A novel fluorescent dye selectively images and kills cancer stem cells by targeting mitochondria: Evidence from a cell line-based zebrafish xenograft model

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Abstract. Numerous agents such as near-infrared dyes that are characterized by specialized cancer imaging and cytotoxicity effects have key roles in cancer diagnosis and therapy via molecularly targeting special biological tissues, organelles and processes. In the present study, a novel fluorescent compound was demonstrated to inhibit cancer cell proliferation in a zebrafish model with slight *in vivo* toxicity. Further studies demonstrated selective staining of cancer cells and even putative cancer stem cells via accumulation of the dye in the mitochondria of cancer cells, compared with normal cells. Moreover, this compound was also used to image cancer cells *in vivo* using a zebrafish model. The compound displayed no apparent toxicity to the host animal. Overall, the data indicated that this compound was worthy of further evaluation due to its low toxicity and selective cancer cell imaging and killing effects. It could be a useful tool in cancer research.

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Introduction

Targeted therapy and immunotherapy are emerging as significant tools that can aid understanding of cancer development and the treatment of cancer in addition to the conventional treatments of surgery, chemotherapy and radiotherapy (1). New methods including genetic techniques and whole genome sequencing are now used to treat cancer. However, drug resistance is a clinical problem in chemotherapy, which is primarily because of the failure to eradicate cancer cells (2).

Chronic myeloid leukemia (CML) is a clonal disorder characterized by the Philadelphia chromosome, which results from aberrant BCR-ABL tyrosine kinase activity (3). BCR-ABL is the fusion oncogene of a chromosome translocation between chromosome 9 and chromosome 22, and promotes the over proliferation of leukemic cells (3). No apparent symptoms which mark the initial stages of CML development have been reported. With the gradual progression of the disease, the chronic phase progresses to a blast crisis often after several years and even evolved into acute leukemia (4). A clinical trial reported that imatinib has strong efficacy in CML, particularly in BCR-ABL-positive CML (5). Moreover, a study using transgenic mice, which were obtained by cloning the promoter of the mouse *tec* gene, demonstrated, through its deletion, that p53 serves a significant role in the development of CML and increases BCL-ABL kinase expression in hematopoietic cells (6). These examples indicate the importance of the research and development of anticancer drugs based on molecular therapy. However, drug resistance and disease recurrence remain as major clinical problems.

In recent years, certain novel methods have been developed to address the early detection and treatment of cancer as well as the development of anticancer drugs (7). For example, near-infrared (NIR) fluorescence probes, fluorescent methods and multispectral imaging technology are well developed (8). A recent study

using a NIR probe for the detection of cancer indicated that the tumor could be observed through protease-activated probes. In the NIR channel, the target-to-background signal ratio of tumor and normal mucosa are relatively high, which indicates the cancer selectivity displayed by such fluorescence reagents (9). Furthermore, a neutral pH NIR fluorescence probe was developed to complete real-time imaging and *in situ* detection of the pH value of cancer cells (10).

The present study, we developed one fluorescent compound and explored its potential biological effects. CML cells were used as the research model together with a zebrafish xenograft organism.

Materials and methods

Reagent and cell culture assay. The tested compound [2-[2-[3-[2-(5-carboxy-1-ethyl-3,3-dimethylindol-1-ium-2-yl) ethenyl]-2-chlorocyclohex-2-en-1-ylidene]ethylidene]-1-ethyl-3, 3-dimethylindole-5-carboxylic acid] was supplied by Tianjin Medical University and was reconstituted in 10 mM stock in DMSO (cat. no. D8418; Sigma-Aldrich) and stored at -20°C. We termed this chemical as compound A02 in this present research. K562 cells were stably transfected with the Kusabira Orange (K562-KOr) fluorescent protein, which could be imaged using the Tritc filter. K562 cells were transfected with 2.5 μg phKO1-MN1 vector (cat. no. AM-V0046M; Amalgaam, Tokyo, Japan) with Kusabira-orange (KOr) fluorescent protein expression using the reagent Lipofectamine 2000 (cat. no. 11668027; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection for 24 h at 37°C, cells were selected using 800 μg geneticin/ml concentration for seven days culture, the positive KOr-expressing cells were sorted by FACSAria flow cytometry (BD Biosciences). The cells are cultured in RPMI-1640 medium (cat. no. 11875119; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS (cat. no. 10099141C; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C in a 5% CO_2 atmosphere. The cell proliferation assay was performed to test the relative cell viability using a CellTiter-Glo luminescent cell viability assay kit (cat. no. G7517; Promega Corporation) according to the manufacturer's instructions. For the cell staining assays, the cells were centrifuge at 200 x g for 5 min for 3 times at room temperature. Zebrafish blood and K562 cancer cells, they were exposed to 20 μM of the compound A02 for 30 min in medium at 37°C in a 5% CO_2 atmosphere, respectively. ALDH(-) and ALDH(+) K562 cells, they were stained using the compound A02 (2 μM) for 24 h in medium at 37°C in a 5% CO_2 atmosphere, respectively.

