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# A novel cancer imaging method using chemi-luminescence-mediated sonosensitization

HE Yonghong, XING Da & WANG Juan

Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China
Correspondence should be addressed to Xing Da (e-mail: xingda@scnu.edu.cn)

Abstract **In this paper, we introduce a novel method named chemiluminescence mediated sono dynamic diagnosis (SDD), which is designed as follows: sonosensitizer is used to localize the cancer tissue, and is sonosensitized by the ultrasound field to produce oxygen free radicals. Then, another agent, FCLA (fluoresceinyl cypridina luciferin analog), reacts with $^1O_2$ to efficiently transform the chemical energy of $^1O_2$ to photons, thus a strong chemiluminescence is emitted. One can then detect this emission with a high sensitive CCD imaging system to localize the tumor. Based on the principle of chemiluminescence mediated SDD, we obtained a clear diagnostic image of a transplanted tumor in a nude mouse in the experiments. This method could have potential applications in clinics for early stage tumor diagnosis.**

**Keywords: cancer diagnosis, sonodynamic, chemiluminescence.**

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Early stage cancer diagnosis attracts more and more attention in recent years. Up to now, cancer diagnosis in clinics depends on the combination of several techniques, such as pathological diagnosis, CT or MRI imaging, and biochemical detection of cancer indicators.

Being noninvasion, nonionization, and functional contrast for bio-medical diagnosis, optical imaging of tissues has become an active research field[1]. Photodynamic diagnosis, also named laser induced drug fluorescence technique, as an early stage cancer diagnostic method was developed rapidly in the 1970s. But there are still some serious limitations because the excitation laser wavelength at the main absorption peak of the photosensitizer (e.g. HpD) is short (near to 400 nm). The first problem is autofluorescence disturbance. There are a large number of chromophores in biological tissues, and these chromophores are excited by the short wavelength laser to emit strong fluorescence disorderly, which greatly disturbs the detection of the emission from photosensitizer. The second one is the problem of excitation depth, short wavelength light has poor penetration ability.

Umemura et al.[2,3] have reported the synergistic effect of ultrasound and hematoporphyrin or gallium-porphyrin derivative ATX-70 on tumor treatment. Compared with "photodynamic therapy", this phenomenon has been termed "sonodynamic therapy (SDT)" and attracts more and more attention[4–8]. SDT appears to be a promising modality for cancer treatment, for ultrasound can penetrate deeply within the tissue and can be focused in a small region of tumor to chemically activate relatively non-toxic molecules thus minimizing undesirable side effects[4].

Recently, we have reported a sonodynamic chemiluminescence (CL) technique[7] and the CL imaging *in vivo* technique[9,10]. Based on these techniques, we use ATX-70, one of the most active sonodynamic agents found[5,8], as sonosensitizer to produce sonodynamic CL for imaging cancer *in vivo* in this work. In *in vivo* experiments, the tumor region of a tumor-bearing mouse was imaged with great contrast during sonosensitization of ATX-70 with FCLA as the CL probe[11]. The results indicate that a novel cancer imaging method, we name it sonodynamic diagnosis (SDD), could be established by sonodynamic action and CL technique.

## 1 Materials and methods

The experimental apparatus is shown as Fig. 1. The setup was similar to that described in detail in our previous study[7] except that a band-pass filter was put in the front of the CL detectors. In short, an ultrasonic transducer was driven by a sinusoidal signal from a function generator (Tektronics, AFG320) and a power amplifier (ENI Co. Ltd, 2100L) to produce a column ultrasound field, which passes through distilled water with black ink to reach the sample for sonodynamic action. The sonodynamic CL light was detected by a photomultiplier tube (PMT) or a cooled intensified charge coupled device (CCD, Princeton Instruments, ICCD-576-S/1) detector. A band-pass filter is centered at 530 nm with a bandwidth of 30 nm, which was used to selectively detect FCLA chemiluminescence to improve image contrast. The PMT worked in photon counting manner was controlled by a computer with a self-designed LabVIEW software (National Instrument)

