Opto-Chemical and Laser Properties of FLT_X1, a Novel Fluorescent Tamoxifen Derivative, and its Potential Applications in Breast Cancer Photodynamic Chemotherapy

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Abstract

Tamoxifen is the most common antiestrogen used in the chronic treatment of breast cancer. In these cells, it mainly binds to intracellular receptors (estrogen receptor alpha, ER α) and antagonizes the binding of its cognate ligand, 17 β -estradiol, thereby preventing uncontrolled hormone-dependent cellular proliferation and growth. In the last decade, in our laboratories we have developed and characterized different tamoxifen derivatives, including a novel fluorescent tamoxifen conjugate: FLT_X1. FLT_X1 is formed by the covalent binding of tamoxifen to a common fluorescent biomarker NBD. This new prodrug was originally designed as a fluorescent biomarker to localize intracellular targets, which not only keeps the pharmacological activity of tamoxifen but also adds a luminescent functionality. Strikingly, the quantum efficiency of FLT_X1 is so high that laser emission has been obtained as an emerging property. In this review, we will show its laser properties under three different configurations. First, as amplified spontaneous emission or mirrorless laser; second, through the evanescent field of WGMs of a ring resonator around an optical fiber; and finally as random laser in uterine tissues impregnated with the prodrug.

Further, we observed another emergent property for FLT_X1: this molecule, but not tamoxifen alone or NBD, was able to generate reactive oxygen species (ROS) upon irradiation. This property is extremely interesting as FLT_X1 might be used for photodynamic therapy. Under this paradigm, FLT_X1 would act as a sensitizer in ER α -overexpressing cells (which feature the most prevalent form of hormone-dependent breast cancer), causing cell death in ER α + cells but reducing damage to other non-cancer (healthy) cells or surrounding tissues. We show here time resolved fluorescence results that suggest molecular aggregations, which could be explanation of the subsequent generation of ROS. This is an original cancer therapy strategy that combines the pharmacological properties of a new tamoxifen derivative and its laser dye features with a highly selective photodynamic therapy.

Introduction

Tamoxifen is a selective modulator of estrogen receptors (SERMs) widely used as coadjuvant therapy in the treatment of breast cancer [1,2]. Tamoxifen is a triphenylethylene molecule that competes with the cognate ligand (17 β -estradiol) for its binding to estrogen receptors (ER), and prevents the transcriptional activation of estrogen-dependent genes which control the proliferation of mammary gland cells [2]. Although it was first introduced in the late 1970s, it is still the first pharmacological strategy for hormone-sensitive breast cancers. In spite of its proven beneficial effects in the treatment of estrogen receptor positive breast cancers (the most prevalent form of hormone-dependent breast cancer), several side effects, some of them life-threatening, like womb cancer, endometrial cancer or thromboembolism, appear exacerbated in patients receiving chronic tamoxifen therapies [3,4]. The current view of these side-effects is that in most cases they are due to the interaction of tamoxifen with either canonical and/or non-canonical molecular targets, whose proper identification has been hampered because the lack of appropriate pharmacological tools. The existence of these adverse secondary effects has boosted many laboratories to search for either modifications in the tamoxifen molecule or novel SERMs structurally unrelated to tamoxifen, which could circumvent its undesirable effects during long-term therapies. In recent years, we have designed and developed a novel fluorescent tamoxifen derivative, FLTX1, which not only allows researchers tracking canonical (i.e. ER) and non-canonical intracellular targets, but also retains most pharmacological properties of tamoxifen (specific ER binding, antagonism of ER-mediated transcriptional activation and cell proliferation), while lacks the trophic worrisome effects of tamoxifen in the uterus [5]. Beyond its pharmacological properties, FLTX1 is endowed with emergent opto-chemical properties of which tamoxifen molecule is devoid [6–8]. Thus, positive gain media based on FLTX1 were prepared and laser emission was demonstrated under optical pulsed excitation. In this article we will review the main properties of FLTX1 with special focus on its laser properties and its potential application as specific site-directed photosensitizer.