Aldehyde dehydrogenase (ALDH)-positive putative CSC preparation. ALDH positive (putative CSCs) and negative cells were sorted using an ALDEFLUOR™ assay kit (cat. no. 01700; Stemcell Technologies, Inc.) followed by FACSAria flow cytometry (BD Biosciences) according to the manufacturer's instructions.

Mitochondrial staining. Mitochondrial staining in viable cells was performed using the Rhodamine 123 dye (cat. no. R302; Thermo Fisher Scientific, Inc.) according to the manufacturer's
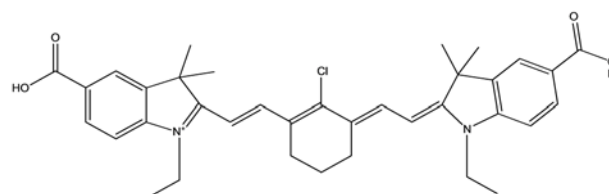

Figure 1. Chemical structure of compound A02 [2-[2-[3-[2-(5-carboxy-1-ethyl-3,3-dimethylindol-1-ium-2-yl)ethenyl]-2-chlorocyclohex-2-en-1-ylidene]ethylidene]-1-ethyl-3,3-dimethylindole-5-carboxylic acid).

instructions. K562 cells were stained using 2 μM Rhodamine 123 and 20 μM tested compound A02 for 30 min at 37°C in a 5% CO_2 atmosphere. The cells were centrifuged at 200 x g for 5 min and washed out for 3 times. Then the cells were observed and imaged in the bright field and fluorescence filters (green and red) using the LSM-510 confocal microscope (Zeiss, Thornwood, USA).

Cancer mass in vivo imaging. ALDH-positive and negative K562 cell populations were injected into the yolk sac (~100 cancer cells per fish) of the transparent 48 h postfertilization transgenic zebrafish line (fli-EGFP; China Zebrafish Resource Center). The xenografted zebrafish were subsequently maintained at 32°C and the successful cancer xenograft models with visible cancer mass were collected the next day [1 day postinjection (dpi)] and obtained the images. Subsequently, the fluorescent compound A02 was subsequently added into the E3 egg water (0.17 mM KCl, 5 mM NaCl, 0.4 mM CaCl2, and 0.16 mM MgSO4 in dd water) and the images were obtained 2 days later, at 3 dpi after washing out using fresh E3 egg water for 3 times. The images for each zebrafish were obtained using an 'angiogenesis based target area best focusimaging acquisition program' (version 4.0, Molecular Devices, LLC) under FITC filter (zebrafish fli-EGFP signal detection), Tritc filter (Kusabira-orange, KOr fluorescent protein signal detection) and Cy5 filter (tested compound A02 signal detection) in an ImageXpressMICRO Device (Molecular Devices, LLC) automatically. The experiments involving zebrafish were performed according to international guidelines and approved by the Laboratory Animal Welfare Ethics Committee of Yunnan University (approval no. YNU20210100). Following the experiments, zebrafish used were sacrificed by an overdose of anesthesia using the final 0.1% tricaine concentration and subsequently stored at -20°C for further professional waste disposal.