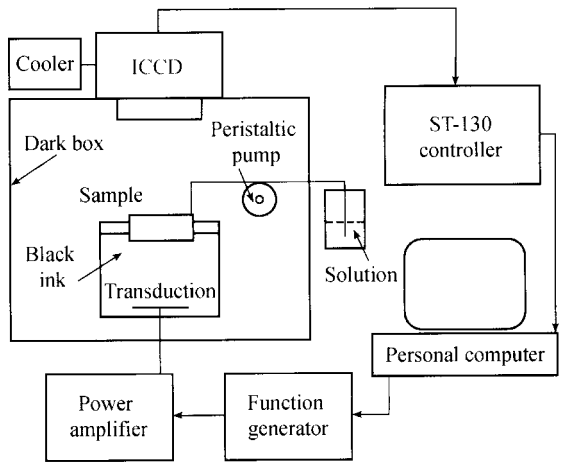


Fig. 1. A schematic diagram of experimental setup.

through an I/O card. A ST-130 controller controls data acquisition of the CCD and transfers the data to the computer in which the digitized image is processed by WINVIEW software.

FCLA, purchased from Tokyo Kasei Kogyo Co. Ltd., was dissolved in double-distilled water and stored at –80℃ until needed. Sonosensitizer ATX-70, purchased from Toyohakka Kogyo LTD. Japan, was diluted in 0.01 mol/L phosphate buffer saline (PBS, pH 7.4) solution before experiments. Cu-Zn superoxide dismutase (SOD, from bovine erythrocytes) and human serum albumin (HSA, 96%) were obtained from the Sigma Chemical Co. Deuterium oxide ($D_2O$, 99.9 atom % D, from Aldrich Chemical Company, Inc.) was stored under nitrogen until needed. Sodium azide ($NaN_3$), mannitol, catalase and other chemicals were all made in China with analytic purity.

In *in vitro* measurement, the volume of samples was maintained at 2 mL with PBS at pH 7.4 during ultrasound exposure. The final concentration of FCLA, HpD and ATX-70 were 10 mol/L, 1 g/mL, 1 mol/L, respectively. All components of the solution were prepared just before use. The sonodynamic chemiluminescence was recorded in real time during ultrasound exposure. For confirming the origin of sonodynamic chemiluminescence, various free radicals quenchers and $D_2O$ were added to the model solution and the effects of quenchers and $D_2O$ on chemiluminescence intensity were recorded and compared. The quenchers used included 10 mmol/L $NaN_3$, 10 mol/L SOD, 10 mmol/L mannitol and 100 g/mL catalase.

In *in vivo* experiments, male nude mice (BALB/c-nu/nu) were used to establish transplanted cancer. The human SWO-38 cell line was established from grade 1—11 glioma tumor. Cell suspension ($4\times10^6$ cells in 0.2 mL PBS) was injected subcutaneously into the right side of the neck of the mice. The volume of the tumor reached approximately 60 $mm^3$ and 2000 $mm^3$ at the time of 11 days and 35 days after the cell injection. ATX-70 (10 mol/L in saline) was injected i.p. to the tumor-bearing mouse for 24 h before measurement. FCLA was dissolved in saline (5 μmol/L) and injected subcutaneously to the mouse for 30 min before measurement. The mouse was anesthetized with pentobarbital sodium (50 mg/kg i.p.), and then was fixed on a metal mesh. An appropriate amount of degassed water with black ink was poured into the pool to soak the abdomen of the mouse, and then the sonosensitization and image acquisition processes start for 15 min.