FLTX1

FLTX1 was developed as a fluorescent derivative of the anticancer drug tamoxifen. It was initially formed from tamoxifen (1, Scheme in Figure 1A) by demethylation (to give 2) and coupling to the fluorophore nitro-2-1,3-benzoxadiazol-4-yl group (NBD), by reaction with commercial NBD-Cl. Alternatively, since tamoxifen is an expensive substrate, a second route was developed from low-cost substrates. Thus, McMurray coupling between ketones (4) and (5) gave the tamoxifen core (6), which was transformed in two steps into demethyltamoxifen (2, R = Me). This second route allows the generation of other tamoxifen derivatives, by using different R groups.

The resulting product, FLTX1, dissolved in methanol, exhibits the fluorescent spectral features illustrated in Figure 1A (right panel). FLTX1 is an optically active molecule, which shows a broad absorption band centered at about 475 nm. It has full width at half maximum (FWHM) around 65 nm. When irradiated at 475 nm, a broad fluorescence emission band (FWHM $\approx$ 80 nm) with maximum emission at about 550 nm.

Pharmacological properties of FLTX1 at a glance

An in-depth study on the pharmacological properties of FLTX1 was published by our group in 2013 [5]. A number of different *in vitro* and *in vivo* methodological approaches were used to demonstrate the usefulness of FLTX1 as a prodrug, from confocal laser scanning microscopy and transcriptional modulation of gene expression in cultured human breast cancer cells (MCF-7 cells) to long-term administration in mice.

Figure 1. A) Synthesis (left panel) and spectral characteristics (right panel) of FLT-X1. B). FLT-X1 and ER α colocalize in MCF7 breast cancer cells. To investigate the potential colocalization of these two molecules, we performed immunocytochemical assays using standard protocols described previously [5]. MCF7 cells were incubated with FLT-X1 (green fluorescence), followed by incubation with MC20, an anti-ER α specific antibody (red fluorescence). The results obtained by confocal microscopy revealed that both fluorescent signals (merged images FLT-X1 and ER α in panel 'Merge'). In the rightmost panel, transmission image overlapped with white spots corresponding to FLT-X1 and MC20 colocalization.

FLT-X1 *in vitro*

In MCF-7 cells, FLT-X1 binds intracellular targets which colocalizes with estrogen receptors [9]. A representative example of FLT-X1 and ER labelling is shown in Figure 1B. FLT-X1 binding was dose-dependent and could be totally displaced by tamoxifen (and partially by 17 β -estradiol), also in a concentration-dependent manner [5]. Quantitation of colocalization between FLT-X1 and MC20 antibody (which specifically recognises ER α) was around 71%, mostly at the perinuclear space (Fig. 1b). The inability of 17 β -estradiol to totally displace FLT-X1 labelling, together with the fact that nearly 30% of FLT-X1 labelling do not colocalize with ER α in MCF7 cells, reinforces the notion that tamoxifen interacts with targets other than ER α , some of which likely underlie some undesirable side effects of tamoxifen [10–13]. Precise determination of binding affinity of FLT-X1 for ER α was determined in competition experiments in rat uterine cytosolic extracts (a classical preparation highly enriched in ER α) showed that the IC₅₀ values (the drug concentration that causes 50% of maximal competition) for FLT-X1 and tamoxifen were 87.5 nM and 123.4 nM, respectively. Thus,

assuming a Relative Binding Affinity (RBA) value of 100 for tamoxifen, the estimated value for FLTXX1 was 141.01, indicating a slightly increased affinity of FLTXX1 for ER than tamoxifen.