Statistical analysis. Data are presented as mean ± SEM. Comparisons between multiple groups and a single control group were calculated using Dunnett's test following one way ANOVA and comparisons between the two groups were performed using unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Compound A02 displays slight toxic effects on biological organisms. To evaluate the novel application of biological chemicals, compound A02 displaying interesting biological characteristics was identified following screening of a series of compounds. The chemical structure is presented in Fig. 1.
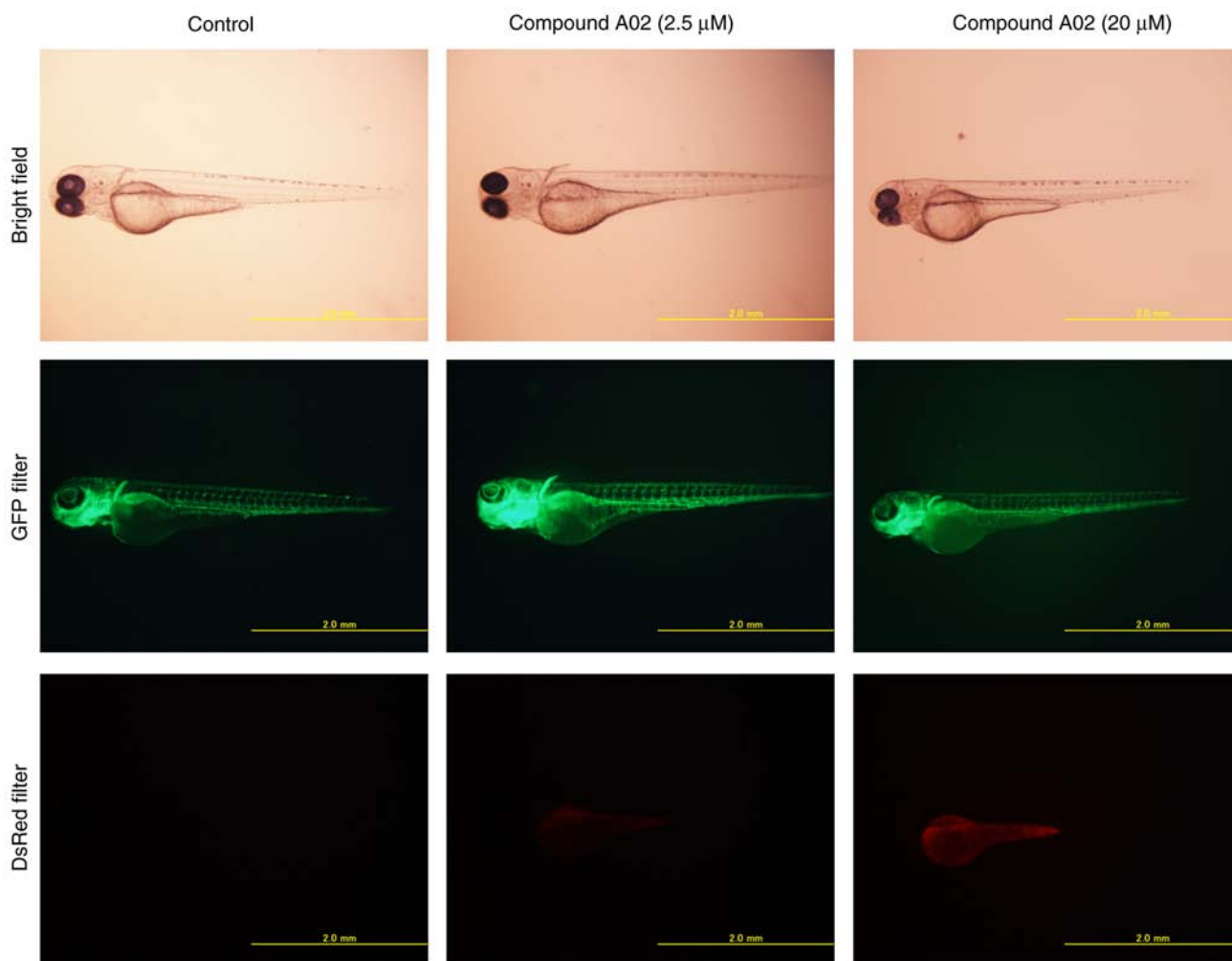
Figure 2. Effects on the morphology and the phenotype of zebrafish. Zebrafish embryos at 5 dpf were imaged using bright field, GFP and DsRed microscopy filters following treatment with compound A02 at different concentrations for 48 h from 3 dpf. Scale bar, 2 mm. dpf, day postfertilization; GFP, green fluorescent protein.