## 2 Results and discussions

Fig. 2 shows the comparison of the sonodynamic CL of the two sonosensitizer, HpD and ATX-70, under the air and nitrogen saturation condition. When air-saturated, the sonochemiluminescence of FCLA has an intensity of 12000 counts per second (cps). The sonodynamic CL intensity of HpD (HpD + FCLA mixture) is 23000 cps, which is consistent with that in our previous experiments[7]. But the intensity of CL from the ATX-70 + FCLA mixture reaches 37000 cps. That means the sonodynamic CL of ATX-70 is two times higher than that of HpD after subtraction of sonochemiluminescence of FCLA. When nitrogen-saturated, the CL intensity from three solutions was 3300 cps, which is about the background level of PBS sonoluminescence intensity measured by the system under the same conditions. ATX-70 was one of the most active sonodynamic agents found up to now. These results indicated that sonodynamic CL detection could be as a fast determination method of sonosensitization efficiency to screen the sonosensitizer.

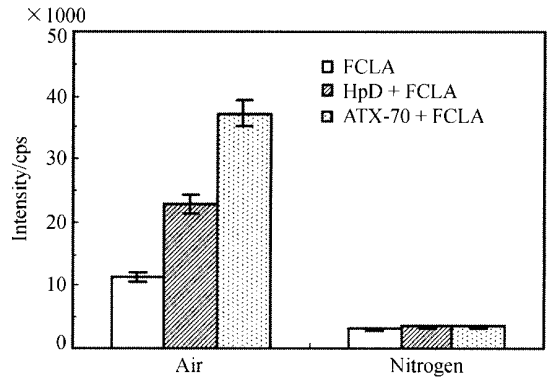


Fig. 2. Chemiluminescence intensity of FCLA solution. FCLA solution in the presence of HpD and ATX-70 under air (left panel) and $N_2$ (right panel) saturation during sonication. Data represent average ± SD from at least three experiments.

In the present work, ATX-70 was used as sonosensitizer. To find out the free radical source of the sonodynamic CL of ATX-70, several free radical quenchers and $D_2O$ were tested in the experiments. Fig. 3 shows the effects of $D_2O$, $NaN_3$ ($^1O_2$ scavenger), SOD ($O_2^-$ scavenger), Catalase ($H_2O_2$ scavenger), and mannitol (·OH scavenger) on sonodynamic CL from ATX-70 + FCLA mixture. Compared with the control, $D_2O$ increases the CL by 23.1%, while the $NaN_3$, SOD, Catalase, and mannitol decrease the CL by 82.2%, 40.8%, 7.6% and 13.3%, respectively. Since the lifetime of $^1O_2$ is approximately 10 times longer in $D_2O$ than in $H_2O$, thus allowing $^1O_2$ more time to exert effects on targets, the substitution of $D_2O$ for $H_2O$ is often used as evidence whether $^1O_2$ was involved in a process. $^1O_2$ and $O_2^-$ can be eliminated by $NaN_3$ and SOD at appropriate amounts, respectively. The effects of $D_2O$, $NaN_3$ and SOD on the sonodynamic CL indicate that both $^1O_2$ and $O_2^-$ play a role in the sonosensitization process. Because FCLA selectively react with both $^1O_2$ and $O_2^-$ to emit CL, in the experiments the generation of $H_2O_2$ in sonosensitization action of ATX-70 cannot be known well from slight decreasing effect of the catalase on the CL. However, the sonodynamic CL intensity inhibited (about 13.3%) by mannitol, one of ·OH scavenger, indicates

that · OH could be produced during the sonosensitization. Considering that the CL came from the reaction of FCLA with only $^1O_2$ and $O_2^-$, the results suggested that · OH might be the cause of $^1O_2$ and $O_2^-$ production. The mechanism of sonosensitization should be further investigated.

