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A Chimeric SERM-Histone Deacetylase Inhibitor Approach to Breast Cancer Therapy

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Abstract

Breast cancer remains a significant cause of death in women and few therapeutic options exist for estrogen receptor negative ER(–) cancers. Epigenetic re-activation of target genes using histone deacetylase (HDAC) inhibitors has been proposed in ER(–) cancers to resensitize to therapy using selective estrogen receptor modulators (SERMs) that are effective in ER(+) cancer treatment. Based upon preliminary studies in ER(+) and ER(–) breast cancer cells treated with combinations of HDAC inhibitors and SERMs, hybrid drugs were designed with computational guidance. Assay for inhibition of four Type I HDAC isoforms and antagonism of estrogenic activity in two cell lines yielded a “SERMostat” with 1–3 μ M potency across all targets. The superior hybrid caused significant cell death in ER(–) human breast cancer cells and elicited cell death at the same concentration as the parent SERM in combination treatment and at an earlier time point.

Keywords

histone deacetylase; estrogen receptor; HDAC inhibitor; SERM; breast cancer

Introduction

Breast cancer is the second leading cause of cancer death in women. The chance of developing invasive breast cancer at some point in a woman's life remains at 12%. About 70–80% of breast cancers are estrogen receptor positive, ER(+), assessed as such because of the presence of ER α that mediates proliferation in response to estrogen. Classical estrogen signaling is mediated by two isoforms acting as liganded nuclear receptors: ER α and ER β .^[1] Ligand binding causes a conformational change leading to receptor dimerization and binding to transcriptional elements in DNA which precedes recruitment of transcriptional coregulators.^[2] Tamoxifen, a first line therapy for patients with ER(+) breast cancer, is sometimes referred to as: i) an antiestrogen, since it opposes the actions of estrogen in the breast; and ii) the prototypical selective estrogen receptor modulator (SERM), because it can also act as an agonist at ER, notably in the uterus. Unfortunately, tamoxifen therapy is ineffective for ER(–) and triple negative breast (TNB) cancers, and roughly half of ER(+) tumors are insensitive or lose response to continued tamoxifen therapy and gain resistance.^[3]

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Current treatment options for ER(–), TNB, and tamoxifen-resistant breast cancers are limited to cytotoxic chemotherapy. A proposed therapeutic approach is the use of histone deacetylase (HDAC) inhibitors for various cancers, which may function in part via epigenetic modulation of ER signaling.^[4] This approach stems from the crucial role of acetylation in regulating gene transcription and the importance of HDACs in suppressing transcription of ER-dependent gene products and of ER itself in ER(–) breast cancer.^[5] Treatment of ER(–) breast cancer cells with DNMT (DNA methyltransferase) and HDAC inhibitors has been reported to induce re-expression of ER α while simultaneously sensitizing these cells to the actions of antiestrogens.^[6] The presence of HDAC1 in a transcriptional co-repressor complex with DNMTs was found to be associated with a silenced ER α promoter in ER(–) cells and was reported to be unsilenced upon HDAC inhibitor treatment leading to activation of ER α gene expression.^[7] Studies on ER(–) cells using HDAC inhibitors (panabinoestat or scriptaid) in combination with tamoxifen, reported enhanced antiproliferative actions and upregulation of ER α .^[8]

In ER(+) cells, knockdown of HDAC1 and HDAC inhibition by either trichostatin A (TSA), butyric acid, or valproic acid (VPA), have independently been reported to decrease ER α levels.^[9] Several papers have shifted focus from ER α , suggesting that increasing levels of ER β in ER(+) cells is most important in relation to the combined interactions of HDAC inhibitors with SERMs and antiestrogens.^[10] The combination of an HDAC inhibitor (TSA, VPA, or vorinostat) with tamoxifen, or the newer generation SERM, raloxifene, was reported to provide antiproliferative or apoptotic actions in ER(+) cell lines.^[9b, 10b, 11] Other researchers have argued against the central importance of ER in inhibition of breast cancer cell growth,^[9a, 9b, 12] however, regardless of the exact mechanism, the strength of the preclinical data has supported clinical trials on tamoxifen/panobinoestat and tamoxifen/vorinostat combination therapy in TNB and endocrine resistant breast cancer, respectively.^[13]

Estrogen signaling requires the displacement of proteins from corepressor complexes, including HDACs, and the recruitment of coactivator proteins to transcription complexes containing liganded ER. Substantial evidence supports the direct interaction of HDACs with corepressor proteins and ER in silenced nuclear transcription complexes in the cell nucleus, as, for example, in interaction of HDAC1 with the activation function 2 (AF2) and DNA binding domain (DBD) of ER α .^[14] Since combination SERM + HDAC inhibitor therapy requires both drugs to be in close proximity in the cell nucleus, the concept of an HDAC inhibitor and SERM hybrid is justified. We report the first test of concept in ER(+) and ER(–) breast cancer cell lines. Drug design was based around a benzothiophene SERM scaffold, hence we term these hybrid HDAC inhibitors, SERMostats.

Results and Discussion

1. Drug Design Rationale: HDAC and ER(–) breast cancer

Endocrine therapy, targeting estrogen signaling through aromatase inhibitors or antiestrogens, such as fulvestrant and tamoxifen, is the cornerstone of breast cancer therapy. However, treatment options for ER(–) and tamoxifen-resistant breast cancers are limited to cytotoxic chemotherapeutics. An alternative therapeutic approach that has been studied in various cancers is the use of HDAC inhibitors. The mechanistic rationale for combination therapy with antiestrogens in ER(–) breast cancer is based upon the use of epigenetic modulators, including DNMT and HDAC inhibitors, to induce re-expression of ER in ER(–) cancer and thus restore susceptibility to antiestrogen therapy.^[6, 15] There is support for this hypothesis, for example: 1) an aberrant methylated ER α promoter is found in more than 25% of ER(–) breast cancers, while in ER(+) tumors, ER promoter methylation was not observed;^[16] and 2) the presence of HDAC1 in a transcriptional co-repressor complex with

DNMTs is associated with a silenced ER α promoter in ER(–) cells.^[9b] It is uncertain if all ER(–) breast cancers would be resensitized to antiestrogen therapy through epigenetic upregulation of ER α , however, enhanced antiproliferative effects of antiestrogens have been correlated with increased ER α in response to HDAC inhibition.^[6b, 7–8, 15]

HDAC and ER(+) breast cancer—Obviously, the same resensitization rationale for ER(–) cancers cannot be applied to mammary tumor cells that already express ER α , but do not respond to antiestrogen therapy. Decreased levels of ER α reported in response to inhibition of HDAC activity,^[9] would attenuate estrogen-dependent proliferation, however, repressed ER α expression on overexpression of HDAC1 in ER(+) cells was reported and observed to be restored by administration of TSA.^[14] An alternate hypothesis for use of HDAC inhibitors in combination in ER(+) cancer cells has been proposed based upon upregulation of ER β by HDAC inhibition and antiapoptotic actions of antiestrogens mediated by ER β .^[10a, 12] However, other mechanisms have been proposed independent of ER β .^[9a] Nevertheless, studies report antiproliferative actions on combination treatment of ER(+) cells.^[14]

HDAC isoforms—HDACs are divided into four families: Class I (HDAC1, -2, -3 and -8), Class II (HDAC 4, -5, -6, -7, -9, -10), Class III (sirtuins) and Class IV (HDAC11).^[17] The presence of HDAC2 and HDAC3 on promoter regions of ER target genes was identified after administration of 4-hydroxytamoxifen (4OHT) to 5azadC/TSA pretreated cells: HDAC2 as a part of the Mi2/NuRD corepressor complex; and HDAC3, a part of the NCoR complex.^[18] Inhibition of HDAC2 activity by siRNA also showed reduced ER α and progesterone receptor (PR) levels in ER(+) cells.^[9b] The vorinostat/tamoxifen phase 2 clinical trial reported response to combination therapy in subjects with a higher level of HDAC2.^[13] All class I HDACs, with the exception of HDAC8, have been implicated as mediators of estrogen signaling in both ER(+) and ER(–) breast cancer. Less evidence exists for the importance of class II HDACs, although: i) HDAC4 was reported to be recruited to target gene promoters as part of corepressor complexes after administration of tamoxifen and raloxifene;^[19] ii) inhibition of HDAC6 has been reported to downregulate ER α levels by cytoplasmic mechanisms;^[9b, 20] and iii) HDAC7 is present on pS2 gene promoter regions.^[9d] Class III & IV HDACs have been least studied in relation to breast cancer.