Treatment of 3 days postfertilization (dpf) zebrafish embryos with the compound A02 (20 μM) for 48 h had little effect on zebrafish survival and morphology; however, a very weak red fluorescence was detected in the yolk sac using the DsRed microscopy filter (Fig. 2). Overall, these data suggested that compound A02 was relatively safe with no apparent toxic effects on the model organism used.

Anticancer effects and staining of cancer cells, particularly CSCs in vitro. The data indicated that the compound A02 could significantly inhibit the proliferation of BCR-ABL+ CML K562 cells at 10 and 20 μM following 24 h exposure compared with the control (Fig. 3A). Exposure of cells to 10 μM compound killed >50% of the cancer cells.

Zebrafish blood and K562 cancer cells were exposed to 20 μM compound A02 for 30 min. The fluorescent signal of compound A02 was clearly detected in K562 cancer cells; however, it was not present in the normal zebrafish blood cells when imaged using the Cyanine-5 (Cy5) filter following washing out of the dyes (Fig. 3B). Subsequently, the zebrafish blood and K562-KOr, expressing Kusabira Orange protein fluorescence, (Fig. S1), cancer cells were mixed and exposed to the compound (20 μM) for 30 min. K562-KOr cancer cells were clearly visible

using the fluorescent signal of the compound following washing out (Fig. 3C). No significant differences were noted between the percentage of positively stained cells detected using the Tritc (KOr) and Cy5 (compound A02 signal) filters (Fig. 3D). Moreover, the ALDH(-) and ALDH(+) K562 cells were sorted (Fig. S2) and stained using compound A02 (2 μM) for 24 h; subsequently, they were imaged using the Cy5 filter (Fig. 3E). The fluorescent intensity of ALDH(-) and ALDH(+) K562 cells was quantified and the results demonstrated that ALDH(+) cells displayed significantly stronger fluorescence compared with the ALDH(-) cells. Taken together, these data suggested that the compound could be used to selectively image cancer cells, notably the ALDH (+) putative CSCs.

