Fluorescent Alkyl Phospholipid Analogs for Fluorescence Imaging of Tumors *in vitro* and *in vivo*

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Introduction

The early detection of cancer has been one of the primary goals of imaging technology, since the identification of a suspected tumor in a localized stage significantly improves the chances for successful treatment and elimination of the cancerous tissue. A large number of imaging strategies have been designed, using a variety of techniques and modalities to aid the physician in making an accurate diagnosis as early as possible. Therefore, the development of more selective and noninvasive tumor diagnostic techniques is a high priority.

Fluorescent imaging has proven to be an efficient tool for preclinical cancer research, antitumor drug discovery and pharmacological developments by providing images of the bio-distribution of fluorescent markers. By tagging regions of interest with tumor-specific fluorescent molecular probes, this technique enables visualization of location and geometries of malignant areas.

A number of synthetic phospholipid ether (PLE) and alkyl phosphocholine (APC) analogs have shown a remarkable ability to selectively accumulate in a variety of animal tumors and human tumor xenografts. The differential clearance rates of PLEs and APCs from normal cells versus viable tumor cells form the basis for their use in tumor imaging. This property makes it possible to image tumors in vivo using radioiodinated versions of PLE and APC analogs. Our SAR studies in this field resulted in discovery CLR1401 (18-p-(iodophenyl)-octadecyl phosphocholine (previously termed NM404), Fig 1), a radioiodinated alkyl phosphocholine analog that has displayed remarkable tumor selectivity and prolonged (> 80 days in mouse models) retention in a wide variety of malignant tumors with tumor-to-background ratios greater than 5[1]. Preliminary results indicate that CLR1401 is sequestered and selectively retained only in viable malignant tumor cells of both primary and metastatic lesions, regardless of location. Selective tumor uptake and retention of radioiodinated CLR1401 is accompanied by background clearance from normal tissues, especially those in the abdomen within 96 h after administration. Unlike FDG, CLR1401 does not localize in inflammatory lesions. On the basis of imaging and tissue distribution studies in several rodent tumor models, CLR1401 was chosen for follow-up evaluation in human cancer patients.

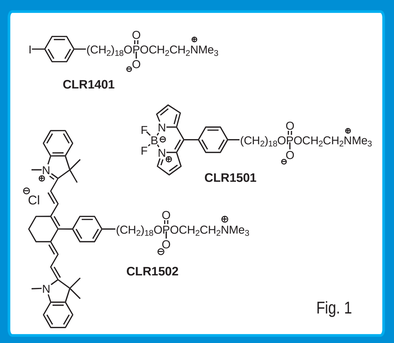


Fig. 1

We have synthesized a number of fluorescent alkyl phospholipid analogs which bear structural resemblance to CLR1401. The fluorophores in these probes are incorporated into the hydrophobic alkyl chain of CLR1401. Here we report results of in vitro and in vivo tumor imaging with two fluorescent analogs, CLR1501 and CLR1502 (Fig 1).

Results and Discussion

CLR1501

Analog CLR1501 is a conjugate of CLR1401 with unsubstituted BODIPY chromophore. Borondipyrromethane (BODIPY) fluorescent dyes have recently received significant attention for the following reasons. Their excitation and emission can be tuned by modification of the pyrrole core; they have sharp absorption and emission spectra, high photostability and high fluorescence quantum yields. Also, fluorescence of the BODIPY chromophores is generally insensitive to solvent and pH[2,3].

The selective tumor uptake of fluorescent alkyl phospholipid analogs was exemplified using CLR1501. In preliminary *in vitro* studies, A375 (melanoma) and 704sk (normal skin) cells were incubated with 7.5 μM of CLR1501 for 24 h. Results are shown in Figs 2a and 2b.

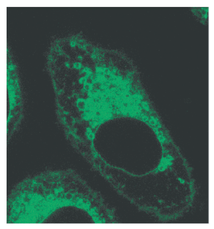


Fig. 2a

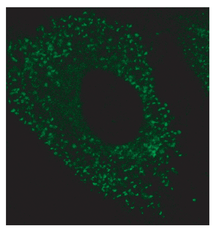


Fig. 2b

Fig. 2. Selective uptake of CLR1501 into A375 melanoma cells (Fig 2a) versus 704sk normal skin cells (Fig 2b) visualized by confocal fluorescence microscopy.
A375 cells showed internalization of CLR1501 into numerous fluorescent vesicles scattered throughout the cytoplasm. In 704sk cells, the CLR 1501 appeared to be transported to the lysosomes. (The samples were excited at 488 nm and emission was visualized at 522 nm. Exposure and image intensity levels are the same for both images.)