Docking of FTLX1 on ER α

In order to get a deeper insight on the reasons for the higher affinity of FLTXX1 over tamoxifen, we performed *in silico* docking studies. The results are shown in Figure 2. The X-ray protein coordinates were extracted from the Protein Data Bank (PDB code 3ERT). The PDB structure was prepared for docking using the Protein Preparation Workflow (Schrodinger, LLC, New York, NY, 2017) accessible from within the Maestro program. The substrate and water molecules were removed beyond 5 Å, bond corrections were applied to the cocrystallized ligands and an exhaustive sampling of the orientations of groups was performed. Finally, the receptor was optimized in Maestro 11.4 by using OPLS2005 force field. The receptor grids were generated using the prepared protein, with the docking grids centered for the ligand bound to the receptor. The ligands were docked using the extra precision mode (XP) [14] without using any constraints. The generated ligand poses were evaluated with empirical scoring function, GlideScore a modified version of ChemScore [15]. GlideScore implemented in Glide, was used to estimate binding affinity and rank ligands [16]. The XP Pose Rank was used to select the best-docked pose for each ligand.

Figure 2. Docking of A) Tamoxifen and B) FLTXX1 into the binding site of ER α (PDB 3ERT).

As we can see in Figure 2, the introduction of the NBD moiety to the tamoxifen (Figure 2A) produces a new hydrogen bond interaction between the -NO₂ group and Cys 530 of helix 12 (XPGLide score - 11.55 Kcal.mol⁻¹) (Figure 2B). This interaction seems to be more effective than those that tamoxifen establishes between the protonated nitrogen and Asp351 (XPGLide score -10.83 Kcal.mol⁻¹). This new interaction with the helix 12 in ER α explains the higher affinity of FLTXX1 over tamoxifen.

FTLX1 in vivo

In vivo, FLTXX1 was able to inhibit MCF-7 cell growth. This cell line is characterized by the fact that its growth and proliferation is dependent on the presence of estrogens. Our findings showed that FLTXX1 was able to reduce estrogen-induced cell proliferation at concentrations as low as 3.0 μ M (which is even lower than the concentration found in patients receiving tamoxifen coadjuvant therapies). As estrogen-dependent cell proliferation is dependent on the activation of estrogen-

responsive genes, we also performed genetic analyses on the effects of FLTXX1 and tamoxifen on transcriptional activation of these genes. We used a strategy based on the manipulation of a specific promotor region (where activated ER binds to the DNA) named estrogen responsive element (ERE). We used a genetic construct where the luciferase gene was engineered to be transcribed under the control of ERE. Thus, whenever estrogens activate DNA transcription, the luciferase protein would be expressed and will emit an amount of luminescence, whose intensity is graded according to the degree of transcriptional activation. The construct was transfected into MCF-7 and TD47 cell lines. The difference between tamoxifen and FLTXX1 was dramatic. Thus, while tamoxifen alone was able to induce transcriptional activation of luciferase, particularly at low concentrations (between 0.01 and 1 μ M), FLTXX1 was completely devoid of this property in the whole range of concentrations assayed. However, as expected, both compounds exhibited similar antiestrogenic behaviours in inhibiting estradiol-induced transcriptional activation [5].

The last set of *in vivo* experiments was aimed to address the actions of FLTXX1 in the uterus. These experiments were particularly interesting, since the uterus is the locus of some of the most important side effects of tamoxifen. Indeed, the major concern in the use of tamoxifen as adjuvant therapy in the treatment of breast cancer is the increased risk of developing uterine cancer and endometriosis [3,4]. In mice, tamoxifen causes a potent uterotrophic effect, being as estrogenic as 17 β -estradiol itself [17]. Tamoxifen caused hypertrophy, endometrial hyperplasia, augmented uterine weight and increased stromal gland number. Strikingly, under similar experimental paradigms, the effects of FLTXX1 were completely negligible, behaving as the placebo [5].

