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Lead optimization of HMBA to develop potent HEXIM1 inducers

Bo Zhong , Rati Lama , Wannarasmi Ketchart , Monica M. Montano , Bin Su

Hexamethylene bis acetamide (HMBA), a small molecule, was investigated by the National Cancer Institute due to its potent anti cancer and cell differentiation activities.^{1,2} The molecule failed at the Phase II clinical trial because of the dose dependent toxicity.¹ HMBA achieves its biological activity via Hexamethylene bis acetamide inducible protein 1 (HEXIM1).³ HMBA significantly induces HEXIM1 expression in various cell lines at millimolar concentrations.³ However, for the agent to reach the active concentration in patients, it has to be administered via infusion of a high dosage,^{1,4} which causes significant toxicity.

HEXIM1 binds to 7SK RNA, a highly abundant non coding RNA. Together they act as potent inhibitors of positive transcription elongation b (P TEFb) and lead to inhibition of transcription.⁵⁻⁸ The regulation of the relative ratio of inactive to active P TEFb in cells by HEXIM1/7SK RNA plays a critical role in a wide range of cellular gene expression such as estrogen and glucocorticoid receptor regulated genes.^{7,9} HEXIM1 also has an important role in heart development and remodeling.^{10,11} HEXIM1 expression is decreased in a model of pathological hypertrophy in the adult heart¹² and decreased HEXIM1 expression augmented angiotensinogen induced pathological hypertrophy.¹³ Recent research suggests that HEXIM1 suppresses cancer metastasis and Human immunodeficiency virus (HIV) replication.^{11,14,15} Another group reported on the inhibitory role of HEXIM1 in prostate tumorigenesis.¹⁶ Thus, drug candidates that can enhance the expression of HEXIM1 will have a great

application in the treatment of cancer, heart disease, and acquired immunodeficiency syndrome (AIDS).

HMBA is the most potent and specific inducer for HEXIM1 thus far. However, this occurs only in in vitro cell cultures at concentrations ranging from one to five millimolars.^{2,3} It is difficult to reach high concentrations of HMBA in blood circulation due to its toxicity effects. There is an urgent need to optimize HMBA and improve its ability to induce HEXIM1 expression.^{11,17} In the previous studies numerous HMBA analogs have been developed to improve the activity for inducing cell differentiation, but their ability to induce HEXIM1 expression has never been evaluated.¹⁸ To obtain better HEXIM1 inducers, we designed and synthesized HMBA symmetrical and unsymmetrical derivatives. The structure of HMBA is represented as three different moieties, A, B and C, (Fig. 1), which was used as the basis for our combinatorial chemistry approach to generate the new analogs. Since the direct binding target of HMBA is still unknown, the derivatives can be explored only via the traditional medicinal chemistry strategy, that is, ligand based modification. Moreover, HMBA is a water soluble molecule, which contributes greatly to its short half life and poor tissue distribution. In the compound design, we introduced functional groups that can increase the hydrophobicity of the molecule.

