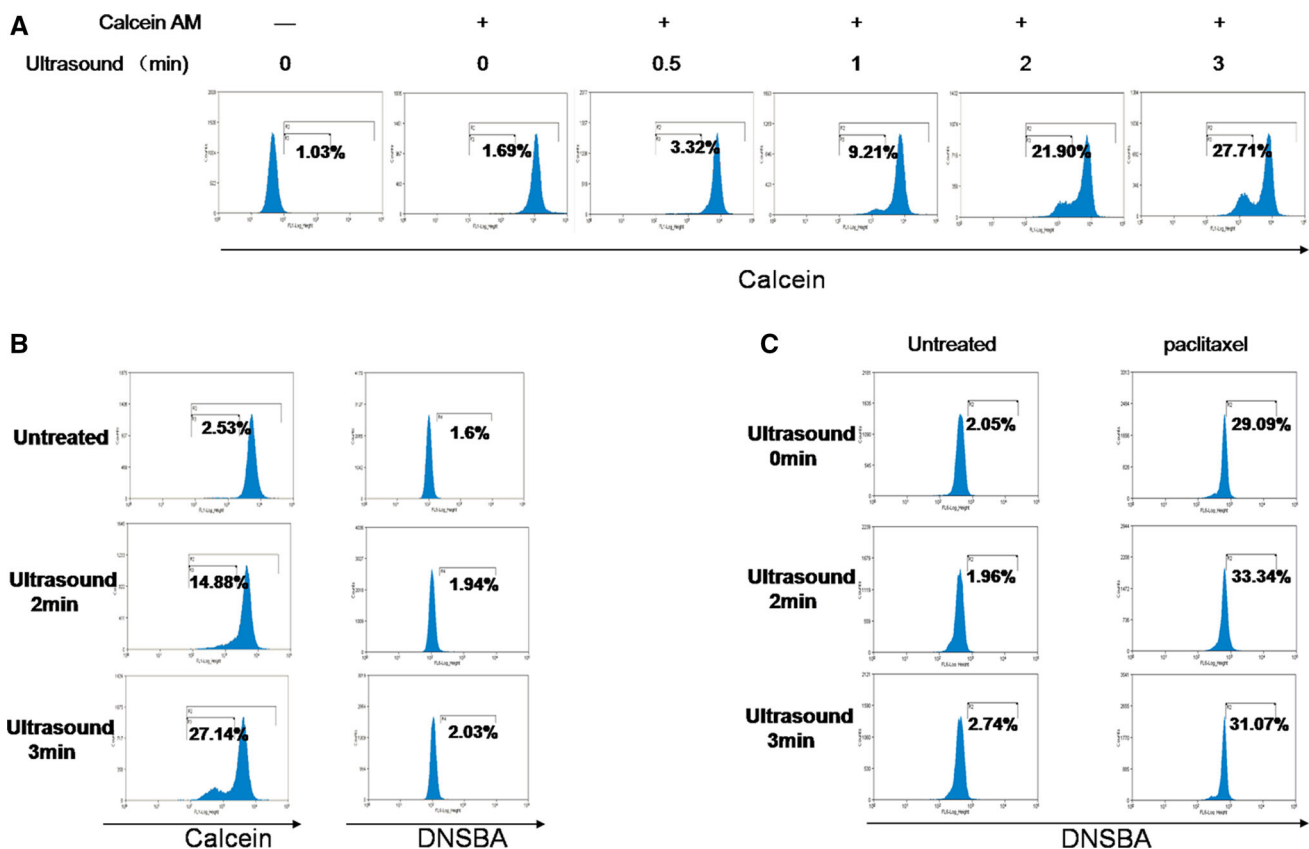
## Discussion

Recently, a number of small molecules based probe has been developed as alternatives to Annexin-V. In our



**Fig. 4** The uptake of DNSBA is dependent on the activation of caspase **a** The histogram shows the uptake fluorescence intensity of  $3 \times 10^5$  cells. CNE1 cells were incubated with paclitaxel in the presence or absence of the pan caspase inhibitor Z-Vad-fmk, DNSBA uptake was determined by FCM. Data are shown as mean  $\pm$  SD. The

untreated group has significant difference compared with the paclitaxel treatment group ( $p = 0.02603$ ). **b** Immunoblot detection of caspase3 and PARP cleavage in CNE1 cells treated with paclitaxel in the presence of the Z-vad.  $\beta$ -actin is shown as a loading control.  $*p < 0.05$