Effects of staining on cancer cells in vivo. As the novel fluorescent dye displayed cancer cell selectivity *in vitro* as indicated by the aforementioned experiments, *in vivo* effects were subsequently examined. K562-KOr cells were injected into zebrafish embryos. The successfully established xenografts (1 dpi) were imaged. Subsequently, these embryos were seeded in egg water either with or without the novel fluorescent compound A02 (2.5 μM) for 48 h and imaged again on 3 dpi. The representative images of the K562-KOr zebrafish xenograft on 1
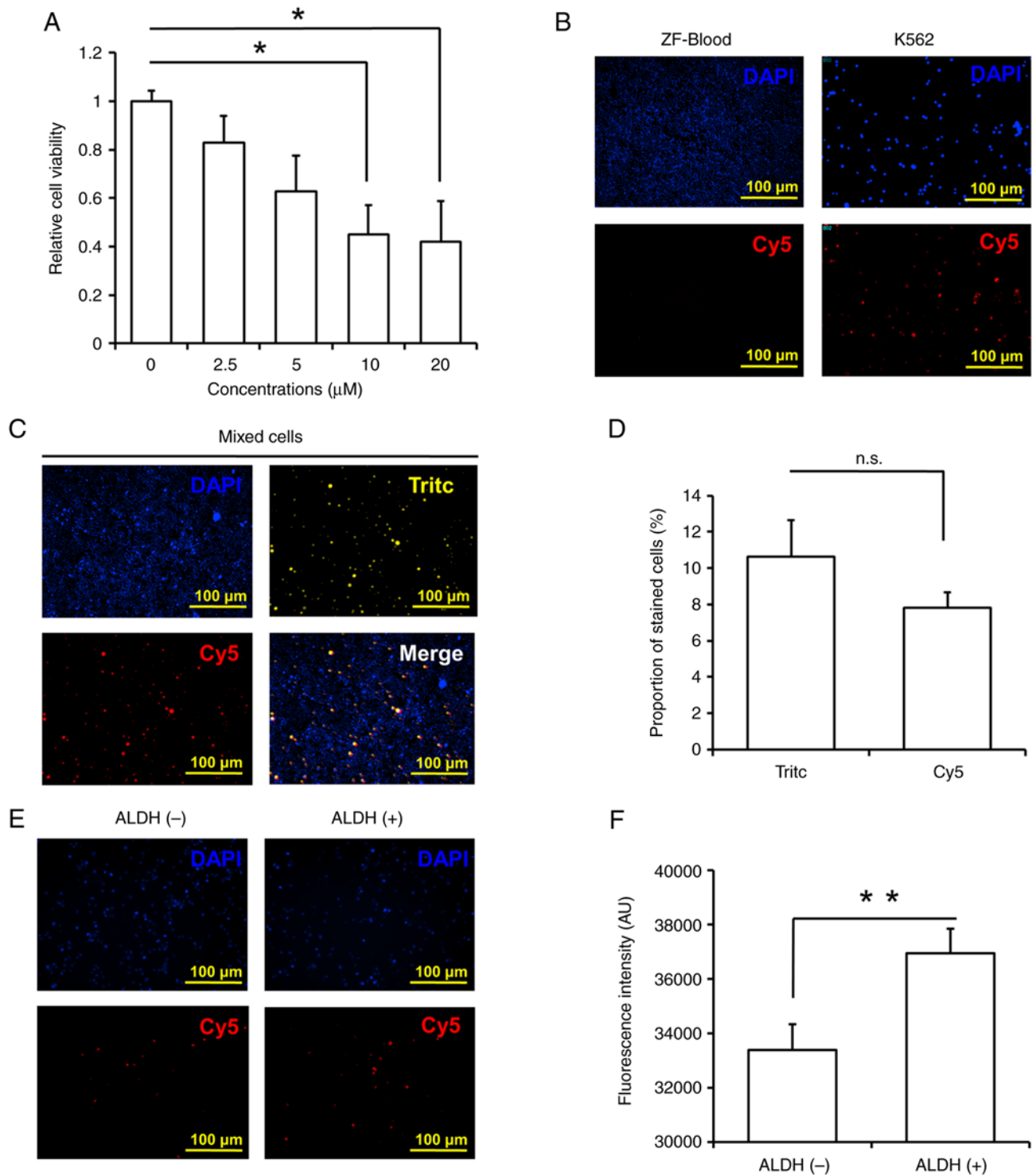
Figure 3. Effects on cancer cell proliferation and cell imaging. (A) Cell proliferative status of BCR-ABL⁺ CML K562 cells, which were treated with the compound for 24 h at different concentrations, n=3. (B) Zebrafish blood cells and K562 cancer cells were treated with 20 μM fluorescent compound A02 for 30 min. The fluorescent signal was detected in K562 cancer cells using the Cy5 filter following washing out of the dyes. DAPI was used to stain the cell nuclei. Scale bar, 100 μm. (C) Zebrafish blood cells and K562-KOr cancer cells were mixed together and subsequently exposed to 20 μM fluorescent dye for 30 min. DAPI was used to stain the cell nuclei. The compound fluorescent signals were detected using the Cy5 filter in these K562-KOr cancer cells and the KOr signal was detected using the Tritc filter, following washing out of the dyes. Scale bar, 100 μm. (D) The percentage of positive K562-KOr cells was quantified. (E) The ALDH(-) and ALDH(+) K562 cells were stained with 2 μM fluorescent dye for 24 h and imaged using the Cy5 filter. DAPI was used to stain the cell nuclei. Scale bar, 100 μm. (F) The fluorescence intensity of ALDH(-) and ALDH(+) K562 cells was quantified using the Cy5 filter. *P<0.05 and **P<0.01. CML, chronic myeloid leukemia; n.s., not significant; Cy5, Cyanine-5; K562-KOr, Kusabira Orange; ALDH, aldehyde dehydrogenase; CML, chronic myeloid leukemia.

and 3 dpi were shown (Fig. 4). The results suggested that this compound could be used to preferentially stain cancer cells *in vivo*.