SERMostat design—Substantial preclinical data, summarized above, and ongoing clinical trials on combination therapies justify study of a hybrid molecule delivering both antiestrogenic SERM activity and HDAC inhibition (SERMostat) to the ER transcriptional complex in the cell nucleus, the target for both pharmacophores. Design of a SERMostat simultaneously binding ER and HDAC has inherent difficulties and is not supported by structural information defining the required geometry. On the other hand, we have shown that a benzothiophene SERM can be used to transport and localize a cargo to the nucleus of ER(+) breast cancer cells.^[21] The SERMostat was designed wherein the cargo was an HDAC inhibitor pharmacophore. We have previously explored extensive modifications of the benzothiophene SERMs, arzoxifene and raloxifene, demonstrating that antiestrogenic activity and ER binding can be maintained with substantial chemical modifications to the C3 side chain.^[21a, 22] The active metabolite of arzoxifene, desmethylarzoxifene (DMA), differs from raloxifene by one C atom and retains the 2-phenylbenzo[b]thiophen-6-ol core. Therefore, decoration of 2-phenylbenzo[b]thiophen-6-ol with a hydroxamate side chain would be predicted to yield a SERMostat. Vorinostat (SAHA) and hydroxamate, **1**, represent control HDAC inhibitors without actions at ER. The slow-binding HDAC inhibitor, entinostat, was the inspiration for design of **10**, incorporating an o-hydroxybenzamide moiety, expected to provide activity against HDAC1/3.^[23] Both approaches incorporate Zn-binding groups to impart inhibition of class I HDACs (Fig. 1A).^[22b, 24]

Computer modeling—Candidate SERMostats were docked to crystal structures of HDAC2 and ER α to select candidates for synthesis. The HDAC2 isoform was prioritized because of data from clinical trials on vorinostat/tamoxifen combination therapy.^[13] After energy minimization in MOE, docking with the crystal structure of HDAC2 (3MAX)^[25], and scoring the obtained binding poses using an HDAC inhibitor training set,^[25] binding affinities were calculated using the Amber 12 forcefield. All hydroxamate ligands were confirmed to bind to HDAC2 in silico in a similar binding orientation to SAHA (Fig. 1C). The highest HDAC2 binding affinity values were calculated for the triazoles, **15a** and **15b**, with the amide-linked hydroxamate **8a** of similar affinity and **8b** marginally lower by 1.7 kcal/mol. The benzamide, **10**, was predicted to be a weaker ligand by 6.3 kcal/mol compared to **15b** (Fig. 1 D). A similar approach was taken for ER α binding using the raloxifene co-crystal structure (1ERR; raloxifene co-crystal; Fig. 1B)^[26]. Hybrids **15a** and **15b** were calculated to have the highest binding affinity, with **8a**, **8b**, and **10** weaker ligands by 1–5 kcal/mol.

2. Synthesis

The synthesis of the amide linked SERMostat candidates **8a** and **8b** was accomplished from commercially available 6-methoxy-2-(4-methoxyphenyl)benzothiophene **2** which was brominated with bromine in hot chloroform to yield 3-bromobenzothiophene **3** in 79% yield.^[27] Bromine-lithium exchange using *n*-BuLi in anhydrous THF at –78°C followed by quenching with solid CO₂ afforded the acid **4** after aqueous workup.^[28] This acid could be coupled to ω -aminoalkanoic acid esters through its chloride to obtain esters **5a** and **5b** which then were demethylated with BBr₃ which resulted in the loss of the methyl ester and the formation of acids **6a** and **6b**. The acids were esterified with MeOH and HCl to the methyl esters **7a** and **7b** before the final conversion to hydroxamic acids **8a** and **8b** with excess NH₂OH.^[29] Acid **4** was converted to the acid chloride using SOCl₂ and coupled with 3-amino-4-methoxybiphenyl (synthesized according to literature procedures)^[30] to obtain amide **9**. This compound could be demethylated with BBr₃ to yield the *o*-hydroxybenzamide **10**.

Friedel-Crafts acylation of the common starting material **2** with 4-iodobenzoyl chloride afforded ketone **11** in 60% yield (Scheme 2).^[31] Sonogashira coupling with ethynyltrimethylsilane and deprotection of the obtained TMS-protected alkyne **12** with KF then demethylation with BBr₃ yielded the alkyne **13a**.^[28] Through copper(I)-catalysed Huisgen [3+2] cycloaddition with ω -azidoalkanoic acid esters, this intermediate was used to obtain the triazoles **14a** and **14b** which could be converted into the SERMostats, **15a** and **15b**, with excess NH₂OH in MeOH.^[32]

3. Inhibition of HDACs and antagonism at ER

The HDAC inhibitory activity for Class I HDACs was evaluated for the synthesized hybrids (Table 1). Both **1** and SAHA are broad spectrum Class I HDAC inhibitors, although **1** demonstrates selectivity for HDAC3 over 1, 2, and 8. Attachment of an alkyl hydroxamate chain to the benzothiophene core of DMA/raloxifene via a simple amide linker led to broad spectrum Type I HDAC inhibitors, generally with submicromolar IC₅₀ values. Extension of the 3-benzothiophene side chain to include more structural components of raloxifene linked via a triazole to the alkyl hydroxamate led to some loss of activity, in particular towards HDAC8. However, **15b** retained reasonable activity towards HDACs 1, 2 and 3. The benzamide derivative **10** demonstrated measurable, time-dependent inhibition, as expected for this chemotype, for HDAC1 and HDAC3, but as predicted by computational studies, activity was weak.

The ability of SERMostats to engage the ER target in a cellular environment was studied using transfection of an ERE-luciferase reporter in MCF-7 breast cancer cells. PPT and DPN, as ER α -selective and ER β -selective agonists, respectively, illustrate the assay as indicative of classical ER α -mediated gene-transcriptional activity (Fig. 2a). The extended side chain of all hybrids prepared is predictive of antiestrogenic as opposed to agonist activity and indeed, **8b** and **15b** showed concentration dependent inhibition of estrogenic response. Other candidate SERMostats showed no significant activity > 25 μ M. Upregulation of alkaline phosphatase in Ishikawa human endometrial cells is a second standard assay for ER α activity. The IC₅₀ of **15b** was determined as 2.9 ± 0.3 μ M in this assay (Fig. 2b). In addition to confirming the potency of **15b** in a second cell system, it was seen as important to provide an indication that the antiestrogenic activity of benzothiophene SERMs in endometrial tissues was retained by **15b**.

4. Activity of combinations in breast cancer cells

Preliminary experiments were carried out on the effect of combination treatments in ER(–) and ER(+) breast cancer cell lines, BT-20 and MCF-7, respectively. To provide comparison with previous reports, the active metabolite of tamoxifen, 4OHT, was studied in combination with SAHA showing modest but significantly increased cell death after 72 h of combination treatment in ER(+) cells, but not ER(–) cells (Supplemental Figure 1). In comparison, Bicaku et al. reported approximately 10% increase in apoptosis of MCF-7 cells after 48 h treatment with 4-OHT (10 μ M) in combination with SAHA over SAHA alone.^[9b] The bioactivity of tamoxifen differs from later generation SERMs, such as raloxifene and arzoxifene, in several important ways, including differing gene profiles and tissue-selective estrogenicity, most notably in uterine proliferation, which is a serious safety concern for tamoxifen.^[33]

Therefore, cell culture experiments were repeated with DMA in combination with either of one of the three HDAC inhibitors: SAHA, MS-275 or **1** (Fig. 3). DMA (10 μ M) was toxic towards MCF-7 cells after 72 h treatment and although the combination was significantly more effective than HDAC inhibitor alone for SAHA and **1**, the combination effect relative to DMA alone was not significant. In contrast, ER(–) cells were less sensitive to both HDAC inhibitors and DMA. Further, the cytotoxicity of DMA combinations with SAHA and **1** were significantly greater than for DMA alone.