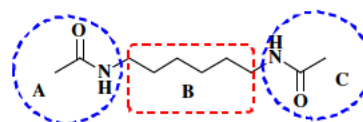
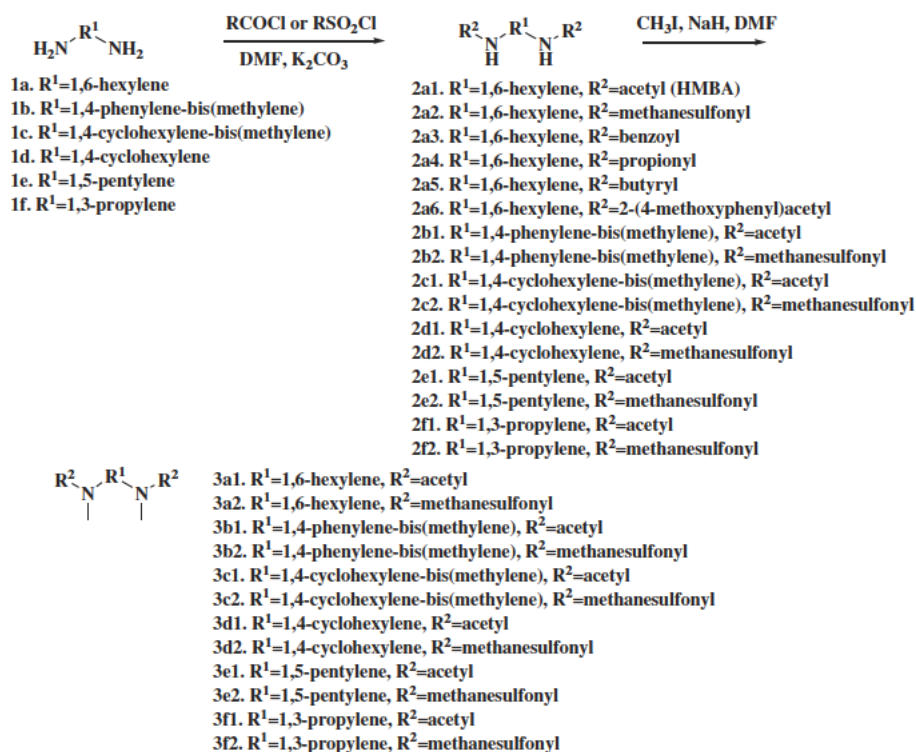
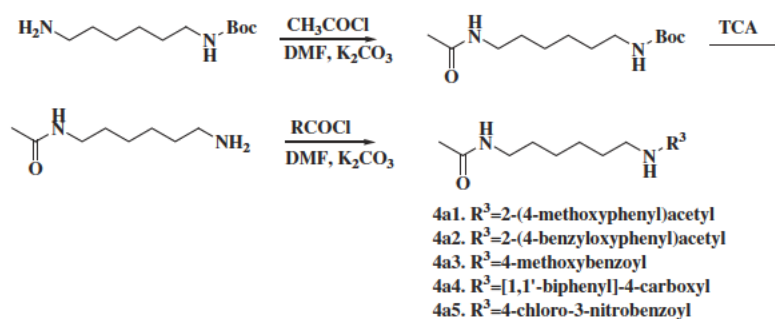


Figure 1. HMBA analog design.



Scheme 1. Synthesis of symmetrical HMBA analogs.



Scheme 2. Synthesis of unsymmetrical HMBA analogs.

The synthesis of the symmetrical and unsymmetrical derivatives is described in Schemes 1 and 2, respectively. For the symmetrical analogs, moieties A and C were kept identical. Besides the acetyl group of HMBA, methanesulfonyl group, propionyl group or butyryl group were introduced. In addition, a bulky benzoyl group was introduced. The active proton in the amide moiety was replaced with methyl group to generate a series of analogs with lower solubility. For the moiety B, various linkers such as 1,3 propylene, 1,5 pentylene, 1,4 cyclohexylene, 1,4 phenylene bis(methylene), 1,4 cyclohexylene bis(methylene) were tested for optimization. Since the starting materials 1,4 di(aminomethyl)cyclohexane **1c** and 1,4 diaminocyclohexane **1d** are mixture of *cis* and *trans* isomers, the corresponding products **2c1**, **2c2**, **3c1**, **3c2**, **2d1**, **2d2**, **3d1**, **3d2** are also mixture of *cis* and *trans* isomers. The further separation of *cis* and *trans* isomers was not carried out in this preliminary study. For the unsymmetrical analogs, the A and B moieties were kept the same as HMBA while the C moiety was modified with aryl or alkyl amide. A total of 32 compounds were synthesized, and these compounds were examined for their potency to induce HEXIM1 in LNCaP prostate cancer cells. HMBA (5 mM) was used as a positive control.