We have also investigated the early time uptake profiles of CLR1501 in A375 cells at 0.5 h and 1 h after incubation (Fig 3).

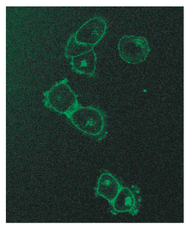


Fig. 3a. Time = 0.5 h

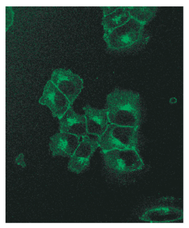
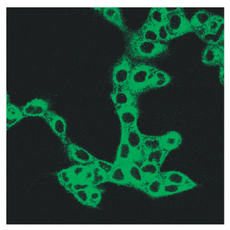
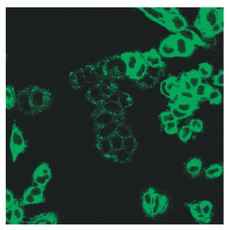


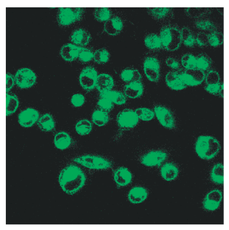
Fig 3b. Time = 1 h

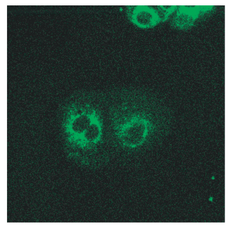
Fig. 3. Early time uptake profiles of CLR1501 in A375 cells at 0.5 h (Fig 3a) and 1 h (Fig. 3b) after incubation.
At 0.5 h, the signals are thin and limited at the plasma membrane. There are some endocytic vesicles formed near the plasma membrane. After 1 h, the compound is not concentrated solely in the plasma membrane, intracellular structures are also observed. More intense and distribited signal associated with the plasma membrane was found after incubation for one hour.

This procedure was repeated with 5 additional tumor cell lines: HCT-116 (colorectal adenocarcinoma), MES SA/Dx-5 (uterine sarcoma), Mia PaCa-2 (pancreatic carcinoma), Ovcar-3 (ovarian adenocarcinoma) and U87-MG (glioblastoma). All 5 tumor cell lines demonstrated significant uptake of CLR1501 similar to CLR1401 (Fig 4).


HCT-116 (Colorectal Carcinoma)


MES SA/Dx5 (Uterine Carcinoma)


Mia PaCa-2 (Pancreatic Carcinoma)


Ovcar-3 (Ovarian Adenocarcinoma)

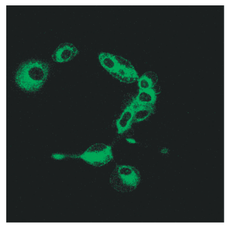

U-87MG (Glioblastoma)

Fig. 4

Fluorescence images of five different tumor cell lines incubated with CLR1501 for 24 h.

Intracellular localization of CLR1501 was investigated in U87-MG (glioblastoma cells) using co-staining of intracellular organelles (Fig 5). Accumulation of CLR1501 was observed primarily in cytoplasm (green signal, Fig. 5). Yellow signals show co-localization of CLR1501 in mitochondria and cyan (blue-green) signals demonstrate co-localization in endoplasmic reticulum (ER). The cyan signals are observed in proximity to the nuclear membrane. This experiment has also demonstrated that CLR1501 does not enter the nucleus.

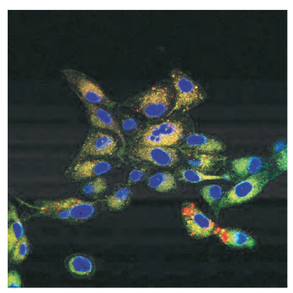


Fig 5.

Fluorescence image of the U87-MG cells incubated with CLR1501 and co-stained with:
Hoechst 33342 (blue) for nucleus;
Blue-White DPX (blue) for endoplasmic reticulum;
Mitotracker (red) for mitochondria.
CLR1501: green.

In a subsequent study, athymic nude mice inoculated with Panc-1 (pancreatic carcinoma) were injected with CLR1501 at 24 and 96 h prior to imaging. In vivo fluorescence imaging has demonstrated pronounced accumulation of CLR1501 in tumors versus non-target organs and tissues (Fig 6a). As compared with other internal organs, CLR1501 was found mostly in the tumors. However, the compound has also accumulated in the skin. The accumulation of CLR1501 in tumor was more pronounced at 96 hours and at the same time the retention of the dye by the rest of the organs decreased tremendously.