Given the observations in combination treatment of ER(–) cells, more detailed comparison was made with SAHA and **1** (1 μ M) in the two ER(–) cell lines, BT-20 and MDA-MB231. In combination with DMA (10 μ M), after 72 h incubation, the combination treatment significantly increased cell death in both cell lines (Fig. 4). Significant upregulation of ER mRNA and re-expression of the protein was reported only after 48 h administration with TSA^[6b]. In another study, administration of HDAC inhibitor caused a time dependent increase in ER expression suggesting that the reexpression of ER by HDAC inhibitors parallels the cytotoxic effect of the combination^[8a, 34]. However, other possible delayed mechanisms of toxicity have been proposed, for example, through growth factor downregulation, or reduction in NF κ B transcription. Our observations in ER(–) breast cancer cells after combination treatments with DMA supported the design of a SERMostat hybrid based upon a DMA core with a hydroxamate side chain.

5. Activity of hybrids in breast cancer cells

The four hydroxamate and one benzamide candidate SERMostats were assayed in MDA-MB-231 cells to measure the influence of combining the two pharmacophores in a hybrid molecule (Fig. 5). Only **15b** showed cytotoxicity towards this ER(–) cell line, compatible with this being the only candidate hybrid that demonstrated both HDAC inhibitory and

antiestrogenic activity, i.e. SERMostat properties. The concentration of the SERMostat required to cause 50% cell death of ER(–) breast cancer cells was 5–10-fold higher than the IC₅₀ values measured for the target proteins, HDAC and ER α . Literature data on combinations of antiestrogens with HDAC inhibitors also report that cytotoxicity requires concentrations of antiestrogens (1 μ M [8, 11a], 2.5 μ M [15], 10 μ M [9b, 10b, 18, 35]), which are 1000-fold higher than the measured antiestrogen IC₅₀ at ER α .

The SERMostat was further compared with combination treatments using raloxifene as the antiestrogen. At 72 h, cytotoxicity was similar for hybrid and combination treatments. Interestingly, it was observed that the hybrid molecule was efficacious at causing cell death at 48 h, in contrast to the combination treatment. Furthermore, cell death at 48 and 72 h was observed to be significant and dependent on hybrid drug concentration.

Conclusion

The need for therapeutic targeting of ER(–) breast cancer and the current use of HDAC inhibitors and antiestrogens as a combination therapy in clinical trials led to the design of candidate hybrid molecules, SERMostats, that had dual actions as class I HDAC inhibitors and antiestrogens. Preliminary experiments were carried out with combinations of classical HDAC inhibitors and antiestrogens/SERMs in ER(+) and ER(–) cell lines. Since it was planned to design a SERMostat based upon a benzothiophene scaffold, comparison with combination treatments including the benzothiophene SERM, DMA, were prioritized. Contradictory literature reports have appeared on the efficacy of combination treatment with HDAC inhibitors and antiestrogens in ER(+) cells.^[12, 36] In our studies, at the concentration of the three HDAC inhibitors tested in combination with DMA, only in ER(–) cells was a significant increase in cell death observed. This combination effect was seen in two cell lines and with two different HDAC inhibitors.

Five candidate SERMostats were designed, synthesized, and assayed for inhibition of HDACs, 1, 2, 3, and 8. One SERMostat proved to be antiestrogenic in cell culture and to inhibit HDACs 1, 2, and 3, with IC₅₀ values of 1–3 μ M. The extent of cell killing of ER(–) breast cancer cells by the SERMostat was equivalent to combination treatments with 4OHT, raloxifene, or DMA, despite these SERMs having antiestrogenic potency higher by 2–3 orders of magnitude. Moreover, the hybrid SERMostat caused significant cell death at earlier time points than combination treatments. A number of interesting approaches to hybrid HDAC inhibitors have been reported, including chimeric nuclear receptor ligands,^[37] and a separate hybrid approach to breast cancer.^[38] This is the first report of a SERMostat approach with efficacy in ER(–) breast cancer cells and serves as a proof of concept.

Experimental Section

Synthetic procedures

All chemicals and reagents were purchased from Sigma Inc. or Cayman Chemicals unless specified otherwise. Unless stated otherwise, all reactions were carried out under an atmosphere of dry argon in oven-dried glassware. Indicated reaction temperatures refer to those of the reaction bath, while room temperature (rt) is noted as 23°C. Dichloromethane (CH₂Cl₂) was distilled over CaH₂, and THF distilled over Na(s). All other solvents were of anhydrous quality purchased from Aldrich Chemical Co. and used as received. Pure reaction products were typically dried under high vacuum in the presence of phosphorus pentoxide. Commercially available starting materials and reagents were purchased from Aldrich, TCI and Fisher Scientific and were used as received unless specified otherwise. Analytical thin layer chromatography (TLC) was performed with glass backed silica plates (5 × 20 cm, 60 Å, 250 μ m). Visualization was accomplished using a 254 nm UV lamp. ¹H and ¹³C NMR

spectra were recorded on either a Bruker Avance 400 MHz spectrometer or Bruker DPX 400 MHz spectrophotometer. Chemical shifts are reported in ppm with tetramethylsilane as standard. Data are reported as follows: chemical shift, number of protons, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, b = broad, m = multiplet, abq = ab quartet), and coupling constants. High resolution mass spectral data were collected on a Shimadzu Q-TOF 6500.

3-bromo-6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene (3)^[27–28]—A

solution of 6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene **2** (0.880 g, 3.26 mmol) in CHCl₃ (35 mL) was heated to 50°C until full dissolution and a solution of elemental Br₂ (0.518 g, 3.24 mmol) in CHCl₃ (5 mL) was added dropwise over 5 min while keeping the temperature constant at 50°C, then heating was continued for 5 min and the yellow solution was left to cool. The beige crystals that had separated were recrystallized from CH₂Cl₂/hexane to white crystals (0.897 g, 79%). ¹H NMR (CDCl₃) δ= 7.73 (d, 1H, Ar-H, J = 8 Hz), 7.70 (d, 2H, 2 Ar-H, J = 8 Hz), 7.28 (s, 1H, Ar-H), 7.09 (d, 1H, Ar-H, J = 8 Hz), 7.02 (d, 2H, Ar-H, J = 8 Hz), 3.99 (3H, s, OCH₃), 3.91 (3H, s, OCH₃). ¹³C NMR (CDCl₃) δ=159.42, 157.77, 138.30, 134.95, 132.89, 130.36 (2C), 125.20, 123.78, 114.65, 113.63 (2C), 104.32, 103.25, 55.32, 54.97.

6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene-3-carboxylic acid (4)^[27]—

A solution of **3** (1.770 g, 5.07 mmol) in anhydrous THF (35 mL) was cooled to –78°C in a dry ice - acetone bath and treated dropwise with 1.6 M *n*-BuLi in hexanes (3.6 mL, 5.76 mmol). After the addition was complete, the solution set up to a suspension of the lithiated compound and stirring at –78°C was continued for 2 h. The reaction mixture was quenched with solid CO₂ (5.0 g, 113.6 mmol) and stirred overnight, allowing the temperature to rise to 20°C. To the white suspension 1M NaOH was cautiously added until all the solid had gone into solution. This aqueous solution was washed twice with CH₂Cl₂ and the washes were discarded. The solution was acidified to pH < 2 with 37% HCl and the product extracted with EtOAc (4×50 mL). After washing with water and brine and drying on Na₂SO₄ followed by evaporation and washing of the crude material with hexane the title compound was obtained as off-white powder (1.068 g, 67%).

¹H NMR (CDCl₃) δ= 8.27 (1H, d, J = 8 Hz), 7.49 (2H, d, J = 8 Hz), 7.27 (1H, s, Ar-H), 7.07 (1H, d, J = 8 Hz, Ar-H), 6.95 (2H, d, J = 8 Hz, 2 Ar-H). ¹³C NMR (CDCl₃) δ= 165.69, 159.53, 156.97, 149.37, 139.25, 132.42, 130.40 (2C), 125.93, 125.01, 114.55, 113.08 (2C), 103.60, 55.08, 54.81.