The biological activities of HMBA analogs were determined by assessing HEXIM1 expression using western blot analyses. The analogs were screened at 500 μ M, a concentration that was 10 times lower than the concentration of HMBA used. Cell morphology was examined after the treatment to exclude compounds that caused cell toxicity. An increment of 2.5 fold in HEXIM1 expression compared to the control was set as a cutoff to select candidates for further analyses. In addition, solubility and structural characteristics were considered when selecting the candidates. Among the derivatives, **3b1**, **3b2**, **3c2**, **2d1**, **3d1**, **2f2**, **2a4** and **2a5** induced HEXIM1 expression above 2.5 fold compared to the vehicle treatment (Fig. 2). Compounds **2c1** and **4a3** also reached this cutoff. However, cell morphology slightly changed after the treatment with these two compounds, which excluded them from further investigation. Compounds **3a2**, **3d2**, **3f2** did not reach the cutoff for HEXIM1. The reason could be that precipitation of these compounds during the treatment hindered entry to cells. Therefore, these three compounds were screened at lower concentrations. Compounds **4a1** and **4a4** have unsymmetrical novel structure, and are selected as candidates for further optimization. These two compounds were included for further analysis even if they

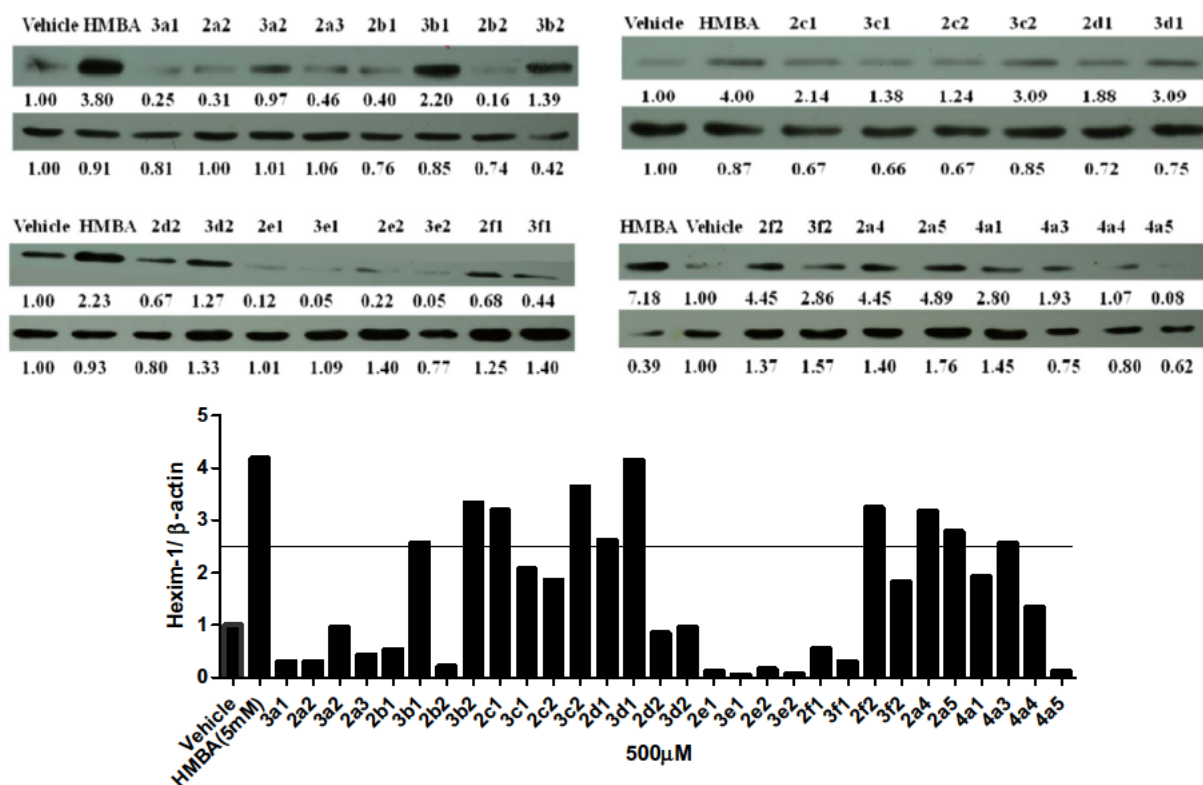


Figure 2. Effect of HMBA analogs on HEXIM1 expression. LNCaP cells were treated with vehicle, HMBA (5 mM) and its analogs at 500 μ M for 24 h. Level of HEXIM1 (upper panel) and β -actin (lower panel) was analyzed by Western blot of cell extracts with HEXIM1 antibody and β -actin antibody respectively. The band intensities of HEXIM1 and β -actin were quantified using ImageJ (NIH) and listed below the bands. The results are the representative of three independent experiments.