To confirm the accumulation of CLR1501 in the tumor, fluorescence imaging was also performed externally as shown in Fig 6b.

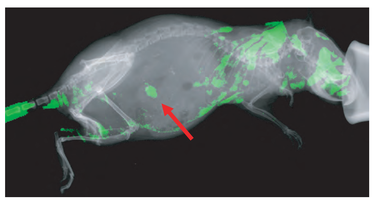


Fig. 6a


Fig. 6b

Fig 6.
Fluorescence image of tumor-bearing athymic nude mouse injected with CLR1501 24 h prior to imaging. The tumor is marked with the arrow. Accumulation of CLR1501 in the skin is also detected by fluorescence (Fig 6a). Fluorescence image of excised tumor from same mouse (Fig. 6b).

CLR1502

The fundamental barriers to optical imaging in tissue are high light scattering, autofluorescence, and high absorption by hemoglobin in the mid-visible band. Use of red and near-infrared light is the most basic step towards improved imaging. Moving to near-infrared wavelengths (700-1100 nm) confers other advantages for imaging mammalian tissues: less background fluorescence is excited, since autofluorescence in tissues is mostly excited by near ultraviolet and blue light; and less autofluorescence interference, since fluorescence from most mammalian tissues peaks in the yellow and is very low beyond 650 nm[4]. The use of near-IR fluorescence improves the performance of fluorescence-based biological assays. For example, near-IR fluorescence provides: 1) significant reduction of background autofluorescence; 2) deeper light penetration; 3) minimal photodamage to biological tissue; 4) less sensitivity to the optical properties of the media. A good fluorescent label should have a large extinction coefficient, high fluorescent quantum yield and high photostability.

With these factors in mind, we have synthesized a NIR alkyl phosphocholine analog conjugated with the indocyanine dye, CLR1502 (Fig 1). Cyanines are popular sources of long-wavelength fluorophores with excitation bands in the range of 600-900 nm[5].

Fluorescence microscopy imaging showed accumulation of CLR1502 in the human skin melanoma cells (A375) in vitro (Fig. 7).

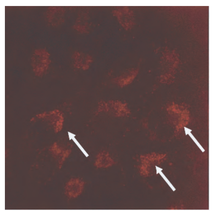


Fig. 7
Fluorescent image of human skin melanoma (A375) incubated with 30 μM of CLR1502 for 24 hours at 37 oC. The agent in the cytoplasm appears as spots or clusters indicated by the arrows.

The CLR1502 analog was evaluated in nude mice for in vivo tumor specificity and body clearance (Fig 8). It has been shown that the probe does not accumulate nonspecifically, and after 24 h post-injection, most of the initial fluorescence was gone from the non-tumoral areas and tumor-to-background ratio continued to increase. Activity in non-tumoral tissues at the early time points is most likely due to blood pool activity associated with the agent (Fig 9)

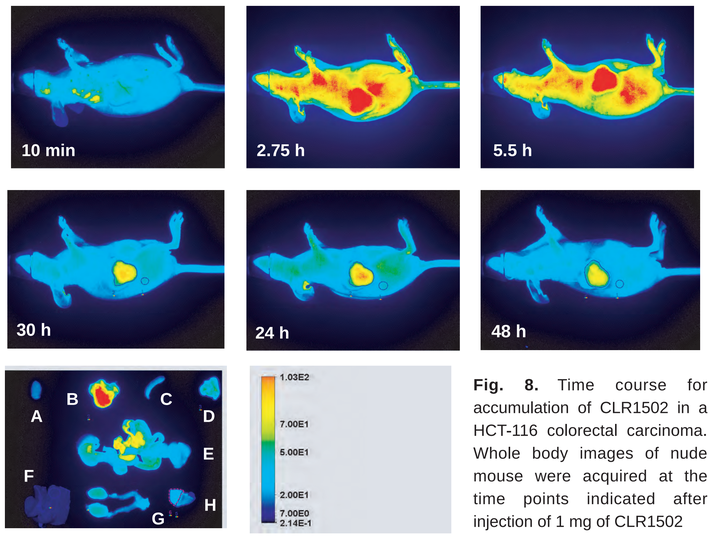


Fig. 8. Time course for accumulation of CLR1502 in a HCT-116 colorectal carcinoma. Whole body images of nude mouse were acquired at the time points indicated after injection of 1 mg of CLR1502
After acquiring the 48 hour image, the animal was sacrificed and the organs were removed.
A: Heart, B: Tumor, C: Spleen, D: Lung, E: GI tract, F: Liver, G: Kidneys and H: Skin.
The color reflects the intensity. The tumor (B) intensity is 200 times as compared to the signal intensity in the liver (F).