Accumulation of mitochondria in cancer cells. The initial experiments indicated that K562 cells were selectively stained by the novel compound A02; therefore the subcellular
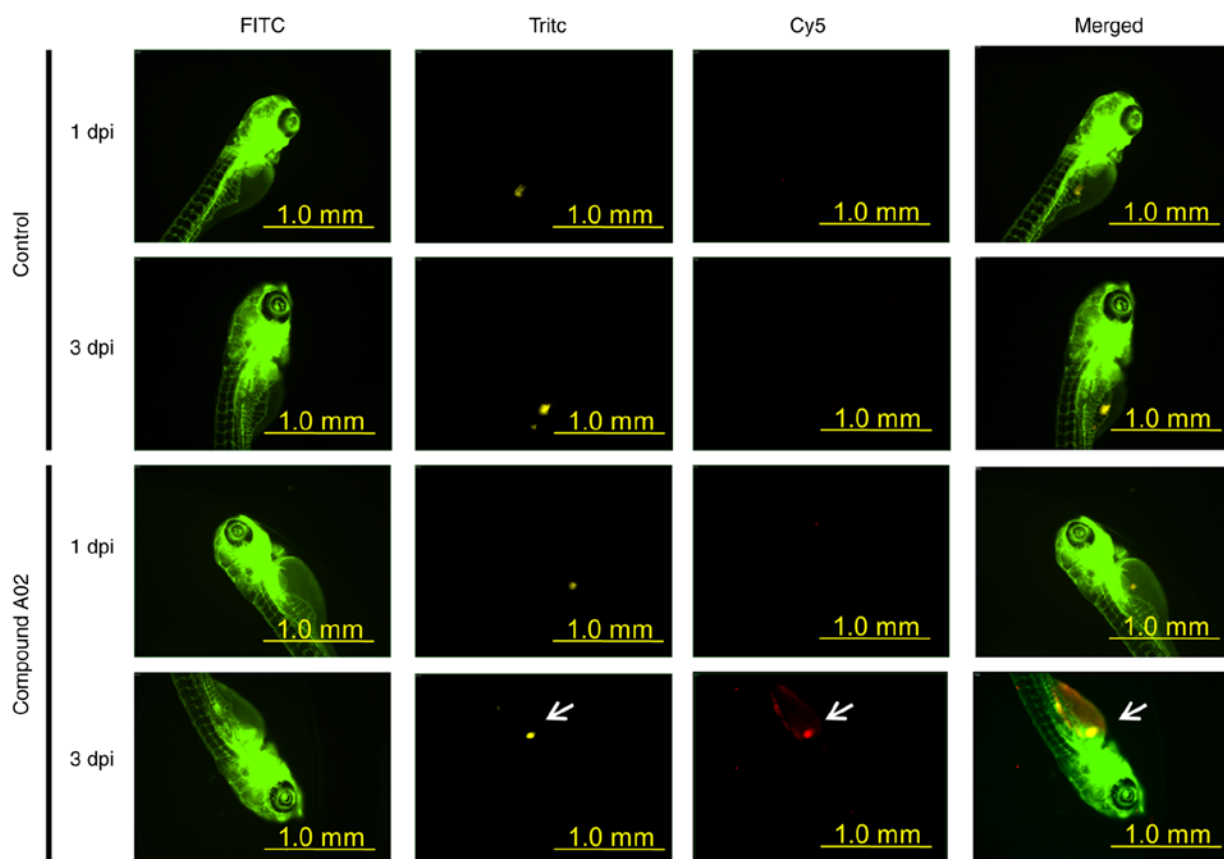
Figure 4. Selective staining of cancer cells *in vivo*. Representative images of the K562-KOr zebrafish xenograft at 1 and 3 dpi (with and without 2.5 μM fluorescent compound A02 exposure for 48 h). The arrow indicates fluorescent compound A02 selectively imaged (fluorescence signal under Cy5 filter) the K562-KOr cancer tumor mass (KOr signal under Tritc filter). Scale bar, 1.0 mm. K562-KOr, Kusabira Orange; dpi, day postinjection.
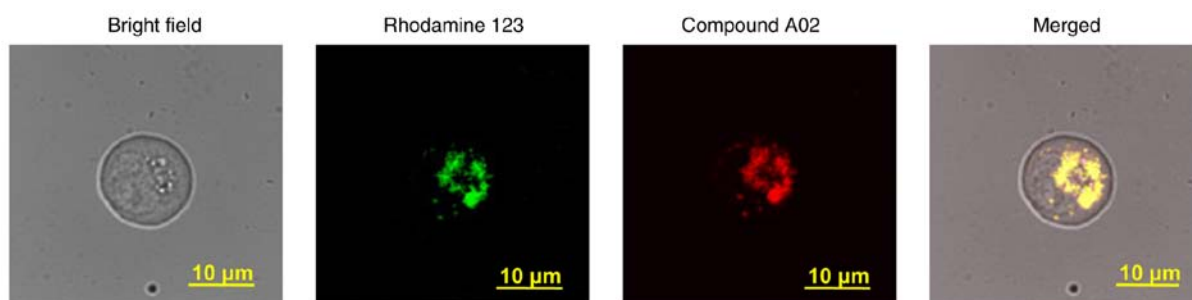