**Fig. 5** Analysis of DNSBA label in cell membrane permeability enhanced model **a, b** FCM analysis of Calcein and molecular probe DNSBA labeled cells with ultrasound treatment for the indicated times in CNE1 cells. Numbers represent the percent of cells in the fraction. **c** FCM analysis of molecular probe DNSBA labeled cells untreated or treated with paclitaxel (150 nM), and ultrasound

treatment for the indicated times in CNE1 cells. R2/R3 was marked to show the percentage of calcein fluorescent (cell membrane permeability) cells and geometric mean of fluorescence intensity of membrane-permeable cells. R4 was marked to show the percentage of DNSBA fluorescent cells

previous studies, dansyl derivant was chosen as the fluorophore to develop imaging probes because of its desirable spectroscopic properties [21]. A series of probes were

developed based on dansyl derivatives and were able to selectively and specifically identify apoptotic breast cancer cell MCF-7. For example, DFNSH was accumulated in the



cytoplasm at different stages of apoptotic cells while Annexin V binds to the membrane surface. The proposed mechanism involved in the intracellular uptake of DFNSH may be related, in part, to the complex membrane alterations that occur in apoptotic cells [14]. A large number of pre-clinical and clinical data showed that the peak effect of radiation and chemotherapy treatment strategy mediated apoptosis occurs in the first 24 h of therapy. Therefore, using a non-invasive imaging technique, real time monitoring of apoptosis induced by treatment, evaluation of individualized treatment strategies is particularly important. Some targeted apoptotic probes had been designed and synthesized based on the biological characteristics of a specific target such as the Annexin V derivatives, caspase inhibitors/substrate conjugations with the well-known mechanism at the beginning of the design [18–20].

Among these available probes for imaging apoptosis, DNSBA is an organic small molecule with good hydrophilicity and chemical stability and is a potential multimodal imaging probe due to its chemical variability. To understand its molecular mechanism for imaging apoptosis, we examined the performance of DNSBA as a tool for monitoring cell apoptosis in various cancer cell models. Detection of cell apoptosis by DNSBA was examined in optical studies on CNE1 cells both *in vitro* and *ex vivo* after its *in vivo* administration. *In vitro*, DNSBA manifested selective uptake and accumulation within apoptotic cells that was highly correlated with Annexin-V binding, and caspase activation. DNSBA selectively targeted cells undergoing apoptosis in nasopharyngeal tumors tissue, while not binding to viable tumor cells. Chemotherapy caused remarkable increase of tumor cell apoptosis, which were in correlation with the increased DNSBA uptake. These data confirmed the usefulness of imaging of cell-death by DNSBA as a tool for early monitoring of tumor response to anti-cancer therapy. In addition, the possibility of DNSBA imaging as the monitoring tool of the therapeutic effect of anti-tumor drugs was evaluated.

Consequently, considerable efforts have been made in developing noninvasive imaging methods to evaluate apoptosis. Although bioluminescence imaging (BLI) in small animal imaging has shown great potential, the application of the human body imaging was limited by the body's complex surface structure and the spatial distance of the tissues and organs due to the limitations of the signal penetration. However, current optical application or equipment is mainly limited by poor sensitivity and specificity. A subset of molecular imaging contrast agents known as “activatable” or “smart” probes provide higher signal-to-background ratio compared to conventional targeted contrast agents [22]. Since positron emission tomography (PET) and single photon emission computed tomography (SPECT) are considered to be the most

sensitive methods in various imaging modalities, radiolabelled by isotopes for PET and SPECT imaging therefore also attracted much attention. It was reported that a promising new imaging PET apoptosis tracer,  $^{18}\text{F}$ -ML-10, was approved for phase II clinical trials. However, the proposed apoptosis detection mechanism is still unclear [23, 24]. Several agents with high selectivity and specificity for activated caspase-3 currently have been developed as positron emitting radiopharmaceuticals for imaging of apoptosis in clinic. For example,  $^{18}\text{F}$ -ICMT-11 used as a caspase-3-specific imaging radiotracer is currently progressing to clinical trial [25]. [ $^{18}\text{F}$ ]-CP18 was cleaved by activated caspase-3, taken up by apoptotic tumor cells and rapidly cleared from non-target tissue, and showed a superior imaging profile [26, 27].

