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Chemistry

A chemically labeled cytotoxic agent: Two-photon fluorophore for optical tracking of cellular pathway in chemotherapy

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ABSTRACT **Chemotherapy is commonly used in the treatment of cancers. However, the mechanism of action of many of these agents is not well understood. We present the synthesis of a two-photon fluorophore (C625) and its biological application when chemically linked to a chemotherapeutic agent (AN-152). By using two-photon laser-scanning microscopy, the drug:fluorophore conjugate can be observed directly as it interacts with receptor-positive cell lines. The results of this project visually show the receptor-mediated entry of AN-152 into the cell cytoplasm and subsequently into the nucleus. These observations will allow for better understanding of the drug's therapeutic mechanism, which is a subject of ongoing research aimed at improving present methods for cancer therapy.**

Two-photon processes have been drawing a great deal of attention in the last decade because of their wide range of applications. Pioneering works by Rentzepis and coworkers (1–3) have established the utility of two-photon processes in three-dimensional data storage and microfabrication. Two-photon laser-scanning microscopy (4–7), introduced by Webb's group, has already been shown to be a powerful technique for probing the three-dimensional structure of a cell and to have inherent optical-sectioning capability without any significant interference from autofluorescence. Our recent efforts on design and synthesis of two-photon chromophores have produced highly efficient two-photon up-converters (which emit photons of a frequency higher than that of the absorbed photons); some of them even exhibit up-conversion lasing (8–10). In this paper, we report the synthesis of a two-photon fluorophore, which was coupled to a chemotherapeutic agent and used in optical tracking of its interaction and entrance in to the target cells by two-photon laser-scanning microscopy.

Chemotherapy has been used widely in the treatment of cancers. However, the cellular mechanism of these agents is often not well understood. For example, whether certain chemotherapeutic drugs attach to the membrane of a cancer cell, enter the cell, or enter the nucleus affecting the DNA replication process is not well understood. Such an understanding at the cellular and molecular level will be a major advancement in biology and will lead to ways of enhancing the efficacy of chemotherapy.

Chemotherapeutic agent AN-152 was made by coupling the cytotoxic agent doxorubicin (Dox) to the luteinizing hormone–releasing hormone (LH-RH) analog, [D-Lys^6]LH-RH (11). Its design is based on the fact that specific high-affinity membrane receptors for LH-RH have been found to be expressed in many sex-linked tissue-derived cancers (12) and expressed *de novo* in many other cancers (13–16). In the last decade, Schally and coworkers (17–20) have developed and tested several cytotoxic LH-RH conjugates in which a wide variety of cytotoxic agents were linked to LH-RH analogs. As one of the most widely used anticancer drugs, Dox has shown to have a broad spectrum of antitumor effects (21). Its complex cytotoxic mechanism has been reported to involve inhibition of enzymes such as topoisomerase II, intercalation into DNA, and generation of cytotoxic radicals (22). By coupling an LH-RH analog with Dox, Schally and coworkers (11, 23) have shown that the product AN-152 has improved selectivity toward cancer cells and reduction in toxicity on normal cells. Additionally, studies *in vivo* show that AN-152 is less toxic and more potent in inhibition of tumor growth than is Dox (23). These tumors include prostatic, mammary, and ovarian cancers possessing LH-RH receptors (24). However, the cellular mechanism of action of this drug is still not well understood. Whether it acts by simply binding onto the membrane to release the cytotoxic radicals or by internalization into the cell or nucleus to interrupt the DNA replication process is of great interest (24). Using our two-photon fluorophore conjugated to AN-152, we have successfully traced its cellular pathway in a human breast cancer cell line expressing LH-RH receptors. The entire process of membrane association, internalization, and intracellular localization of the chemotherapeutic drug AN-152 was visualized in real time with no loss of viability of targeted cells.

MATERIALS AND METHODS

Materials and Instrument. The [D-Lys^6]LH-RH was purchased from California Peptide Research (Napa, CA). Images were obtained with a commercial confocal laser-scanning microscope head (Bio-Rad MRC-1024), which was attached to an upright microscope (Nikon Eclipse E800). A water-immersion objective lens (Nikon Fluor-60X; numerical aperture = 1.0) was used for cell imaging. The system was adapted for two-photon excitation by using dichroic mirror sets from Bio-Rad. A broad bandpass filter (460–600 nm) was used as an emission filter. The excitation light was provided by a home-built mode-locked Ti-Sapphire laser tuned to 800 nm, operating at a 80-fs pulse width and a 90-MHz repetition rate. After passing through the scanning head, the average power at the sample was about 15 mW.