FLTXX1 and its optical properties

Fluorescence decay properties

The decay of the fluorescence was measured under pulsed excitation at 475 nm and detection at 550 nm as a function of the concentration of the FLTXX1 solution using acetone as solvent. The decay curves were fitted to a double exponential decay of the form:

$$I(t) = A_1 e^{(-\frac{t}{\tau_1})} + A_2 e^{(-\frac{t}{\tau_2})}$$

Where τ_1 and τ_2 are the decay constants and A_1 and A_2 the pre-exponential factors. The best fitting was obtained for short and long decay constants of $\tau_1 = 0.3$ ns and $\tau_2 = 7$ ns, respectively. The pre-exponential factor of the short decay component was several orders of magnitude higher than the long decay one and, in all the cases, the ratio of A_1 was more than 99%. Moreover, the contribution of the long decay component decreases as the concentration increases and it is negligible at the highest concentration of 10 mM, (Figure 3). This behavior can be related to the tendency of FLTXX1 to aggregate in the form dimers. The lifetime of dimers is expected to be lower than that of isolated molecules due to the interaction of the molecules in the aggregated form and the formation of triplet excited states, which introduce additional relaxation paths. Therefore, the short decay component would be assigned to dimers, while the short one to isolated molecules, which are more abundant at lower concentrations. The aggregation into dimers might have a strong influence in the production of ROS, as it will be shown later.

Figure 3. Decay curves at different concentration of FLTX1 dissolved in acetone. IRF: Instrumental Response Function

We were interested in the determination of the capability to produce laser emission out of the fluorescent drug complex, FLTX1. Consequently, we designed three different laser configuration setups.

Amplified Spontaneous Emission

An easy and convenient way to check the laser properties of organic materials is through Amplified Spontaneous Emission (ASE) [18–21]. ASE is a cooperative luminescent effect, which occurs by successive stimulated emission of radiation from the molecules in the excited state in the presence of photons with an energy that coincides with the energy gap between the excited and the fundamental state of the molecules. ASE is observed when optical gain in the active medium is higher than the optical losses through it. It is sometimes referred to as *Mirrorless Laser* because it occurs without any mirror nor resonator to provide optical feedback [22]. In order to prove if ASE can be produced in a solution of FLTX1, an OPO pulsed laser tuned at 475 nm was used as the excitation source [8]. The pump beam was focused on a stripe line at the front surface of the probe cuvette using a cylindrical lens (Figure 4A). At relatively low pump power the typical broad emission band of FLTX1 was detected. However, when the pump power was increased above a certain pump threshold the emission spectrum narrowed and the emission intensity increased abruptly (Figure 4B). These are the two characteristic features of ASE which confirm that the medium had a positive optical gain [20–22]. The pump power threshold and the ASE efficiency were measured as a function of the FLTX1 concentration (Figure 4C). The highest efficiency was found for the 10 mM FLTX1 solution with a relatively low pump threshold of $7.5 \mu\text{J}/\text{mm}^2$.

Figure 4. Amplified Spontaneous Emission of FLTX1. A) Experimental setup; B) Narrowing of the emission spectrum as a function of the pump density; C) ASE threshold and efficiency as a function of the FLTX1 concentration.

Once we had shown that the radiative emission efficiency of FLTX1 was enough to produce optical gain, we planned to obtain laser emission using a microresonator to provide the required optical feedback.

Whispering Gallery Mode Laser

Spherical or cylindrical dielectric microresonators have shown the ability to support whispering gallery modes (WGM) lasers without external mirrors. They have found interesting applications in

optofluidic devices [23,24]. A simple way to obtain WGM laser emission was proposed in reference [25]. These authors used an optical fiber as a cylindrical microresonator and it was surrounded by the optical gain medium. The evanescent wave of the WGM of the microresonator couples the gain molecules that surround the resonator and stimulate the emission of radiation. In our case, a segment of silica 125 μm diameter optical fiber was dipped into the FLTX1 solution, resting vertically against the cuvette front wall. A cylindrical lens focused the pump beam on the cuvette front face to form a horizontal line (300 μm long by 210 μm wide), which homogeneously illuminated the optical fiber and surrounding dye solution. Clock-wise and anticlock-wise WGMs couple to the cuvette wall through the contact area and produce laser beams in both directions. As the pump power is increased the broad emission spectrum suddenly becomes a series of narrow lines at a given threshold (Figure 5). In addition to this, the emission intensity abruptly increases. Both the sudden increase of the emission intensity and the narrow lines of the emission spectrum are evidences of laser oscillation [7]. The observed pump energy density threshold for WGM laser emission was 19 $\mu\text{J}/\text{mm}^2$.