Methyl 7-(6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene-3-carboxamido)heptanoate (5a)—

A suspension of acid **4** (0.690 g, 2.20 mmol) in anhydrous toluene (15 mL) was refluxed with SOCl₂ (2.0 mL) for 2 h, then the solvents were evaporated and the greenish-brown crystals of the acid chloride were taken up in CH₂Cl₂ and added dropwise to a solution of the HCl salt of methyl 6-aminoheptanoate (0.510 g, 2.61 mmol) and Et₃N (3 mL) in anhydrous CH₂Cl₂ (25 mL) under Ar at 0°C. The reaction mixture was stirred for 24 h, washed with H₂O and brine, dried on Na₂SO₄ and evaporated. The residual oil was purified by column chromatography on silica using hexane/EtOAc 7:3 as eluent affording the product as slightly yellowish oil that set up as crystals (0.746 g, 75%). ¹H NMR (CDCl₃) δ= 7.98 (1H, d, J = 8.8 Hz), 7.50 (2H, d, J = 8.4 Hz), 7.26 (2H, s), 7.04 (1H, dd, J₁ = 2.0 Hz, J₂ = 8.8 Hz), 6.97 (2H, d, J = 8.4 Hz), 5.55 (1H, b, NH), 3.89 (3H, s, OCH₃), 3.87 (3H, s, OCH₃), 3.67 (3H, s, OCH₃), 3.32 (2H, m), 2.29 (2H, m), 1.57 (2H, m), 1.40 (2H, m), 1.26 (2H, m), 1.16 (2H, m). ¹³C NMR (CDCl₃) δ= 174.07, 165.22, 160.17, 157.70, 140.78, 139.96, 133.06, 130.45 (2C), 127.62, 125.46, 124.55, 114.82, 114.29 (2C), 104.29, 55.59, 55.37, 51.45, 39.53, 33.90, 29.04, 28.73, 26.48, 24.72.

HRMS-ESI: m/z $[M+Na^+]$ calculated for $C_{25}H_{29}NO_5S$: 478.16590, observed: 478.1657 ($M+Na^+$)

Methyl 6-(6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene-3-carboxamido)hexanoate (5b)—

A suspension of **4** (0.260 g, 0.83 mmol) in anhydrous toluene (15 mL) was refluxed with $SOCl_2$ (1.0 mL) for 2 h, then the solvents were evaporated and the acid chloride was taken up in CH_2Cl_2 and added dropwise to a solution of the HCl salt of methyl 6-aminohexanoate (0.366 g, 2.02 mmol) and Et_3N (1.0 mL) in anhydrous CH_2Cl_2 (7.5 mL) under Ar at $0^\circ C$. The reaction mixture was stirred for 24 h, washed with H_2O and brine, dried on Na_2SO_4 and evaporated. The residual oil was purified by column chromatography on silica using gradient elution from hexane/ CH_2Cl_2 2:8 to CH_2Cl_2 to $CH_2Cl_2/MeOH$ 20:1 as eluent affording brownish crystals (0.300 g, 82%). 1H NMR ($CDCl_3$) δ = 7.99 (1H, d, J = 8.5 Hz), 7.51 (2H, d, J = 8 Hz), 7.28 (2H, s), 7.05 (1H, dd, J_1 = 2.2 Hz, J_2 = 8.8 Hz), 6.98 (2H, d, J = 8.5 Hz), 5.63 (1H, b, NH), 3.89 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.33 (2H, m), 2.26 (2H, m), 1.58 (2H, m), 1.41 (2H, m), 1.18 (2H, m). ^{13}C NMR ($CDCl_3$) δ = 198.82, 173.94, 165.26, 160.22, 157.73, 140.85, 139.99, 133.10, 130.51 (2C), 127.65, 125.48, 124.57, 114.85, 114.32 (2C), 104.32, 55.62, 55.40, 51.50, 39.41, 33.80, 28.96, 26.34, 24.50.

6-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-carboxamido)hexanoic acid (6a)—

A solution of **5a** (0.231 g, 0.52 mmol) in anhydrous CH_2Cl_2 (7 mL) was placed under a blanket of Ar and cooled to $0^\circ C$. A solution of BBr_3 in CH_2Cl_2 (2.5 mL of a 1M solution, 2.5 mmol) was dripped in and stirring was continued for 5 h letting the solution attain $20^\circ C$. The orange-brown solution with yellow precipitate was diluted with EtOAc (40 mL) and the product was extracted into saturated $NaHCO_3$ solution (4×20 mL). The organic phase was colorless after the extractions. The combined aqueous layers were washed with EtOAc, the pH adjusted to 1 by the cautious addition of 5M HCl and the solution was extracted with EtOAc (4×30 mL). The pooled organic solutions were dried on Na_2SO_4 and evaporated to obtain the title compound as brownish clear glass (0.151 g, 72%). 1H NMR ($CDCl_3$) δ = 7.71 (1H, d, J = 8.7 Hz), 7.32 (2H, d, J = 8.4 Hz), 7.15 (1H, d, J = 1.8 Hz), 6.90 (1H, dd, J_1 = 8.7 Hz, J_2 = 1.8 Hz), 6.83 (2H, d, J = 8.4 Hz), 3.36 Hz (3H, s), 3.24 (2H, m), 2.21 (2H, t, J = 7.5 Hz), 1.51 (2H, m), 1.39 (2H, m), 1.12 (2H, m). ^{13}C NMR ($CDCl_3$) δ = 176.58, 166.85, 166.77, 157.70, 154.74, 140.82, 139.88, 132.15, 130.15 (2C), 126.81, 126.77, 124.30, 123.70, 115.70 (2C), 114.90, 106.88, 49.60, 39.56, 39.43, 33.79, 28.52, 26.22, 24.40.

6-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-

carboxamido)heptanoic acid (6b)—A solution of (0.629 g, 1.38 mmol) of **5b** in anhydrous CH_2Cl_2 (15 mL) was placed under a blanket of Ar and cooled to $0^\circ C$. A solution of BBr_3 in CH_2Cl_2 (1M, 5.5 mL, 5.5 mmol) was dripped in and stirring was continued for 20 h letting the solution warm up to $20^\circ C$. The colorless solution with yellow precipitate was quenched by the dropwise addition of saturated $NaHCO_3$ solution, acidified to pH < 2 with 37% HCl and extracted with EtOAc (4×30 mL). The combined organic solutions were washed with brine, dried on Na_2SO_4 and evaporated. After gradient elution on silica gel using $CHCl_3$ to $CHCl_3/MeOH$ 8% in 4 steps, the title compound was obtained as yellowish viscous oil (0.263 g, 46%). 1H NMR ($CDCl_3$) δ = 7.77 (1H, d, J = 8.7 Hz), 7.37 (2H, d, J = 8.5 Hz), 7.20 (1H, d, J = 2.0 Hz), 6.94 (1H, dd, J_1 = 2.2 Hz, J_2 = 8.8 Hz), 6.88 (2H, d, J = 8.5 Hz), 3.29 (m, 2H, CH_2), 2.28 (2H, m, CH_2), 1.56 (2H, m), 1.39 (2H, m), 1.27 (2H, m), 1.14 (2H, m). ^{13}C NMR ($CDCl_3$) δ = 166.89, 158.06, 155.10, 141.09, 140.21, 132.47, 130.49 (2C), 127.18, 124.58, 124.10, 116.00 (2C), 115.17, 107.11, 39.81, 34.26, 28.99, 28.96, 26.72, 24.95.

Methyl 6-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-

carboxamido)hexanoate (7a)—The acid **6a** (0.292 g, 0.73 mmol) was refluxed in a solution of MeOH (15 mL) and CH₃COCl (1 mL) overnight, evaporated and the crude oily product purified on a column of silica using hexane/EtOAc 1:2 as eluent. The ester was obtained as colorless oil (0.159 g, 53%).

¹H NMR (CDCl₃) δ= 7.76 (1H, d, J = 8.8 Hz), 7.36 (2H, d, J = 8.4 Hz), 7.20 (1H, d = 2.1 Hz), 6.93 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.1 Hz), 6.87 (2H, d, J = 8.8 Hz), 3.66 (3H, s), 3.28 (2H, t, J = 7.5 Hz), 2.27 (2H, t, J = 7.8 Hz), 1.56 (2H, m), 1.41 (2H, m), 1.14 (2H, m). ¹³C NMR (CDCl₃) δ=178.37, 170.24, 161.32, 158.35, 144.332, 143.46, 135.73, 133.72, 127.84, 127.30, 119.23, 118.45, 110.40, 99.60, 55.06, 42.94, 37.29, 32.10, 29.77, 27.94.