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Abbreviations: Dox, doxorubicin; LH-RH, luteinizing hormone–releasing hormone; AN-152, conjugate of [D-Lys^6]LH-RH and Dox; C625, 4-(*N*,*N*-diphenylamino)-4′-(6-*O*-hemiglutarate)hexylsulfinyl stilbene.

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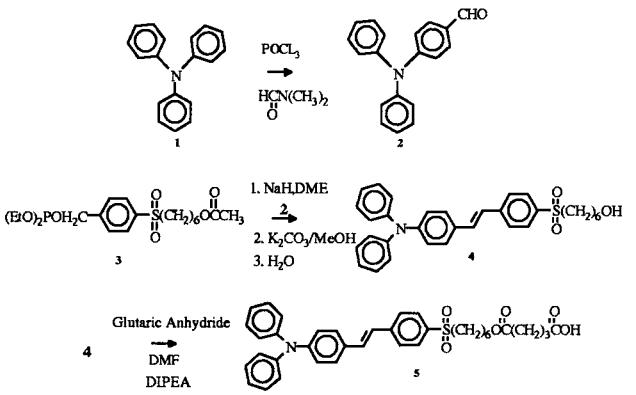

FIG. 1. Synthetic pathway of C625. DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide.

Synthesis of AN-152:C625. The preparation of the cytotoxic LH-RH conjugate AN-152 has been described (11). The detailed synthesis of the fluorophore C625 [4-(*N*,*N*-diphenylamino)-4′-(6-*O*-hemiglutarate)hexylsulfinyl stilbene] is shown in Fig. 1. For the present work, *N*,*N*-diphenylamino-4-benzaldehyde (2) was prepared from triphenylamine. The strategy for synthesis of compounds 3 and 4 has been described (25, 26). The probe with a carboxylic acid end was made by mixing compound 4 with glutaric anhydride in the presence of *N*,*N*-diisopropylethylamine in anhydrous *N*,*N*-dimethylformamide (11). The final product, C625, was purified by column chromatography and identified by NMR and MS [^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (br, 4H), δ 1.58 (qui, 2H), δ 1.73 (m, 2H), δ 1.93 (qui, 2H), δ 2.39 (m, 4H), δ 3.08 (t, 2H), δ 4.03 (t, 2H), δ 7.18, δ 6.98 (d, 2H), δ 7.05, δ 7.11, δ 7.38 (br, 12H), δ 7.38 (d, 2H), δ7.62, δ 7.83 (d, 4H); fast-atom bombardment MS: calculated for $C_{37}H_{39}NO_6S$ 625.2 and found 625.5]. The excitation and emission spectra are shown in Fig. 2.

C625 was then conjugated to the 3′ amino group of the Dox part of the cytotoxic LH-RH conjugate AN-152 by using a benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate reagent in the presence of *N*,*N*-diisopropylethylamine in anhydrous *N*,*N*-dimethylformamide (Fig. 3; ref. 11). The product was purified by reverse-phase HPLC and analyzed by two-dimensional NMR and MS (fast-atom bombardment MS: calculated for $C_{128}H_{154}N_{20}O_{31}S$ 2499.1 and found 2500.9). C625 was also coupled to the primary amino groups on the LH-RH analog and Dox separately. The success in coupling separately with the LH-RH analog (LH-RH:C625) and Dox (Dox:C625) made it possible to compare the cellular uptake of AN-152 to the uptake of its components.

Cells and Cell-Culture Conditions. MCF-7 human breast cancer cells (from the American Type Culture Collection; LH-RH receptor-positive; ref. 11) were maintained at 37°C in a 5% CO_2, humidified environment in improved MEM with

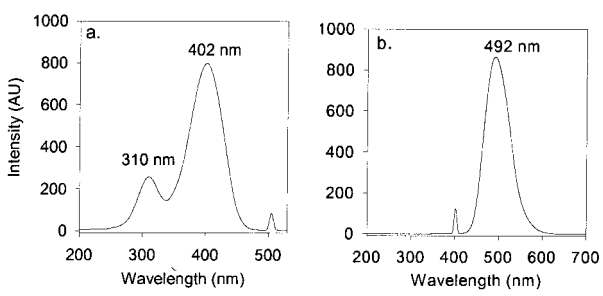

FIG. 2. Excitation spectrum (measured at emission wavelength of 500 nm) (*a*) and emission spectrum (measured at excitation wavelength of 400 nm) (*b*) of C625 in chloroform. AU, atomic units.

