Bioorganic & Medicinal Chemistry Letters 26 (2016) 4879–4883

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A new selective fluorescent probe based on tamoxifen

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ARTICLE INFO

Article history:
Received 30 July 2016
Revised 8 September 2016
Accepted 10 September 2016
Available online 12 September 2016

Keywords:
Tamoxifen
Fluorescent probe
Fluorescent tamoxifen derivative
Estrogen receptor

ABSTRACT

Developing targeted validation probes that can interrogate biology is of interest for both chemists and biologists. The synthesis of suitable compounds provides a means for avoiding the costly labeling of cells with specific antibodies and the bias associated with the interpretation of biological validation experiments. The chemotherapeutic agent, tamoxifen has been routinely used in the treatment of breast cancer for decades. Once metabolized, the active form of tamoxifen (4-hydroxytamoxifen) competes with the binding of estrogens to the estrogen receptors (ER). Its selectivity in ER modulation makes it an ideal candidate for the development of materials to be used as chemical probes. Here we report the synthesis of a fluorescent BODIPY®FL conjugate of tamoxifen linked through an ethylene glycol moiety, and present proof-of-principle results in ER positive and ER negative cell lines. Optical microscopy indicates that the fluorescent probe binds selectively to tamoxifen sensitive breast cancer cell lines. The compound showed no affinity for the tamoxifen resistant breast cancer lines. The specificity of the new compound make it a valuable addition to the chemical probe tool kit for estrogen receptors.

Estrogens, such as 17β-estradiol, are well-known steroid hormones that play a central role in regulating a number of normal cell processes including proliferation and differentiation.[1,2] These hormones bind to a number of different receptors in cells, including the estrogen receptor (ER). The binding of the steroid to this nuclear receptor leads to a conformational change of the ER which results in the formation of a docking site for the binding of cofactors needed for transcription.[3] Overexpression in the ER is associated with 75–80% of breast cancers.[4,5] Hence ER ligands have found widespread clinical use in modern medicine. Tamoxifen (Fig. 1) is one such example that is widely administered for the treatment of breast cancer. This non-steroidal small molecule modulates estrogen receptors (ER).[6] By competing for binding with the 17β-estradiol hormone it prevents the conformational change of the ER, and hence the associated transcription no longer occurs.[7,8]

Despite the clinical success of Tamoxifen, like other estrogen therapies it has side effects for non-target tissues, including hot flushes as well as more serious conditions such as endometrial cancer.[9] These adverse side affects are likely the result of the tamoxifen acting through different targets.[5,10] It is believed that the endometrial cancer could be due to the formation of DNA adducts.[11] However, the exact mechanisms are still unknown, which in part is due to a lack of experimental tools to identify targets. Fluorescently tagging bioactive molecules through the conjugation of a fluorophore to a bioactive compound is one solution to this problem. These compounds are known to provide an invaluable tool for cellular-based microscopy.[12]

The development of new chemical probes based on Tamoxifen is one strategy for further understanding the mechanisms of estrogen signaling and the specific physiological responses associated. Tamoxifen and the active metabolite 4-hydroxytamoxifen (OHT) represent ideal candidates to base new ER targeted compounds on. Despite the simplicity of this approach, relatively few examples are found for these compounds.[13–15] Although these probes would be useful in ER detection and imaging,[14] they are unlikely to be useful for investigating the much debated membrane associated ERs.[16,17] This is because these probes either have no specificity for breast cancer cell lines,[13] or do not involve the addition of a linker molecule,[14,15] known to be a useful strategy for developing conjugates that target membrane receptors.[18] The loss of specificity and complex uptake pattern seen by others,[13] was attributed to several factors including the hydrophobic nature of the linker and/or fluorophore tags.[19] In this work we have attempted to simplify the cellular localization while maintaining selectivity of the tamoxifen probe through the introduction of a more hydrophilic linker shown to maintain ER selectivity.[20] We herein report the synthesis of a fluorescent analogue of tamoxifen, based on conjugating the BODIPY®FL fluorophore through an ethylene glycol

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http://dx.doi.org/10.1016/j.bmcl.2016.09.028
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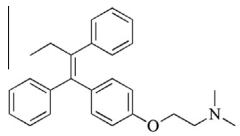


Figure 1. Tamoxifen.

moiety. We find that our new probe appears to be both cell permeable and selective.

Tamoxifen represents an ideal small molecule to base ER probes on. However, to date only three examples of fluorescent tamoxifen probes have been developed.[13–15] Two of these were based on the direct attachment of dyes to tamoxifen or its metabolite.[14] Although these probes have good binding affinity and selectivity, they would not be useful for interpreting the contentious membrane receptor problem. To date the only tamoxifen probes developed for membrane associated ER studies have had poor affinity and loss of specificity compared to the parent compound.[13] The design of our conjugate has been in response to this.