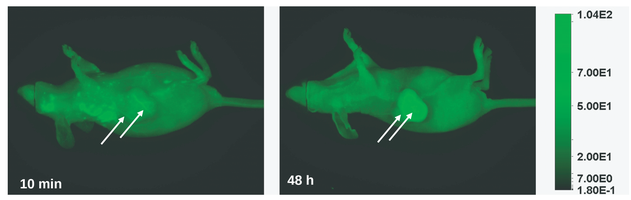


Fig. 9
Fluorescent image of nude mouse bearing HCT-116 colorectal carcinoma 10 min and 48 h post injection with 1 mg of CLR1502. The arrows show blood vessels. At 10 min, the vessels are highlighted with fluorescence while at 48 h only tumor shows fluorescence and not the blood vessel.

Materials and Methods

In vitro studies. All cells were purchased from ATCC. Cells were maintained at 37°C in appropriate media supplemented with 10% FBS and 5% CO_2. Before imaging, the cells were removed from flasks with 0.25% trypsin and were allowed to grow overnight on the slides. The next day, the media was gently replaced with Phosphate Buffered Saline (PBS) and the cells were incubated with 7.5 μM of CLR1501 or 30 μM CLR1502 in appropriate media for 24 h. CRL1501 and CLR1502 were formulated with 0.4% Polysorbate 20, 2% ethanol and saline. The cells were washed with PBS and fluorescence images were obtained with: Bio-Rad Radiance 2100 MP Rainbow fluorescent microscope for cells labeled with CLR1501; and multiphoton microsope at Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison.

In vitro co-staining of U87MG cells organelles. U87MG cells were incubated with CLR1501 for 48 hours in Eagle MEM media at 37 ^oC with 10% FBS and 5% CO_2. Co-stained dyes: Hoechst 33342 (1 μg/mL), Mitotracker (25 nM) and Blue-White DPX (100 nM) were diluted in PBS and added to the cells for 15 minutes. After washing thoroughly with PBS, the cells were imaged using Bio-Rad Radiance 2100 MP Rainbow fluorescent microscope.

In vivo tumor imaging with CLR1501. Panc-1 bearing athymic nude mice (Crl: Athymic Nu-Fox1^{nu}) were injected with 150 μL of 6 mg/mL CLR1501 formulated in 0.4% Polysorbate 20, 2% ethanol and saline at 24 hour and 96 hours prior to imaging. The whole body fluorescence images were obtained Kodak in-vivo multispectral imaging system FX, Carestream Health, New Haven, CT.

In vivo tumor imaging with CLR1502. Colorectal tumor bearing athymic nude mouse (Crl: Athymic Nu-Foxn^{nu}) was injected with 1 mg/150 μL of CLR1502 (6.7 mg/ml) formulated in 0.4% Polysorbate 20, 4% ethanol and saline. The mouse was imaged using Pearl Imager System, Li-Cor Biosciences, Lincoln, NE.

Conclusions.

Preliminary results of in vivo tumor imaging with fluorescent alkyl phosphocholine analogs have indicated that NIR dye - alkyl phosphocholine conjugates are promising candidates for the further development as optical tumor imaging agents.

References

[1] Pinchuk AN; Rampy MA; Longino MA; Skinner RWS; Gross MD; Weichert JP; Counsell RE. *J Med Chem*, **2006**, *49*, 2155-2165.
[2] Loudet A, Bugess K. *Chem Rev,* **2007**, *107*, 4891-4932
[3] Ulrich G, Ziessel R, Harriman A. Angew. *Angew Chem, Int Ed*, **2008**, 4891-4932
[4] Ballou B, Ernst LA, Waggoner AS. *Curr. Med. Chem*., **2005**, *12*, 795-805
[5] a) Goncalves MS. *Chem. Rev*., **2009**, *109*, 190-212; b) Frangioni JV. *Curr. Opin. Chem. Biol*., **2003**, *7*, 626-634

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