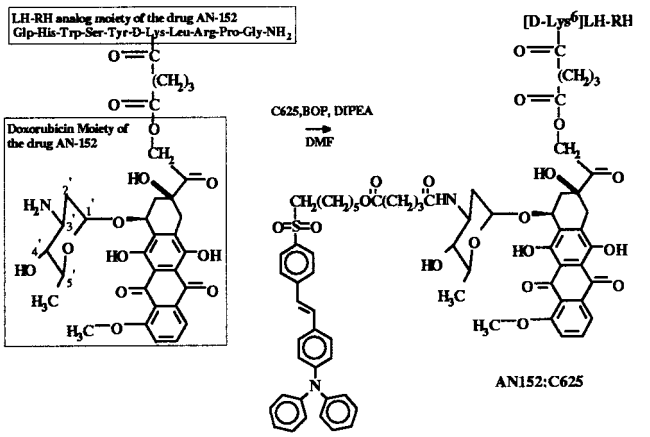

FIG. 3. Coupling of C625 to AN-152. DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; BOP, benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate.

10% (vol/vol) FBS. UCI-107 human ovarian cancer cells (LH-RH receptor-negative; ref. 23), which were provided by P. Carpenter (University of California, Irvine, CA), were maintained as described above but in improved MEM with 5% (vol/vol) heat-inactivated FBS. The cells were pretreated with epidermal growth factor (10 nM). The entry of the agents into the cells was tracked immediately after adding the labeled drug (0.6 μM AN-152:C625) or the labeled components (0.6 μM LH-RH:C625 or 0.6 μM Dox:C625) by two-photon laser-scanning microscopy.

RESULTS AND DISCUSSION

The synthesized probe showed very good two-photon efficiency. By using 10-ns pulses of 800 nm, the two-photon absorption cross section of the dye in chloroform was measured as 744 GM (1 Göppert–Mayer = 10^{-50} cm^4·s per photon). The linear excitation spectrum for fluorescence of C625 shows its λ_{max} at 402 nm, which makes 800-nm two-photon excitation very suitable (Fig. 2*a*). Even after conjugation to the cytotoxic agent, the sufficient fluorescent efficiency was maintained, which allows for tracking of the entry of the drug into the cells by using two-photon laser-scanning microscopy. There have been reports of autofluorescence in several kinds of cells with two-photon excitation (27–29). However, at the power we used to generate two-photon induced fluorescence of the labeled drug, there was no significant autofluorescence in MCF-7 or UCI-107 cells at 800 nm. After adding the labeled drug to the medium (0.6 μM) at room temperature (25°C), we took time-sequence images of the cells every minute for more than 1 h. Because the near-infrared excitation is a two-photon process, the average energy absorbed by the cells is small, and the viability of the cells is maintainable during continuous scanning (4, 7, 30). After continuous tracking for more than 1 h, no significant photobleaching was observed, and the viability of most of the cells was maintained (as determined by their microscopic morphology). The first fluorescence outline of the cell appeared in about 5 min, followed by entry of the drug into the cytoplasm. After 20 min, the drug appeared to have entered the nucleus in most cells under microscopic observation (Fig. 4). The three-dimensional reconstruction created from different optical sections also showed the distribution of the drug in the cells and its entry into the nucleus.

The entry studies performed with LH-RH:C625 and Dox:C625 on the MCF-7 cell line showed that uptake of the drug is facilitated by the presence of LH-RH. Dox:C625 has poor solubility in water; therefore, we used 0.2% DMSO to