Figure 5. Mitochondrial staining in cancer cells. Confocal images of K562 cells, which were stained using 2 μM Rhodamine 123 (green) and 20 μM compound A02 (red) for 30 min. Scale bar, 10 μm.

localization of the compound was assessed. To label the organelles, the Rhodamine 123 fluorescent dye was used to stain the mitochondria and microscopic imaging indicated that the staining of the fluorescent compound A02 signal (red) colocalized with the Rhodamine 123 labelled mitochondria green signal (Fig. 5). These data demonstrated that the main intracellular dye accumulation site of this novel compound A02 was the cancer cell mitochondria.

Discussion

CSCs refer to cancer cells that can self-renew and eventually form heterogeneous malignant tumors (11). CSCs are associated with tumor recurrence, metastasis and chemoresistance, and are associated with certain abnormal signaling pathways, such as the Hedgehog, WNT and Notch signaling pathways (12). Cluster of differentiation (CD) 44, CD24, ALDH1A1, CD133 and CD13 have been reported to be cell surface markers of CSCs (13-15). Leukemia stem cells (LSCs) are the root cause of the occurrence, development and recurrence of leukemia (16). A previous study using a mouse model reported that CD27, a TNF receptor family member, promoted the development of CML in LSCs (17). Compounds, such as WNT signaling inhibitors, may serve as a potential therapeutic agent for CML (18). Therefore, targeted therapy is a method to eliminate LSCs and prevent their self-renewal, which could be used in the clinical therapy in future.
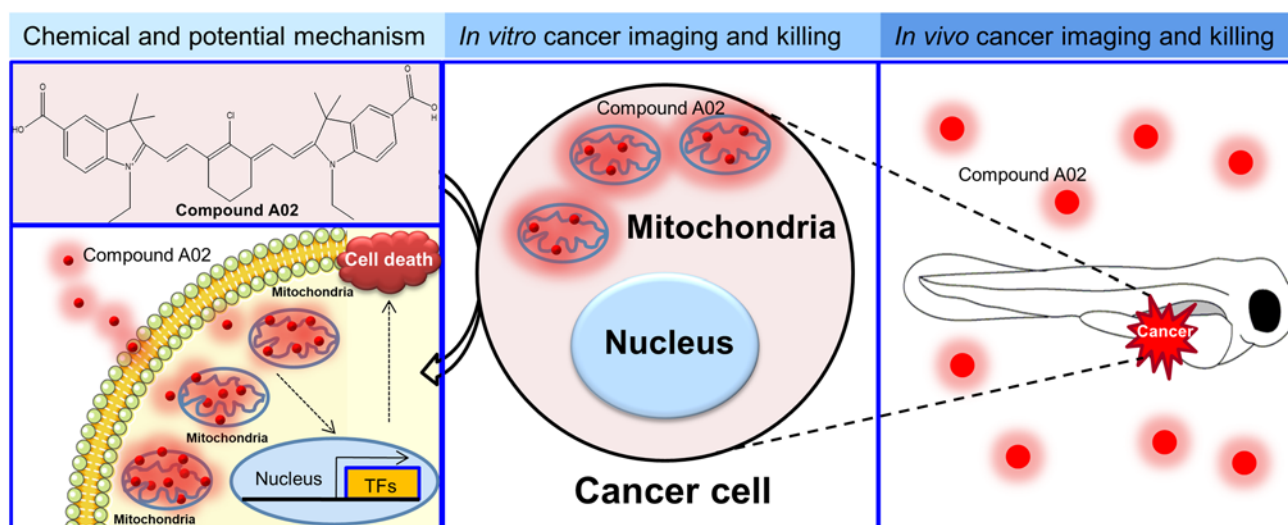
Figure 6. Working model for the application of the fluorescent compound A02 in cancer.