Figure 5. Whispering Gallery Mode Laser of FLTX1. A) Laser emission spectrum of the WGMs; B) Emission intensity as a function of pump energy density.

Random Laser

In the last two sub-sections, we have shown the ability of FLTX1 to provide laser emission without (ASE) or with (WGM) optical feedback using a solution of the fluorescent drug in a cuvette. However, we wanted to demonstrate the laser emission capability of the prodrug in biological systems. For that purpose, we tested random laser (RL) emission because it happens when the optical gain medium produces scattering of the light. RL occurs without external resonators, since optical feedback, necessary for laser oscillation, is provided by a random collection of scatters [26,27]. RL has been reported in biological tissues impregnated with a concentrated laser dye solution [28–31]. Therefore, we planned to use FLTX1 as a fluorescent biomarker to embed tissues, such as uterine tissues, in which ER α , the main molecular targets of FLTX1, are particularly abundant [9].

Uterine tissues from mice and rats were fixed with paraformaldehyde and processed as previously described [9]. After staining, preparations were mounted in glass slides with a drop of 10% glycerol and covered with coverslips for RL studies.

The OPO pulsed pump laser was tuned at the maximum of the FLTX1 absorption band. A cylindrical lens focused the pump beam on the tissue with normal incidence to form a homogeneous

horizontal line 4mm long and 210 μm wide.

At low pump power, the characteristic broad luminescence band of FLTX1 centered at about 550 nm was detected. However, under high pump power excitation, the emission spectrum dramatically narrowed and the emission intensity sharply increased. These are the two typical features of a transition from spontaneous emission to laser emission. Nevertheless, two different types of spectra were recorded depending on the excitation region of the sample. In the first case, the FWHM dropped to about 12-15 nm at the pump threshold (Figure 6A). However, in the second case, the FWHM dropped to about 0.5 nm, which was limited by the spectral resolution of the acquisition setup (Figure 6B). The first type of laser emission corresponded to incoherent RL, while the second to coherent RL. The appearance of incoherent or coherent RL depended on the region of the tissue under optical excitation. A change in the position of the irradiated tissue by a few hundreds of micrometers could be enough to vary from coherent RL to incoherent RL. In the case of coherent RL the stimulated emitted light wave experiences phase coherence, which produces additive wave interference, giving rise to the sharp resonances observed in the coherent RL spectra [32]. The inhomogeneities of the tissue act as scatters of the light (Figure 6C). Those regions with a high density of scatters could give rise to close loops of the scattered light, which experiences optical amplification due to stimulated emission, and would produce the narrow peaks of coherent RL. The fact that some regions show incoherent RL, while others coherent one could be related to the different density of scattering centers in the tissue.

In both incoherent and coherent RL, the pump density threshold was about 120 – 140 $\mu\text{J}/\text{mm}^2$, depending on the region of the tissue. A detailed analysis of the spectral position of the RL coherent peaks could provide information about the length of the closed loops of the stimulated emission light paths [28]. In our case, we deduced a closed loop length of about 18 μm , which is in close according to average cell diameter. Taking into account that typical sizes of the cells in uterine tissues under study were about 10-20 μm long, RL could occur inside a single cell. However, this possibility has not been proved yet.

Figure 6. Random Laser properties of FLTX1. Emission spectra as a function of pump density showing the transition from spontaneous emission to A) incoherent RL and B) coherent RL. C) Confocal microphotographs of mouse uterine wall showing the different cell layers (el: epithelial lining; st: stroma; ec: columnar epithelial cells; mg: mucous gland) labelled with FLTX1 solutions. Illustrated are, from left to right, FLTX1, propidium iodide, merged and phase contrast transmission images.