Methyl 7-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-

carboxamido)heptanoate (7b)—The acid **6b** (0.235 g, 0.57 mmol) was dissolved in a solution of MeOH (15 mL) and CH₃COCl (0.9 mL) and refluxed overnight. After evaporation of the solvent, the remaining brown oil was purified by column chromatography using hexane/EtOAc 1:2 as eluent, yielding colorless oil as the main fraction that set up as crystals (0.146 g, 60%). ¹H NMR (CDCl₃) δ= 7.78 (1H, d, J = 8.8 Hz), 7.32 (2H, d, J = 8.5 Hz), 7.15 (1H, d, J = 2.2 Hz), 6.90 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz), 6.83 (2H, d, J = 8.5 Hz), 3.64 (3H, s, OCH₃), 3.23 (2H, t, CH₂, J = 6.8 Hz), 3.06 (3H, b), 2.25 (2H, t, J = 7.5 Hz), 1.50 (2H, m), 1.32 (2H, m), 1.24 (2H, m), 1.05 (2H, m). ¹³C NMR (CDCl₃) δ= 208.49 (COO), 175.16 (CO), 166.12, 157.73, 154.74, 140.89, 139.89, 132.18, 130.32 (2C), 129.92, 124.27, 124.05, 115.75 (2C), 114.92, 106.84, 51.63, 39.44, 33.89, 30.78, 28.74, 26.43, 24.61.

6-hydroxy-N-(6-(hydroxyamino)-6-oxohexyl)-2-(4-

hydroxyphenyl)benzo[b]thiophene-3-carboxamide (8a)—A suspension of NH₂OH * HCl (4.418 g, 63.6 mmol) in anhydrous MeOH (35 mL) was cooled to 0°C under Ar and a solution of 85% KOH (4.206 g, 63.7 mmol) in MeOH was added slowly with good stirring. The stirring and cooling were maintained for 5 min then the solution was filtered to remove precipitated KCl. To the filtrate a solution of ester **7a** (0.155 g, 0.37 mmol) was added dropwise with cooling to 0°C under Ar. After 45 min, the ester had disappeared as indicated by TLC. Stirring was continued for 15 min and the clear yellowish solution was poured into crushed ice (200 g). The pH was adjusted to 7 with acetic acid and the now colorless solution was extracted with EtOAc (4×100 mL). The extracts were dried on Na₂SO₄ and evaporated yielding compound **8a** as off-white gum (0.152 g, 98%). ¹H NMR (CDCl₃) δ= 7.77 (1H, d, J = 8.8 Hz), 7.37 (3H, m), 7.20 (1H, d, J = 1.1 Hz), 6.94 (1H, dd, J₁ = 8.6 Hz, J₂ = 1.1 Hz), 6.88 (2H, d, J = 8.3 Hz), 6.44 (1H, b, NH), 3.24 (2H, m), 2.02 (2H, m, CH₂), 1.55 (2H, m, CH₂), 1.37 (2H, m), 1.26 (1H, m), 1.08 (2H, m). ¹³C NMR (CDCl₃) δ= 174.76, 170.16, 161.17, 158.37, 144.40, 143.50, 135.63, 133.85, 130.53, 127.96, 127.38, 119.32, 118.46, 110.40, 42.83, 36.12, 32.06, 29.76, 28.64. HRMS-ESI: m/z [M+H⁺] calculated for C₂₁H₂₂N₂O₅S: 415.1322, observed: m/z 415.1329 (M+H⁺).

6-hydroxy-N-(7-(hydroxyamino)-7-oxoheptyl)-2-(4-

hydroxyphenyl)benzo[b]thiophene-3-carboxamide (8b)—A suspension of NH₂OH * HCl (4.422 g, 63.6 mmol) in anhydrous MeOH (35 mL) was cooled to 0°C under Ar and a solution of KOH (4.183 g, 74.6 mmol) in MeOH was added slowly with good stirring. The stirring was maintained for 5 min then the solution was filtered. To the filtrate consisting of a solution of NH₂OH in MeOH a solution of **7b** (0.140 g, 0.34 mmol) was added dropwise with cooling to 0°C under Ar. Stirring was continued for 24 h allowing the temperature to rise to 20°C. The clear yellowish solution was poured into crushed ice (200 g) and the pH was adjusted to 7 with acetic acid. The now colorless solution was extracted with 4× 100 mL

EtOAc. The extracts were dried on Na₂SO₄ and evaporated yielding 105 mg of an off-white gum. This was dissolved in acetone containing a few drops of MeOH and treated with Et₂O until persistent cloudiness and then with hexane. After cooling to –40°C for 15 min the suspension was filtered. The filtrate was diluted with hexane and cooled to –40°C again, filtered for a second time and evaporated. The hydroxamate **8b** was obtained as nearly colorless oil (42.2 mg, 30%). ¹H NMR (CDCl₃) δ= 7.67 (1H, d, Ar-H), 7.26 (1H, d, J = 8.0 Hz), 7.25 (1H, d, J = 8.0 Hz), 7.10 (1H, s), 6.84 (1H, d, J = 8.2 Hz), 6.79 (2H, d, J = 8.1 Hz), 6.25 (1H, b, NH), 3.15 (2H, m), 1.97 (2H, m), 1.42 (2H, m). ¹³C NMR (CDCl₃) δ= 193.28, 158.49, 155.99, 143.80, 139.78, 137.35, 132.41, 132.28, 130.61, 129.95, 126.74, 123.98, 116.06, 107.57, 84.72, 83.11. HRMS-ESI: m/z [M+H⁺] calculated for C₂₂H₂₄N₂O₅S: 429.1479, observed: m/z 429.1484 (M+H⁺).

6-methoxy-N-(4-methoxy-[1,1'-biphenyl]-3-yl)-2-(4-methoxyphenyl)benzo[b]thiophene-3-carboxamide (9)—A suspension of **4** (0.441 g, 1.40 mmol) in anhydrous toluene (20 mL) was treated with SOCl₂ (1.3 mL) and refluxed for 3 h. After 0.5 h a clear solution was obtained. The solvents were evaporated and the evaporation was repeated with the addition of fresh toluene to drive off residual traces of SOCl₂. The acid chloride that was obtained as a brown solid in quantitative yield was taken up in CH₂Cl₂ (4 mL) and dripped into a solution of 4-methoxy-(1,1'-biphenyl)-3-amine (0.711 g, 3.57 mmol) and Et₃N (1.5 mL) in anhydrous CH₂Cl₂ (6 mL) held at 0°C under Ar. The reaction was stirred for 3 days at 20°C, diluted with CH₂Cl₂ (15 mL), washed with 0.5 M NaOH solution, 0.5 M HCl, brine and finally dried on Na₂SO₄ and evaporated. The brown oil was purified by column chromatography on a silica column using hexane/EtOAc 7:3 as eluent. The product was obtained as light amber foam (0.40 g, 58%). ¹H NMR (CDCl₃) δ= 8.94 (b, NH), 8.26 (d, 1H, J = 8.2 Hz), 8.00 (1H, s), 7.68 (d, 2H, J = 6.8 Hz), 7.57 (d, 2H, J = 6.8 Hz), 7.45 (d, 2H, J = 6.8 Hz), 7.35 (d, 1H, J = 6.5 Hz), 7.30 (m, 2H), 7.10 (d, 1H, J = 8.8 Hz), 6.97 (2H, d, J = 6.8 Hz), 6.83 (1H, d, J = 8.0 Hz), 3.91 (3H, s), 3.84 (3H, s), 3.57 (3H, s). ¹³C NMR (CDCl₃) δ= 162.31 (CO), 160.01, 157.41, 147.07, 142.24, 140.36, 139.63, 133.78, 132.81, 130.57 (2C), 128.29 (2C), 127.66, 127.06, 126.90 (2C), 126.63, 124.82, 124.68, 121.72, 118.04, 114.62, 113.99 (2C), 109.66, 103.89, 55.22, 55.12, 54.98.