As a witness of recent progress in medicine, biology, and physics, the field of diagnostic imaging is shifting from conventional anatomical imaging to the sphere of functional and molecular imaging, with the aim of imaging biological processes related to both health and disease. In this study, we evaluated the feasibility of using DNSBA, a specific apoptosis-targeting imaging agent, as a probe for molecular imaging in three classical apoptotic pathways cell models characterized by different apoptosis-inducing agents. The three experimental models of apoptosis pathways used in the present study complemented each other regarding the applicability of DNSBA technology in the detection of apoptosis. The ability to detect multiple pathways or even all apoptotic stages gave rise to some advantages in the evaluation of cancer treatment response. The implementation of the new molecular probe DNSBA technology, for *in vivo* imaging of apoptosis, would represent a major addition to this emerging field. It is highly desired to develop potential non-invasive imaging of cell death processes *in vivo* as means to both the diagnosis of disease and monitoring of treatment efficacy in a broad spectrum of disease states. An inherent fluorescent property of DNSBA was utilized for detection of cancer cell death in three different apoptosis pathways of apoptosis (Figure 1, 2). Those results in accordance with *in vitro* specific detection of DNSBA towards apoptotic cells suggested DNSBA has the capability to imaging and monitor cancer apoptotic cells *in vitro*. Additionally, the probe provides quantitative analysis of the extent of cancer cell damage based on the extent of DNSBA accumulation in these cells (Fig. 3a). However, in consideration of the photospectrum of probe DNSBA visible in UV region, the *in vivo* studies can be carried out in both cells and tissue sections level. The uptake of probe DNSBA significantly increased in paclitaxel treated tumor tissues, which is similar to the positive position of TUNEL assay detection (Fig. 2d). Histological analysis of formalin fixed tumor tissues showed that paclitaxel treatment significantly increased

apoptosis based on increased apoptotic bodies stained with HE, and increased fragmented DNA staining. These results suggested that DNSBA can be considered as a good pharmacodynamic candidate for imaging apoptosis in tissue. It suggested that the molecular probe has characteristics of targeting apoptotic cells in tissue sections and provides strong experimental evidence for in-depth study of the precursor synthesis multimode probe, also brings out new approach to transforming DNSBA into multi-modal apoptotic imaging probe.

This study demonstrated that DNSBA may provide a sensitive mean for real-time diagnosis and/or monitoring of the extent of apoptosis in cancer, utilizing selective targeting, binding, uptake, and accumulation within tumor tissue apoptotic and/or necrotic cells. Moreover, as a non-invasive method, DNSBA imaging would allow longitudinal studies in a single individual, rendering important information on the optimal timing and dosing of drugs and on the efficacy of therapeutic interventions.

## Conclusion

Built on model of nasopharyngeal carcinoma cells CNE1, we designed and screened molecular probes targeting apoptosis in tumor cells. Based on the study of different apoptotic pathways and tumor cells, we established apoptotic molecular imaging detection platform. Meanwhile, we proved DNSBA detection of apoptosis has covered three types of apoptotic pathway and phases of apoptotic cell and tissue levels. It provided meaningful experimental evidence for DNSBA as a novel apoptotic molecular probe. In our study, we successfully introduced a novel molecular imaging probe DNSBA for noninvasive apoptosis detection. The biological evaluation of DNSBA showed that this probe selectively binds to paclitaxel-induced apoptotic cancer cells, and exhibits intracellular uptake and accumulation in apoptotic cells. DNSBA has the ability of detecting multiple pathways resulting in cell death, and may be advantageous in the evaluation of cancer treatment, particularly indicative of a positive therapeutic outcome.

In conclusion, this study showed that DNSBA is a useful probe with the potential to address the preclinically important goal of the early detection of tumor response to radiotherapy. Ongoing studies in multiple tumor types are now being performed. It is hoped that DNSBA may serve as a molecular imaging probe for noninvasive imaging of cell death for the prediction of tumor response, thereby assisting in the transition to a more personalized approach in oncology. Further ongoing studies are focused on applying apoptosis imaging of DNSBA into monitoring treatment response of various chemotherapeutic agents and

radiotherapy. DNSBA cellular imaging is useful for the early assessment of treatment-induced apoptosis and, thus, may be used as a substitute for Annexin V for assessing treatment response.

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