FLTX1 as photosensitizer

In order to evaluate FLTX1 as a photosensitizer drug, and its ability to produce reactive oxygen species or radicals, a colorimetric tetrazolium/formazan assay was implemented, using nitro blue tetrazolium (NBT). NBT is a water-soluble, yellow compound that upon reaction with oxidizing radicals is transformed into a chromogenic formazan dyes with a characteristic, intense blue colour [33]. Thus, when solutions of FLTX1 and NBT are irradiated, reactive species produced by FLTX1 oxidize NBT and yield the blue formazan dye, which displays a broad absorption band at 500-600 nm. Under this paradigm, the amount of oxidized formazan will depend on the photosensitizer activity of FLTX1.

In our assays we measured changes of formazan absorbance produced by 1 mL samples of 50 μM FLTX1 and NBT in DMSO (dimethyl sulfoxide) irradiated at 473 nm during 0, 4, 8, 12, 16 and 20 minutes. The curves resulting from subtraction of the absorbance at $t = 0$ (control) are shown in Figure 7A (left panel). The absorbance spectra due to formazan generation between 500 and 600 nm was observed at all times, indicating ROS generation in an irradiation time-dependent manner. The increase in absorbance is totally attributable to the presence of FLTX1, since in the presence of NBT alone, only small changes of absorbance in the range 500-600 nm were detected in the absence of FLTX1 (Figure 7, right panel)

Figure 7. A) Absorbance spectra of formazan produced after irradiating a 50 μM FLTX-NBT solution @ 475 nm at different times (Left). B) Typical absorbance curves with respect to irradiated NBT as control (Right).

Conclusions

In the present article, we have delineated some biological, pharmacological and optochemical characteristics of a novel anticancer prodrug developed in our laboratories. This compound, named FLTX1, is endowed with excellent properties so as to be considered a potential chemotherapeutic agent against estrogen receptor positive breast cancer. FLTX1 specifically binds estrogen receptors and antagonizes estrogen-dependent cell growth, while lacks main adverse effects on uterus exhibited by the parent molecule: tamoxifen. Additionally, FLTX1 exhibits surprising optical properties and behaves a laser dye, which can be demonstrated under different configurations, namely ASE, WGM and RL. Strikingly, FLTX1 also displays an emergent property as photosensitizer in situ, generating reactive species when irradiated at wavelengths which induce laser emission.

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Figure legends.

Figure 1. A) Synthesis (left panel) and spectral characteristics (right panel) of FLTX1. B). FLTX1 and ER α colocalize in MCF7 breast cancer cells. To investigate the potential colocalization of these two molecules, we performed immunocytochemical assays using standard protocols described previously [5]. MCF7 cells were incubated with FLTX1 (green fluorescence), followed by incubation with MC20, an anti-ER α specific antibody (red fluorescence). The results obtained by confocal microscopy revealed that both fluorescent signals (merged images FLTX1 and ER α in panel 'Merge'). In the rightmost panel, transmission image overlapped with white spots corresponding to FLTX1 and MC20 colocalization.

Figure 2. Docking of A) Tamoxifen and B) FLTX1 into the binding site of ER α (PDB 3ERT).

Figure 3. Decay curves at different concentration of FLTX1 dissolved in acetone. IRF: Instrumental Response Function

Figure 4. Amplified Spontaneous Emission of FLTX1. A) Experimental setup; B) Narrowing of the emission spectrum as a function of the pump density; C) ASE threshold and efficiency as a function of the FLTX1 concentration.

Figure 5. Whispering Gallery Mode Laser of FLTX1. A) Laser emission spectrum of the WGMs; B) Emission intensity as a function of pump energy density.

Figure 6. Random Laser properties of FLTX1. Emission spectra as a function of pump density showing the transition from spontaneous emission to A) incoherent RL and B) coherent RL. C) Confocal microphotographs of mouse uterine wall showing the different cell layers (el: epithelial lining; st: stroma; ec: columnar epithelial cells; mg: mucous gland) labelled with FLTX1 solutions. Illustrated are, from left to right, FLTX1, propidium iodide, merged and phase contrast transmission images.

Figure 7. A) Absorbance spectra of formazan produced after irradiating a 50 μ M FLTX-NBT solution @ 475 nm at different times (Left). B) Typical absorbance curves with respect to irradiated NBT as control (Right).