6-hydroxy-N-[4-hydroxy-(1,1'-biphenyl)-3-yl]-2-(hydroxyphenyl)benzo[b]thiophene-3-carboxamide (10)—A solution of **9** (0.32 g, 0.65 mmol) in anhydrous CH₂Cl₂ (3 mL) was cooled to 0°C under Ar and treated dropwise with 1M BBr₃ solution in CH₂Cl₂ (4.4 mL, 4.4 mmol). The brownish yellow suspension was stirred for 18 h while letting the temperature rise slowly to 20°C. The reaction mixture was then quenched by the dropwise addition of saturated NaHCO₃ solution and extracted with EtOAc (3× 20 mL). The pooled organic solutions were washed with brine, dried on Na₂SO₄ and evaporated. Purification by column chromatography on silica using hexane/EtOAc 6:4 as eluent afforded a light beige solid (0.160 g, 54%). ¹H NMR (DMSO-*d*⁶) δ= 9.89 (1H, bs, OH), 9.82 (1H, bs, OH), 9.76 (1H, s, OH), 9.27 (1H, s, NH), 8.08 (1H, d, J = 2.1 Hz), 7.77 (1H, d = 8.7 Hz), 7.57 (d, 2H, J = 7.4 Hz), 7.45 (4H, m), 7.31 (3H, m), 6.85–6.96 (2H, m), 6.84 (2H, d, J = 8.6 Hz). ¹³C NMR (DMSO-*d*⁶) δ= 163.85, 158.13, 155.40, 148.20, 140.11, 139.09, 131.81, 129.91, 128.97, 127.18, 126.73, 126.14, 123.78, 123.57, 120.91, 116.16, 115.84, 107.02. HRMS-ESI: m/z [M+H⁺] calculated for C₂₇H₁₉NO₄S: 454.11076, observed 454.1122 (M+H⁺)

(4-iodophenyl)(6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophen-3-yl)methanone (11)^[31]—To a suspension of 6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene **2** (6.5 g, 24.0 mmol) and 4-iodobenzoyl chloride (6.5 g, 24.0 mmol) in anhydrous CH₂Cl₂ (100 mL) anhydrous AlCl₃ (4.83 g, 36.2 mmol) was

added in 3 portions and the deep red solution was stirred for 24 h then poured into ice water and extracted 4 times with CH₂Cl₂. The pooled organic extracts were washed with H₂O and brine, evaporated and the crude product was purified by column chromatography using hexane/EtOAc 8:2 as eluent. The title compound was obtained as yellow solid (7.24 g, 60%).

[6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophen-3-yl][4-((trimethylsilyl)ethynyl)phenyl] methanone (12)—To a degassed solution of **11** (7.06 g, 14.01 mmol) in anhydrous THF (100 mL) and Et₃N (20 mL) there was added (Ph₃P)₂PdCl₂ (0.101 g, 0.14 mmol) and CuI (0.086 g, 0.45 mmol) followed by ethynyltrimethylsilane (5.3 mL, 37.5 mmol) and the solution was stirred at ambient temperature for 3 days. The resulting dark suspension was filtered, evaporated and purified on a silica column using hexane/EtOAc 75:25 as eluent yielding protected alkyne **12** as brownish solid (5.1 g, 77%). The compound was deprotected without characterization.

[6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophen-3-yl][4-((trimethylsilyl)ethynyl)phenyl] methanone (13a)—A solution of protected alkyne **12** (3.704 g, 7.85 mmol) in anhydrous CH₂Cl₂ (60 mL) was cooled to 0°C in an ice bath under inert gas atmosphere and an 1M solution of BBr₃ in CH₂Cl₂ (36 mL) was added dropwise. The solution turned dark and was stirred for 20 h, poured cautiously into a mixture of sat. NaHCO₃ solution and ice and extracted with EtOAc (3×100 mL) after stirring for 20 min. The combined organic solutions were washed with H₂O and brine then evaporated to dark oil that was purified on a column of silica using CH₂Cl₂/hexane 8:2 as eluent obtaining protected alkyne **13a** as yellow solid (2.580 g, 74%). ¹H NMR (CDCl₃) δ= 7.72 (2H, d, J = 8.4 Hz, Ar-H), 7.65 (1H, d, J = 8.9 Hz, Ar-H), 7.33 - 7.37 (3H, m), 7.31 (2H, d, J = 8.8 Hz), 7.00 (1H, dd, J₁ = 8.9 Hz, J₂ = 2.4 Hz), 6.74 (2H, d, J = 8.8 Hz), 3.88 (3H, s, OCH₃), 3.75 (3H, s, OCH₃), 0.26 (9H, s, SiMe₃). ¹³C NMR (CDCl₃): 193.41, 159.94, 157.77, 144.45, 140.11, 136.95, 133.73, 131.80, 130.46, 129.86, 129.65, 127.75, 125.75, 124.13, 114.97, 114.09, 104.50, 104.20, 55.60, 55.22, 0.28.

(4-ethynylphenyl)[6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophen-3-yl]methanone (13b)—A solution of **13a** (2.570 g, 5.81 mmol) in 50 mL MeOH was treated with a solution of KF · 2 H₂O (1.797 g, 19.10 mmol) in H₂O (15 mL) then stirred at 20°C for 4 days. After extractive workup with CH₂Cl₂ and H₂O and column chromatography using hexane/EtOAc 6:4 as eluent the title compound was obtained as dark yellow solid (1.959 g, 91%). ¹H NMR (DMSO-*d*⁶) δ= 9.81 (1H, s, OH), 9.75 (1H, s, OH), 7.63 (2H, d, J = 8.4 Hz, Ar-H), 7.43 (1H, d, J = 8.4 Hz), 7.42 (1H, d, J = 8.4 Hz), 7.36 (1H, d, J = 2.1 Hz), 7.12 (2H, d, J = 8.6 Hz), 6.89 (2H, dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz), 6.64 (2H, d, J = 8.6 Hz), 4.45 (1H, s, CH). ¹³C NMR (DMSO-*d*⁶) δ= 193.28, 158.49, 155.99, 143.80, 139.78, 137.35, 132.41, 132.28, 130.61, 129.95, 126.74, 123.98, 116.06, 107.57, 84.72, 83.11. HRMS-ESI: *m/z* [M+H⁺] calculated for C₂₃H₁₄O₃S: 371.0742, observed: *m/z* 371.0735 (M+H⁺).

Ethyl 5-(4-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-carbonyl)phenyl)-1H-1,2,3 triazol-1-yl)pentanoate (14a)—A solution of alkyne **13b** (0.340 g, 0.92 mmol), ethyl 5-azidopentanoate^[32c] (0.274 g, 1.60 mmol), CuSO₄ (7.4 mg, 46 μmol), ascorbic acid (29 mg, 165 μmol) in THF (3 mL) and H₂O (2 mL) was stirred at ambient temperature for 24 h, at which point some unreacted alkyne was detected by TLC. Stirring was continued for 20 h at 50°C then the reaction mixture extracted with CH₂Cl₂ (3×5 mL). The organic solutions were washed with H₂O, dried on Na₂SO₄ and evaporated. After column chromatography (hexane/EtOAc 6:4) a bright canary yellow solid was obtained (0.239 g, 50%). ¹H NMR (DMSO-*d*⁶) δ= 9.79 (1H, s, OH), 9.69 (1H, s, OH), 8.67

(1H, s, triazole H), 7.82 (2H, d, J = 8.5 Hz, Ar-H), 7.74 (2H, d, J = 8.5 Hz, Ar-H), 7.38 (1H, d, J = 8.8 Hz), 7.36 (1H, d, J = 2.1 Hz), 7.16 (1H, d, J = 2.1 Hz), 7.12 (2H, d, J = 8.6 Hz), 6.88 (2H, dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz), 6.64 (2H, d, J = 8.6 Hz), 4.40 (2H, t, J = 6.6 Hz, CH₂), 4.02 (2H, q, J = 7.2 Hz, CH₂), 2.33 (2H, t, J = 6.6 Hz, CH₂), 1.86 (2H, m, CH₂), 1.52 (2H, m, CH₂), 1.15 (3H, t, J = 7.2 Hz, CH₃). ¹³C NMR (DMSO-*d*⁶) δ = 173.00, 158.64, 155.93, 145.55, 139.78, 135.81, 132.59, 130.67, 130.46, 129.69, 125.45, 124.14, 123.93, 123.15, 116.10, 115.76, 107.58, 60.20, 49.71, 33.16, 29.32, 21.80, 14.52.