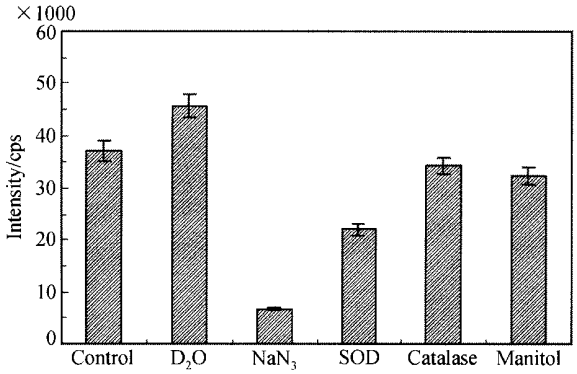


Fig. 3. Effects of $D_2O$ and quenchers on sonodynamic chemiluminescence mediated by FCLA solution. The quenchers and $D_2O$ were added before ultrasound exposure. Quenchers used are $NaN_3$ (10 mmol/L), SOD (10 μmol/L), catalase (100 μg/mL) and mannitol (10 mmol/L). Data represent average ± SD from at least three experiments.

Fig. 4(a) shows the two-dimensional image of sonodynamic CL of ATX-70 mediated by FCLA in a tumor-bearing nude mouse. Fig. 4(b) is a two-layer image for location comparison. The bottom layer of the image is the nude mouse obtained on illumination with dim white light. The tumor can be observed in the right shoulder. The upper layer is the image (a). The bright area exactly corresponds to the tumor region, including the shape and the location. The contrast of image is largely improved. In the cases where only ATX-70 or FCLA was injected into the mouse body, there was no significant difference between the emission intensities from the tumor and non tumor region.

ATX-70 should accumulate in tumor tissues at the time of measurement, and ultrasound could make sonosensitization. The mechanism of sonosensitization was studied in considerable detail in recent years[3,8]. It is shown that sonosensitization is related to cavitation of acoustic field[8]. When cavitation occurs in biological tissues, the extremely high temperature and atmospheric pressure in bubbles provide appropriate environments for chemical reaction. Researches on sonoluminescence shows that water vapor molecules in the cavity are split apart into · H and · OH[12]. These free radical molecules are in high-energy states, and its energy could transfer to the ATX-70 molecules to form the excited states ATX-70*. Umemura et al.[3] reported that the cytotoxicity of sonosensitization is intermediate by singlet oxygen because the cytotoxicity was inhibited by histidine, a known singlet oxygen and hydroxyl radical scavenger. Miyoshi et al.[5]

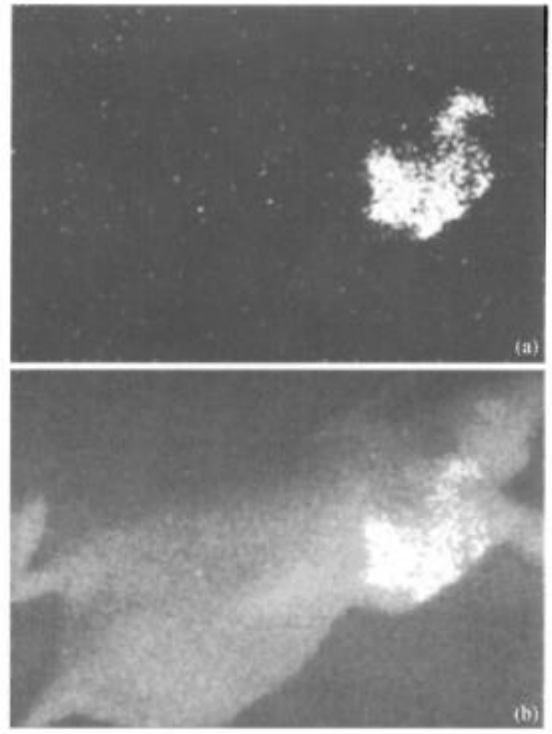


Fig. 4. (a) Sonodynamic chemiluminescence image of the mouse with ATX-70 and FCLA as sonosensitizer and chemiluminescence probe; (b) two-layer image. The bottom layer is a photograph of the mouse obtained on illumination with dim white light. The top layer is the image (a). The bright area corresponds to the tumor region.

reported that sonosensitization effect is in connection with the gas composition, and 20% of oxygen concentration in the gas mixture is very important to sonosensitization. They also confirmed that the singlet oxygen is involved in the sonosensitization process. These results indicate that the energy of ATX-70* has been further transferred to oxygen to form singlet oxygen $^1O_2$. This process is like photosensitization. Then $^1O_2$ reacts with FCLA, which was diffused into tumor tissues after being injected, to efficiently emit chemiluminescence.

