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HMBA is a putative HSP70 activator stimulating HEXIM1 expression that is down-regulated by estrogen

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ABSTRACT

Hexamethylene bis-acetamide inducible protein 1 (HEXIM1) is identified as a novel inhibitor of estrogen stimulated breast cell growth, and it suppresses estrogen receptor-α transcriptional activity. HEXIM1 protein level has been found to be downregulated by estrogens. Recently, HEXIM1 has been found to inhibit androgen receptor transcriptional activity as well. Researchers have used Hexamethylene bis-acetamide (HMBA) for decades to stimulate HEXIM1 expression, which also inhibit estrogen stimulated breast cancer cell gene activation and androgen stimulated prostate cancer gene activation. However, the direct molecular targets of HMBA that modulate the induction of HEXIM1 expression in mammalian cells have not been identified. Based on HMBA and its more potent analog 4a1, we designed molecular probes to pull down the binding proteins of these compounds. Via proteomic approach and biological assays, we demonstrate that HMBA and 4a1 are actually heat shock protein 70 (HSP70) binders. The known HSP70 activator showed similar activity as HMBA and 4a1 to induce HEXIM1 expression, suggesting that HMBA and 4a1 might be putative HSP70 activators. Molecular target identification of HMBA and 4a1 could lead to further structural optimization of the parental compound to generate more potent derivatives to stimulate HEXIM1 expression, which could be a novel approach for hormone dependent breast cancer and prostate cancer treatment.

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1. Introduction

Hexamethylene bis-acetamide inducible protein 1 (HEXIM1) was initially identified as an estrogen receptor transcription inhibitor and also down regulated by estrogen, therefore was named EDG1 (estrogen down regulated gene 1) (Fig. 1) [1]. It could be induced by a small molecule Hexamethylene bis-acetamide (HMBA), which leads to the current name HEXIM1 [2,3].

HMBA is a small molecule that has been investigated by the National Cancer Institute due to its potent anti-cancer and cell differentiation activities [4]. HMBA induces cell differentiation via upregulation of HEXIM1 in breast cancer cells [1–3]. However, HMBA failed at the Phase II clinical trial because of a dose-dependent toxicity [4]. The main toxicity is the thrombocytopenia, which is due to the very short biological half-life of HMBA that necessitates administration of the agent through infusion at a high dosage [4–6].

HEXIM1 binds to 7SK snRNA, a highly abundant non-coding RNA [7]. This complex acts as a potent inhibitor of positive transcription elongation b (P-TEFb) and prevents elongation of RNA Pol II generated transcripts [2,8,9] (Fig. 1). The ratio of inactive to active P-TEFb in cells is regulated by HEXIM1/7SK snRNP, which plays a critical role in the regulation of expression of a wide range of genes such as estrogen and glucocorticoid receptor regulated genes [10–12]. We also reported the inhibitory role of HEXIM1 in

Abbreviations: HEXIM1, hexamethylene bis-acetamide inducible protein 1; HMBA, hexamethylene bis-acetamide; HSP70, heat shock protein 70; SAHA, suberanilohydroxamic acid; HDAC, histone deacetylases; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FBS, fetal bovine serum; PVDF, polyvinylidene diflouride.

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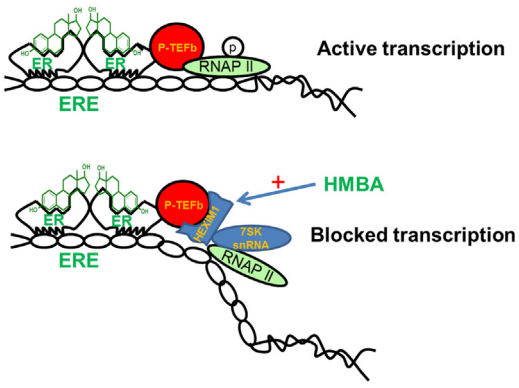

Fig. 1. HMBA induces HEXIM1 expression and blocks estrogen receptor transcription.

prostate tumorigenesis [13,14]. HEXIM1 functions as an androgen receptor co-repressor as it physically interacts with the androgen receptor and is required for the ability of anti-androgens to inhibit androgen-induced target gene expression and cell proliferation [14]. Stimulating HEXIM1 expression is a novel strategy for the treatment of hormone dependent breast and prostate cancer.

The induction of HEXIM1 transcription by HMBA likely occurs through the recruitment of P-TEFb to HEXIM1 promoter. Peterlin and colleagues reported that AKT/PI3K is involved in the phosphorylation of the elongation factor inhibitory complex, which causes the complex to dissociate and HEXIM1 expression to be transiently induced before the recruitment of newly formed elongation factor inhibitory complexes [15,16]. However, the direct molecular targets of HMBA that transiently induce HEXIM1 are not well-defined.