Methyl 6-(4-(4-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-carbonyl)phenyl)-1H-1,2,3-triazol-1-yl)hexanoate (14b)—A solution of alkyne **13b** (0.549 g, 1.61 mmol), methyl 6-azidohexanoate (0.493 g, 2.88 mmol), CuSO₄ (0.044 g, 0.28 mmol) and ascorbic acid (0.181 g, 1.03 mmol) in THF (6 mL) and H₂O (1 mL) was stirred at ambient temperature for 24 h. After the addition of water (10 mL) the solution was extracted with EtOAc (3×8 mL). The organic solutions were combined, washed with H₂O, dried on Na₂SO₄ and evaporated. After column chromatography (hexane/EtOAc 6:4) the ester was obtained as yellow gum (0.623 g, 76%). ¹H NMR (CDCl₃) δ = 9.80 (1H, s, OH), 9.70 (1H, s, OH), 8.67 (1H, s, NH), 7.82 (2H, d, J = 8.5 Hz), 7.75 (2H, d, J = 8.5 Hz, Ar-H), 7.39 (1H, d, J = 8.8 Hz), 7.37 (1H, d, J = 2.1 Hz), 7.17 (2H, d, J = 8.6 Hz), 6.89 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz), 6.66 (2H, d, J = 8.6 Hz), 4.38 (2H, t, J = 7.2 Hz), 4.10 (2H, q, J = 5.2 Hz), 1.94 (2H, t, J = 7.3 Hz, CH₂), 1.85 (2H, m, CH₂), 1.53 (2H, m, CH₂), 1.23 (2H, m, CH₂). ¹³C NMR (CDCl₃) δ = 193.56, 169.37, 158.37, 155.94, 145.54, 142.73, 139.79, 136.46, 135.85, 132.60, 130.67, 129.69, 125.42, 124.15, 123.93, 123.07, 116.10, 115.76, 107.58, 49.93, 49.04, 32.45, 29.69, 25.84, 24.90.

N-hydroxy-5-(4-(4-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-carbonyl)phenyl)-1H-1,2,3-triazol-1-yl)pentanamide (15a)^[29]—A solution of hydroxylamine freebase in anhydrous methanol was generated from 85% KOH (4.90 g, 74.2 mmol) and hydroxylamine hydrochloride (5.16 g, 74.2 mmol) in 100 mL MeOH and the solution was filtered free of the formed KCl. A solution of **14a** (0.142 g, 0.26 mmol) is added and the red reaction mixture was stirred for 24 h. The solution was poured into 300 mL ice water, the pH was adjusted to 2 with 1M HCl and the light yellow solution was extracted with EtOAc (3×80 mL). The pooled extracts were washed with brine then dried on Na₂SO₄ and evaporated. The crude product was purified by gradient elution on a column of silica gel using CH₂Cl₂/MeOH 10%–15%–20% as eluent affording the title compound as bright canary yellow crystals (0.099 g, 72%). ¹H-NMR (CDCl₃) δ = 10.37 (1H, s, OH), 9.82 (1H, s, OH), 9.75 (1H, b, NH), 8.67 (1H, s, Ar-H), 7.83 (2H, d, J = 8.5 Hz), 7.75 (2H, d, J = 8.5 Hz, Ar-H), 7.39 (1H, d, J = 8.8 Hz), 7.36 (1H, d, J = 2.1 Hz), 7.17 (2H, d, J = 8.6 Hz), 6.89 (1H, dd, J₁ = 8.7 Hz, J₂ = 2.2 Hz), 6.65 (2H, d, J = 8.6 Hz), 4.39 (2H, t, J = 7.2 Hz), 4.10 (2H, d, J = 5.2 Hz), 1.99 (2H, t, J = 7.3 Hz, CH₂), 1.83 (2H, m, CH₂), 1.48 (2H, m, CH₂). ¹³C-NMR (CDCl₃) δ = 193.55, 169.37, 158.38, 155.95, 145.53, 142.75, 139.78, 136.47, 135.82, 132.59, 130.67, 129.68, 125.42, 124.14, 123.92, 123.14, 116.10, 115.77, 107.58, 49.76, 49.03, 31.98, 29.57, 22.52. HRMS-ESI: m/z [M+H⁺] calculated for C₂₈H₂₄N₄O₅S: 529.1546, observed: m/z 529.1555 (M+H⁺).

N-hydroxy-6-(4-(4-(6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thiophene-3-carbonyl)phenyl)-1H-1,2,3-triazol-1-yl)hexanamide (15b)—A solution of hydroxylamine in anhydrous methanol was generated from 85% KOH (5.96 g, 90.3 mmol) and hydroxylamine hydrochloride (5.91 g, 85.0 mmol) in MeOH (150 mL) and the solution was filtered free of the formed KCl. A solution of ester **14b** (0.545 g, 1.01 mmol) was added and the red reaction mixture was stirred for 24 h. After the addition of 300 g ice the pH was adjusted to 5 with 1M HCl and the light yellow solution was extracted with EtOAc (3×80 mL). The pooled extracts were washed with 0.5M HCl, H₂O and brine then dried on

Na₂SO₄ and evaporated leaving the crude product as an oil weighing 0.441 g. Purification by gradient elution on a column of silica gel using CH₂Cl₂/MeOH 10%–15%–20% as eluent afforded yellow oil that set up as crystals (0.371 g, 68%). ¹H NMR (CDCl₃) δ= 10.33 (1H, s, OH), 9.81 (1H, s, OH), 9.72 (1H, s, NH), 8.67 (1H, s, Ar-H), 7.83 (2H, d, J = 8.4 Hz), 7.75 (2H, d, J = 8.4 Hz, Ar-H), 7.39 (1H, d, J = 8.8 Hz), 7.37 (1H, d, J = 2.1 Hz), 7.17 (2H, d, J = 8.6 Hz), 6.89 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz), 6.66 (2H, d, J = 8.6 Hz), 4.38 (2H, t, J = 7.2 Hz), 4.10 (2H, q, J = 5.2 Hz), 1.94 (2H, t, J = 7.3 Hz, CH₂), 1.85 (2H, m, CH₂), 1.53 (2H, m, CH₂), 1.23 (2H, m, CH₂). ¹³C NMR (CDCl₃) δ= 193.56, 169.37, 158.37, 155.94, 145.54, 142.73, 139.79, 136.46, 135.85, 132.60, 130.67, 129.69, 125.42, 124.15, 123.93, 123.07, 116.10, 115.76, 107.58, 49.93, 49.04, 32.45, 29.69, 25.84, 24.90. HRMS-ESI: m/z [M+H⁺] calculated for C₂₉H₂₆N₄O₅S: 543.1702, observed: m/z 543.1718 (M+H⁺).

Biology

Cell lines and cell culture conditions—MCF-7, BT20 and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA). MCF-7 were cultured in phenol red containing RPMI 1640 media supplemented with 10% fetal bovine serum, 1% glutamax, 1% non-essential amino acids, insulin (6 µg/ml), 1% antibiotic-antimycotic and 5% CO₂ at 37°C. Treatment media was prepared by supplementing phenol red free RPMI 1640 media with charcoal-dextran treated fetal bovine serum while other supplements remained the same. BT20 and MDA-MB-231 cells were cultured in phenol red containing DMEM media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and were maintained in 5% CO₂ at 37°C. Treatment media for both cell lines was prepared by supplementing phenol red free DMEM media with 10% charcoal-dextran treated fetal bovine serum and 1% penicillin-streptomycin.

Cytotoxicity assay—The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Inc. BT20 and MDA-MB-231 cells were plated at a density of 5 × 10³ cells/well in 96 well plates overnight. Cells were treated with test compounds on the next day and cell viability was measured at 24, 48 and 72 h using the MTS reagent (Promega) according to the manufacturer's instructions. MCF-7 cells were plated at a density of 1 × 10⁴ cells/well in 96 well plates and the above procedure was repeated. Absorbance at 490 nm was measured using the Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-Tek). Data is representative of three individual experiments.

HDAC activity assay—Carried out as described in published detailed experimental procedures using recombinant HDAC1, 2, 3 and 8. [29, 39]. Stock solutions of human recombinant HDAC 1, 2 and 3 (BPS Bioscience) and HDAC8 (purified from E. coli) were prepared in assay buffer 1 (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and 1 mg/mL BSA). Serial dilutions of the probes were made in assay buffer 2 (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂). Enzyme was added to the probes in 96-well opaque half-area microplates (Corning) and preincubated for different preincubation times. It was observed the Z-factor for the assays remained above 0.7 up to 3 h for HDAC1, 3 and 8 and up to 24 h for HDAC2, hence these preincubation times were chosen for the assays. After preincubation, HDAC fluorescent substrate Boc-L-Lys (Ac)-AMC (Chem-Impex) in case of HDAC1, 2 and 3 and BML-KI-178 (Biomol Inc.) in case of HDAC8 was added, and the mixture incubated for 35 min (HDAC1, 3, 8) or 60 min (HDAC2) at room temperature. The reaction was quenched with 1 mg/mL trypsin and 5 µM trichostatin A in assay buffer 1 (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂) and further incubated at room temperature for 35 min. Plates were read on the Synergy microplate reader at excitation wavelength 360 nm and emission wavelength 460 nm. The IC₅₀ values were determined using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA).