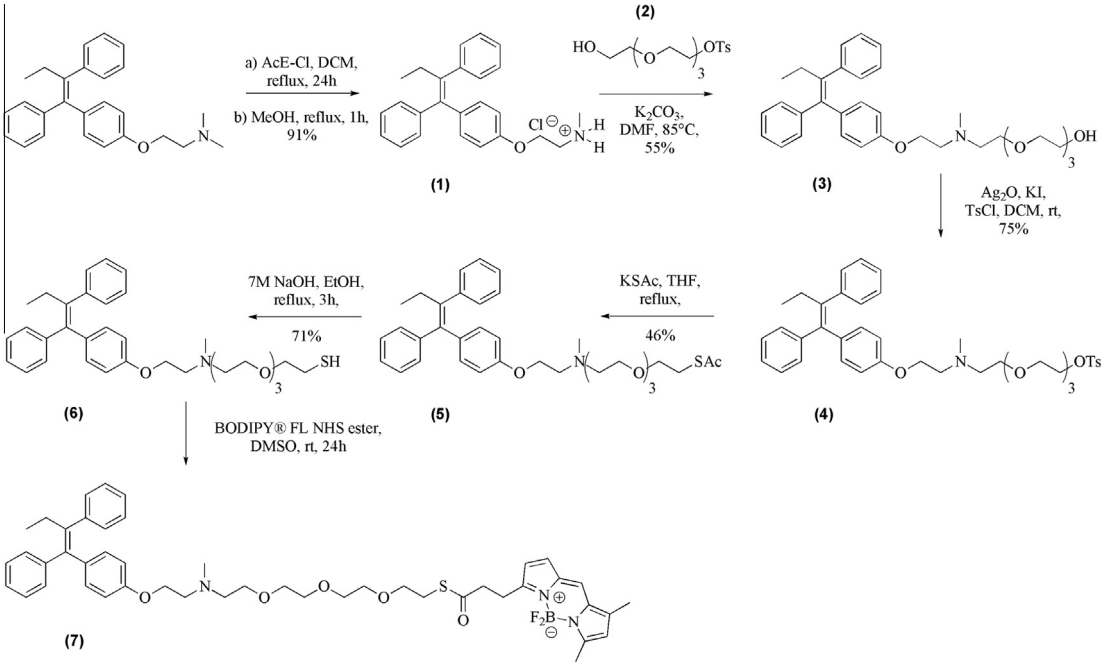
Since the triphenylethylene core is the motif required to bind to the ER, the attachment of the dye has been made through the basic alkylaminoethoxy side chain, which is accepted to protrude out of the receptor binding pocket.[21] A short ethylene glycol linker was thought to be the best approach for the conjugation of the dye to the tamoxifen. Bulky hydrophobic side chains have been proposed for reduced specificity and affinity in conjugated fluorescent tamoxifen.[13] It has been predicted that the highest binding affinities are observed for bivalent 4-hydroxytamoxifen compounds with very short ethylene glycol linkers.[22,23] Cell specificity was achieved for an optical probe based on a nanoparticle conjugated to tamoxifen through a short ethylene glycol.[20]

Although the metabolite of the drug, OHT has 30–100 times more affinity for the ER,[24] we have chosen to tether the fluorophore directly to tamoxifen. In addition to being commercially available, sufficient binding has been found for probes based on tamoxifen rather than the metabolite.[15] A large number of commercial dyes exist, we have chosen to use the BODIPY®FL fluorophore as it has been well characterized as a cell permeable fluorophore. Although not cell specific the BODIPY®FL conjugated OHT has displayed unusual localization in the cytoplasm making it an ideal choice for our dye.[19,13]

The synthesis of tamoxifen conjugated with BODIPY®FL is outlined in Scheme 1. We have used a traditional conjugation approach to develop the probe compound based on tamoxifen.[25] Briefly, the tertiary amine of commercially available tamoxifen was demethylated using α-chloroethyl chloroformate to produce the quaternary ammonium salt **1**.[26] Subsequent nucleophilic substitution between monotosylated polyethylene glycol **2**[27] and the amine hydrochloride **1** afforded tertiary amine **3**. The terminal hydroxyl group of **3** was then tosylated to give compound **4**, and then substituted with thioacetate to give **5**. Thioacetate **5** was then hydrolyzed to give the thiol **6** and subsequently conjugated with the thiol active maleimide BODIPY®FL fluorophore NHS ester to give the BODIPY®FL conjugated tamoxifen **7**.

The cellular localization of **7** in ER-positive MCF7 and ER-negative MDA231 breast cell lines was visualized by fluorescent confocal microscopy.[28] Following incubation of the cells with the conjugate, uptake studies found that **7** was internalised in the ER-positive but not ER-negative cells (Fig. 2). The specific uptake pattern suggests a receptor-mediated mechanism of uptake. No difference in degree of uptake or localization is found with an increase in concentration of **7**. We did not find any localization of the BODIPY®FL conjugate in the cytoplasm.

Tamoxifen has been administered as a chemotherapeutic agent for the treatment of breast cancer for over three decades. The active form of the drug competes with estrogen in the binding of ER sites. The selectivity of this small compound makes it an ideal candidate to develop fluorescent-based detection tools. Despite the potential of this small compound there are very few examples of fluorescent-based analogues found in the literature, and those that have been reported have associated drawbacks. Hence, this project has involved the development of a fluorescently labeled analogue of tamoxifen by conjugating a fluorescent BODIPY®FL group via an ethylene glycol linker unit. Preliminary cell testing shows that the molecule has maintained its cell specificity in



Scheme 1. Synthesis of BODIPY®FL conjugate of tamoxifen **7**.