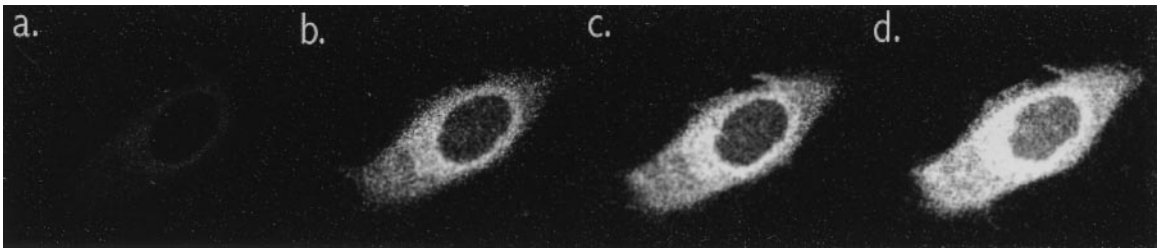

FIG. 4. Fluorescence images of a single MCF-7 cell (LH-RH receptor-positive), taken 4 min (*a*), 20 min (*b*), 40 min (*c*), and 50 min (*d*) after treatment with AN-152:C625 (0.6 μM). The staining of the cell occurred within 5 min, and the entry into the nucleus occurred after 20 min, with an increase of the fluorescence intensity over time.

deliver it to the cell medium. The cells treated with LH-RH:C625 showed an entry pattern similar to that of AN-152:C625, with the staining becoming visible within 5 min. The staining of Dox:C625-treated cells was visible only after 15 min. After 30 min, the fluorescence intensity of LH-RH:C625-treated cells was dramatically higher than that of Dox:C625-treated cells, as was seen with AN-152:C625. These results confirm the previous observations that the LH-RH analog plays the role as a carrier to facilitate the delivery of the cytotoxic agent to the specific cell lines.

To show that the penetration of the AN-152:C625 into the cells is not caused by the presence of the chromophore, we compared the entry of the labeled drug into receptor-positive cells (MCF-7) and receptor-negative cells (UCI-107). In UCI-107 cells, which were shown to lack detectable levels of LH-RH receptors (23), the uptake of the drug was much less (Fig. 5 *a* and *b*). The fluorescence uptake was first detected approximately 30 min after the administration of the drug (0.6 μM), whereas in MCF-7 cells, entry was visible in less than 5 min (Fig. 4). In addition, we compared the MCF-7 cells before and after LH-RH receptors were blocked by adding free [D-Trp^6]LH-RH. The LH-RH receptors on the cells were saturated with LH-RH analog 10 min before addition of AN-152:C625. The treated cells showed significantly lower uptake of the drug, indicated by a lower intensity of the fluorescence image. Further, as the concentration of LH-RH in the medium increased (0.1 μM to 1 μM), the uptake of the drug into the cells decreased, as visualized by a decrease in the fluorescence intensity (data not shown).

All these results indicate that the entry of the drug into an LH-RH receptor-positive cell is facilitated by receptor-mediated entry rather than by the lipophilic nature of the attached chromophore moiety.

CONCLUSION

We have developed a highly efficient two-photon chromophore and demonstrated its flexibility as a probe in a biological application. Chemical conjugation of the chemotherapeutic drug AN-152 to a two-photon probe allows for the optical tracking of the agent's cellular pathway in living cells in real time. We have shown in this work that the entry of AN-152 into the cell is accomplished by receptor-mediated endocytosis. Subsequently, the drug appears to enter the nucleus of the targeted cell. These observations will help us to understand the mechanism of action of this drug better, which is a subject of ongoing research aimed at improving present methods for cancer therapy. This approach can be extended further by coupling this efficient two-photon probe to many other biologically active molecules (proteins, peptides, nucleic acids, etc.) and optically tracking them *in vitro* (or *in vivo*) in real time with minimal photodamage to the cells.

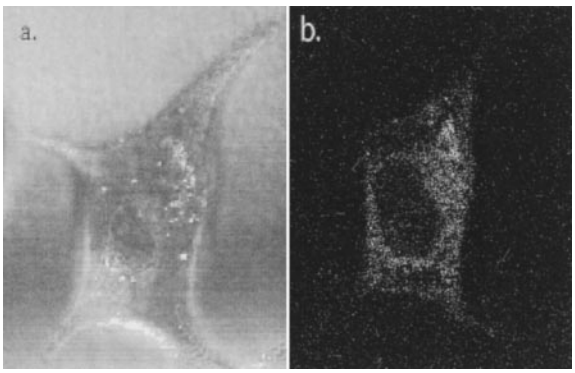

FIG. 5. Reflection image (*a*) and fluorescence image (*b*) of a UCI-107 cell (LH-RH receptor-negative) 40 min after treatment with AN-152:C625 (0.6 μM). Compared with the MCF-7 cell, the UCI-107 cell showed very little drug uptake.

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