Ishikawa assay—The procedure described previously was used to run this assay.^[22b] Briefly, Ishikawa cells (5×10^4 cells/mL) were plated in 96 well plates and incubated overnight in estrogen free media. Cells were treated with the hybrid molecules with 17β estradiol and the appropriate controls at 37°C for 4 days. Alkaline phosphatase enzyme activity was measured by reading the liberation of p-nitrophenol from Mp-nitrophenylphosphate at 340 nm every 15 secs for 16–20 readings using the Synergy microplate reader. The maximum slope of the lines generated by the kinetic readings was calculated using a Kinecalc computer program (Bio-Tek Instrument). The reduction in percent induction as compared to DMSO control was used to obtain antiestrogenic activity according to the following formula:

$$[1 - ((\text{slope}_{\text{sample}} - \text{slope}_{\text{cells}}) / (\text{slope}_{\text{estrogen}} - \text{slope}_{\text{cells}}))] \times 100.$$

Inhibition of 17β estradiol induced luciferase activity—Antiestrogenic activity for $\text{ER}\alpha$ was measured using MCF7 cells which were kept in stripped media three days prior to treatment. Cells were plated (4×10^5 cells) in 12 well plates and were transfected with 3 μg of the pERE-luciferase plasmid, which contains three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of firefly luciferase. To normalize for cell viability and transfection efficiency, 1 μg pRL-TK plasmid (Promega, Madison, WI) containing a cDNA encoding Renilla luciferase was co-transfected along with ERE plasmid. Transfection was performed overnight using Lipofectamine 2000 transfection reagent (Invitrogen), in Opti-MEM media according to the manufacturer's instructions. Cells were treated with test compounds and the luciferase activity was measured in cell lysates using the Dual Luciferase assay system (Promega) with FLUOstar OPTIMA (BMG LABTECH, Durham, NC). Antiestrogenic activity of the active hybrid molecule was studied by cotreating the hybrid molecule with E_2 to observe inhibition of E_2 response. Data is representative of three individual experiments reported as the relative luciferase percentage with 1 nM E_2 treatment set as 100% and initial luciferase activity values were calculated by dividing the firefly luciferase (ERE) reading by the renilla luciferase (pRL-TK) reading.

Modeling

Coordinates of HDAC2 and $\text{ER}\alpha$ protein structures were downloaded from the protein data bank (PDB).^[26, 40] Visual inspection of the crystal structures of 1ERR and 3MAX was performed using Chimera.^[41] Conserved water molecules near the binding site were included and allowed to be toggled on and off during docking. MOE was used to prepare ligands and for protonation of the water oxygen atoms after fixing of protonation states of the protein. After energy minimization in MOE, the docking of 10 conformations per molecule to the crystal structure of HDAC2 (3MAX)^[25] was completed, scoring the obtained binding poses with MOE using standard parameters and calculating binding affinities for the highest-scoring poses using the Amber 12 forcefield.^[42] A training set consisting of 20 molecules with known HDAC2 inhibition was used to benchmark the obtained scores.^[25] The same approach was applied to $\text{ER}\alpha$ binding using crystal structure 1ERR.^[26]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) General design of SERMostat hybrid molecules. B) Overlay of raloxifene (blue) and **15b** (magenta) docked to the crystal structure of ER α (PDB structure 1ERR). C) Overlay of SAHA (magenta) and **15b** (blue) docked to the crystal structure of HDAC2 (PDB structure 3MAX). D) Overlay of the N-(2-aminophenyl)benzamide ligand LLX (magenta) and **15b** (blue) docked to the crystal structure of HDAC2 (3MAX) ^[26]

Figure 2.

Antiestrogenic activity of SERMOstat hybrids. a) Concentration dependent antiestrogenic activity of the SERMOstats **8b** and **15b** tested in an ERE-luciferase reporter assay. b) The antiestrogenic activity of SERMOstat **15b** was confirmed in endometrial cells tested in the alkaline phosphatase assay: $\text{IC}_{50} = 2.9 \pm 0.3 \mu\text{M}$. Data shown are mean $\pm$ s.e.m.

Figure 3.

Decreased cell viability measured in ER(-) cells on treatment with combinations of HDAC inhibitor and DMA after 72 h treatment. HDACi (SAHA, **1**, and entinostat (MS-2-75)) were screened in combination with and without DMA in: a) ER(-) BT20; and b) ER(+) MCF7 cells. **p<0.001 compared to DMA treatment. Data shown is mean $\pm$ s.e.m analyzed by one way ANOVA with Dunnett's post test.

Figure 4. Time dependence of cell death caused by treatment with HDAC inhibitors (SAHA or **1**) and DMA in combination and alone, observed in two ER(-) cell lines, BT20 (a, b), and MDA-MB-231 (c, d). Combination treatment (inverted triangle) led to significant cell death at 72 h, in contrast to treatment with SAHA alone (triangle) and DMA alone open (square) (a, c). Combination treatment (inverted triangle) led to significant cell death at 72 h, in contrast to treatment with HDAC inhibitor **1** (solid square) and DMA alone (open square) (b, d). Data shown is mean $\pm$ s.e.m analyzed by one way ANOVA with Dunnett's post test: **p<0.001 compared to DMSO ctrl (circle).

Figure 5.

Cell death in response to treatment with hybrid molecules, combinations, and HDAC inhibitor and antiestrogen components assayed in MDA-MB-231 ER(-) cells, showing superiority at 48 h of SERMostat. a) Of the hybrid molecules (10 μ M) assayed at 24, 48 and 72 h, only SERMostat **15b** caused significant cell death. b) Comparison of SERMostat with combination treatment and component treatment with **1** or raloxifene. c) Dose response (0.1 μ M, 1 μ M, 10 μ M, 25 μ M) for SERMostat **15b** at 24, 48 and 72 h. ***p<0.001 compared to DMSO ctrl. Data show mean and s.d. analyzed by one way ANOVA with Tukey's post test.

Scheme 1.

Reagents and conditions: (a) Br₂, CHCl₃; (b) 1) *n*-BuLi, THF, -78 C, 2) CO₂; (c) SOCl₂, PhMe, reflux; (d) H₂N(CH₂)₄COOMe or H₂N(CH₂)₅COOMe, Et₃N, CH₂Cl₂; (e) BBr₃, CH₂Cl₂; (f) MeOH, HCl; (g) NH₂OH, MeOH; (h) 3-amino-4-methoxybiphenyl, Et₃N, CH₂Cl₂; (i) BBr₃, CH₂Cl₂.

Scheme 2.

Reagents and conditions: (a) 4-iodobenzoyl chloride, AlCl_3 , CH_2Cl_2 (b) Me_3SiCCH , $(\text{Ph}_3\text{P})_2\text{PdCl}_2$, CuI , Et_3N , THF, reflux; (c) KF , MeOH , H_2O ; (d) BBr_3 , CH_2Cl_2 ; (e) ethyl-5-azidopentanoate or methyl-6-azidohexanoate, CuSO_4 , sodium ascorbate, THF, H_2O ; (f) NH_2OH , MeOH .

Table 1IC₅₀ values (μM) for inhibition of recombinant HDAC isoforms.

Structure and #	HDAC1	HDAC2	HDAC3	HDAC8
 vorinostat ¹	0.0224 ± .0047	0.200 ± .014	0.027 ± .001	0.44 ± .02
 1	0.33 ± 0.01	1.50 ± .02	0.008 ± .0004	0.480 ± 0.009
 8a	0.596 ± .051	2.44 ± .02	0.214 ± .006	0.719 ± .063
 8b	0.168 ± .013	0.665 ± .052	0.128 ± .007	0.400 ± .036
 15a	8.39 ± .30	19.2 ± .65	5.74 ± .53	23.5 ± 1.5
 15b	1.03 ± .08	1.82 ± .11	0.748 ± .034	29.9 ± 1.3
 10	% inhibition ² n.d.(5 min) 21–34% (3 h)	% inhibition ² n.d.(5 min) n.d.(3 h) n.d.(24 h)	% inhibition ² n.d.(5 min) 0–12% (3 h)	% inhibition ² n.d.(5 min) n.d.(3 h)

Data show mean and S.D; n.d. inhibition not detectable.

¹ From reference (Neelapapu et al., 2011)² Inhibition % measured after pre-incubation for different times.