Mitochondria are an important target for cancer therapy due to their role in the occurrence, metastasis and recurrence of tumors (19). Mitochondria are subcellular organelles serve key roles in biological process. For example, mitochondria serve an active role in the efficacy of anticancer drugs and regulate cellular processes by releasing cytochrome C (20). Cancer treatment may interrupt the mitochondrial respiratory chain, reduce ATP production and disrupt redox homeostasis. Reactive oxygen species (ROS), a highly reactive chemical species with oxygen free radicals, are thought to be involved in the pathogenesis of different cancers. Apoptosis is induced by increasing ROS levels and destroying mitochondrial DNA. Hypoxia leads to increased ROS levels, which regulates the TGF-β signaling pathway to promote self-renewal of CSCs and protects them from cell death caused by exposure to drugs or radiation (21). Therefore, mitochondria have become an important target in cancer research and target drug development.

In the present study, the potential toxicity or side effects of compound A02 were assessed. The data indicated that exposure of zebrafish to 20 μM of A02 demonstrated no apparent effects on their embryonic development; which suggested that the compound was relatively safe within a possible therapeutic window, given that this concentration had already demonstrated antiproliferative ability against cancer cells under certain conditions. The data further demonstrated cancer cell mitochondrial accumulation. The applied concentrations of the dye in the present study were similar with those used for certain other dyes (such as IR-780 iodide) reported in published studies, which were relatively safe for *in vivo* applications (22). For example, a previously reported near-infrared fluorescent heptamethine indocyanine dye indicated accumulation by *in vivo* imaging with no apparent side effects at a dose as high as 0.2 mg/kg in a mouse model and displayed cancer cell preference at a dose of 10 μM in *in vitro* studies (22). Additional tests should be performed in future studies to assess the *in vivo* profile of this compound by using other animal models. Furthermore, the optimization of the compound structure could enable the identification of additional candidates for future medical or diagnostic applications. Possible models for the application of the fluorescent compound A02 in cancer are presented (Fig. 6). The compound A02 permeated into cancer cells and targeted the mitochondria, which may induce mitochondrial damage and subsequent cell death by regulating the associated transcription factors. Although the underlying mechanism requires further investigation, the present study suggests that this compound will be further explored due to its low toxicity to the host and its selective cancer cell imaging and killing abilities. In conclusion, the present study demonstrated a novel fluorescent dye that could selectively image and kill cancer cells, including CSCs, by invading the mitochondria. The future development of this reagent may provide an alternative strategy in cancer therapy, including CML and other cancer types.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TR, YJH, NX and BBZ contributed to the study conception and experimental design. TR, MZY, LJQ, SCZ, NNC and JS performed measurements and drafted the manuscript. TR,

WMZ, YJH, HBS, NX and BBZ aided in the composition of the manuscript, performed the experiment analysis, interpretation of data. NX, HBS and BBZ confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Experiments involving zebrafish were approved by the Laboratory Animal Welfare Ethics Committee of Yunnan University (approval no. YNU20210100).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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