Briefly, the strong emission from the tumor region in Fig. 4(b) is produced through the following process: ATX-70 selectively accumulated in the tumor region, ultrasound field sensitized ATX-70 to excited states ATX-70*, and then the energy transferred from ATX-70* to oxygen molecules $O_2$ to form $^1O_2$. Finally, $^1O_2$ reacts with FCLA to emit strong chemiluminescence from tumor region.

The chemiluminescence observed in the present experiments mainly comes from the superficial tissues because of the absorption by tissues. However, if we select another chemiluminescence reagent that has similar properties to FCLA and can emit longer wavelength photons (800—1200 nm[13]), and utilize the confocal scanning technique, it is possible to make deep-layer tumor diagno-

sis. This method could be further combined with endoscopy to become a practical technique in clinical diagnosis. The ultrasonic power density in the experiment is about 0.18 W/cm$^2$, which is higher than the cavitation threshold but much lower than the damage threshold of the tissue lesion[10]. The side effect of sonosensitization could be much smaller than that of photodynamic diagnosis.

## 3 Conclusion

In this paper, we proposed a sonodynamic diagnosis of cancer with CL probe. Using ATX-70, the most active sonodynamic agent, sonosensitizer with FCLA as the CL probe, the tumor-bearing mice were imaged with good contrast. This technique could have potential applications in clinic early stage cancer diagnosis.

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# Heat shock response down-regulates IL-18 expression in the murine macrophage cell line, RAW264.7

WANG Yun[1,2], LI Changlin[1], WANG Xingyu[1], ZHANG Jinsong[1] & ZHANG Zongliang[1]

1. Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China;
2. Department of Cell Biology, The Third Military Medical University, Chongqing 400038, China

Correspondence should be addressed to Zhang Zongliang (e-mail: immusig@sunm.shcnc.ac.cn)

Abstract **Heat shock response is a self-defense mechanism for protection of cells and organisms from a wide range of harmful stressors. Recent studies revealed that it is involved in the regulation of cytokines expression. IL-18 is an important cytokine in mediating immune response. We studied LPS-induced IL-18 expression in heat shock treated RAW264.7 murine macrophages. Our results show that the heat shock response significantly inhibited the expression of LPS-induced pro-inflammatory cytokine IL-18. Further research on the down-regulation mechanism shows that this inhibitory effect is correlated to the great suppression of the binding activity of AP-1, which is a transcription factor binding to the promoter of IL-18 (-1120 to -1083) and regulates the transcription of IL-18. Meanwhile, we observed that the phosphorylation of JNK, which is AP-1 upstream kinase, was greatly decreased. These results confirmed that the down-regulation effect on IL-18 production in heat shock response is related to the suppression of the JNK/AP-1 signaling pathway.**

**Keywords: heat shock response, heat shock protein, IL-18, JNK/AP-1, macrophage.**

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Exposure of cells to the conditions of environmental stress, including heat shock, UV light, or the pathologic conditions such as inflammation and tissue damage, leads to the cell stress response, also termed as the heat shock response (HSR)[1]. The HSR through activation of heat-shock transcription factors (HSFs) and the elevated expression of heat shock proteins (HSPs) that function as molecular chaperones protects the cells against further damage[2].

Macrophages are important effectors and immunoregulatory cells. They play an important role in spanning innate and adoptive immunity. After stimulation by lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, macrophages secrete a series of cytokines such as IL-1, IL-6, IL-12, IL-18,