In breast cells, HEXIM1 suppresses cancer metastasis by inhibiting cell invasion, angiogenesis, and the premetastatic niche [17]. However, HEXIM1 expression is lost during breast and prostate tumor progression [11,14]. To enhance HEXIM1 expression, we developed polymer-mediated delivery of HMBA to mammary tumors that resulted in increased HEXIM1 expression and inhibited tumor metastasis. This delivery strategy did not result in thrombocytopenia, the toxicity associated with HMBA in clinical trials. If the druggable features of HMBA could be improved through structural optimization, more promising drug candidates may be developed to increase HEXIM1 expression. These new drug candidates potentially have a broad application in hormone dependent breast and prostate cancer treatment.

To enhance the potential translational impact of our studies, we conducted a study that focused on the lead optimization of HMBA, which resulted in the generation of more potent inducers of HEXIM1 expression such as compound 4a1(Fig. 2) [18]. The finding provides a unique unsymmetrical molecular scaffold that can induce HEXIM1 expression in prostate and breast cancer cells, and has opened a new lead optimization direction for HMBA [18,19]. Our results support the potential of both symmetrical and unsymmetrical HMBA derivatives as new drug candidates. However before we can conduct further lead optimization of HMBA to generate more potent HEXIM1 inducers, a critical step that needs to be taken is the identification of the molecular targets of HMBA and the derivatives.

The detail mechanism of the induction of HEXIM1 expression by HMBA is poorly understood, with no any known molecular candidates for direct interaction with HMBA. The elucidation of the target of HMBA is necessary in order to further optimize its therapeutic potential and minimize the side effects. Target identification will be valuable in understanding how HMBA and derivatives act in the cells. Moreover, there may be existing chemotherapeutic approaches or drugs that have the same molecular target as HMBA, and thus may substitute for HMBA in the induction of HEXIM1 expression for the treatment of cancer. In addition, HMBA and 4a1 induce cell differentiation and is considered less cytotoxic than the existing cancer chemotherapeutic agents.

In the current study, we designed molecular probes based on the structures of HMBA and its more potent analog 4a1 to pull down the binding proteins of these compounds. Via proteomic approaches and biological assays, we found that HMBA and 4a1 bind to heat shock protein 70 kDa (HSP70) to induce HEXIM1 expression. To the best of our knowledge, HSP70 is the first potential molecular target of HMBA identified, which sheds light on the upstream signaling involved in the induction of HEXIM1 expression. This finding provides a solid foundation for further optimization to improve the therapeutic potential of HMBA, and minimize its side effects.

2. Materials and methods

2.1. Biotinylation of 4a1 and HMBA

Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in pre-dried glassware. Thin-layer chromatography was performed on pre-coated silica gel F254 plates (Whatman). Silica gel column chromatography was performed using silica gel 60A (Merck, 230–400 Mesh), and hexane/ethyl acetate was used as the elution solvent. Mass spectra were obtained on the Micromass Quadrupole Time-of-Flight (QTOF) Electrospray mass spectrometer at Cleveland State University MS facility Center. All the NMR spectra were recorded on a Bruker 400 MHz in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^{1}H NMR spectra are reported in parts per million to residual solvent protons. The probe synthesis is illustrated in Schemes 1 and 2. The final product characterization is as follows.

4a1 probe: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.907 (1H, t, J = 5.2 Hz), 7.742 (2H, m), 7.137 (2H, d, J = 8.4 Hz), 6.834 (2H, d, J = 8.4 Hz), 6.408 (1H, s), 6.343 (1H, s), 4.297 (1H, m), 4.126 (1H, m), 3.915 (2H, t, J = 6.8 Hz), 3.293 (2H, s), 3.096 (1H, m), 3.001 6H, m), 2.816 (1H, dd, J = 5.2, 12.4 Hz), 2.575 (1H, d, J = 12.4 Hz), 2.047 (2H, m), 1.779 (3H, s), 1.719–1.229 (22H, m); ESI–MS calculated for $C_{32}H_{50}N_5O_5SNa$ $[M+Na]^+$: 639.3, found: 640.1

HMBA probe: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.791 (1H, t, J = 5.2 Hz), 7.749 (1H, t, J = 5.6 Hz), 6.445 (1H, s), 6.374 (1H, s), 4.298 (1H, m), 4.115 (1H, m), 3.092 (1H, m), 2.997 (4H, m), 2.817 (1H, dd,

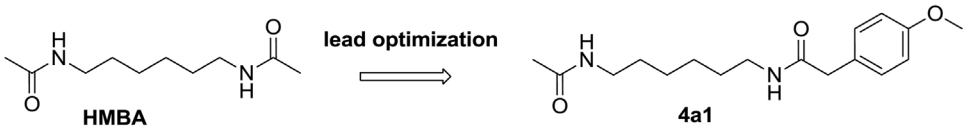

Fig. 2. Structures of HMBA and 4a1.

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