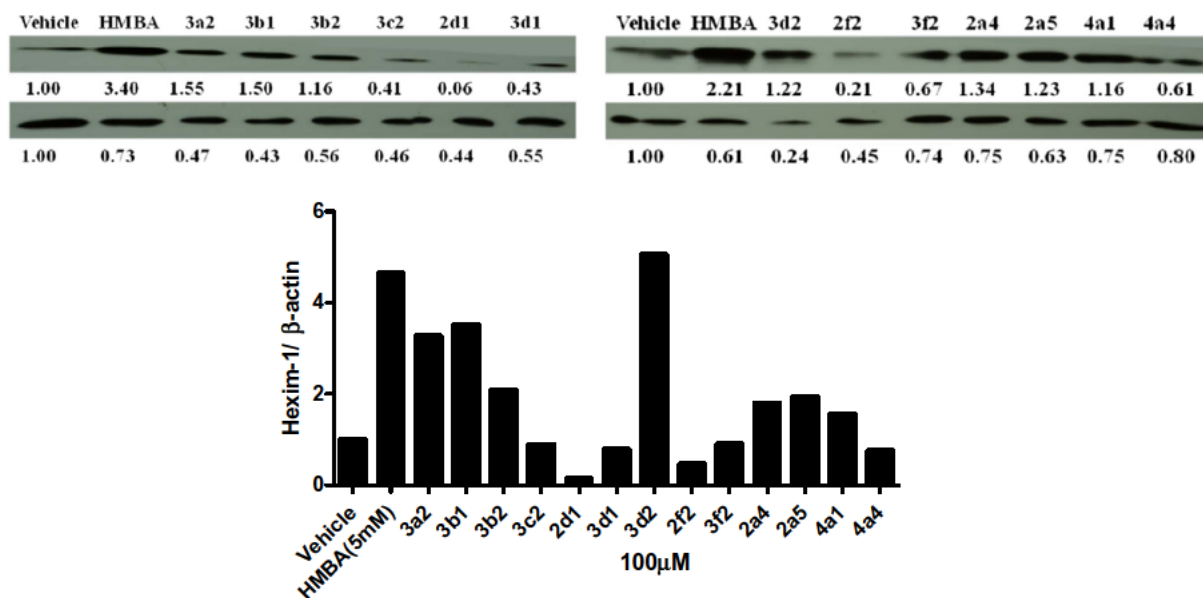


Figure 3. Lower concentration of HMBA analogs on HEXIM1 expression. LNCaP cells were treated with vehicle, HMBA (5 mM) and identified analogs at 100 μ M for 24 h. Level of HEXIM1 (upper panel) and β -actin (lower panel) was analyzed by Western blot of cell extracts with HEXIM1 antibody and β -actin antibody respectively. The band intensities of HEXIM1 and β -actin were quantified using ImageJ (NIH) and listed below the bands. The results are the representative of three independent experiments.

did not reach the cutoff. Structurally, the aromatic and cyclohexylene moiety in compounds **3b1**, **3b2**, **2c1**, **3c2**, **2d1**, and **3d1** resulted in enhanced activity. Compounds **2a4** and **2a5** with terminal propionyl and butyryl moieties exhibited better potency as well, suggesting that the bigger alkyl group at the terminal moiety allowed for enhanced activity. It is also possible that due to the

higher hydrophobicity of these analogs when compared to HMBA, there is enhanced distribution of these compounds in the cells leading to improved biological activity (Fig. 2).

Some compounds were further evaluated at a lower concentration of 100 μ M (Fig. 3). The compounds did not precipitate in cell culture, suggesting they were water soluble at 100 μ M.

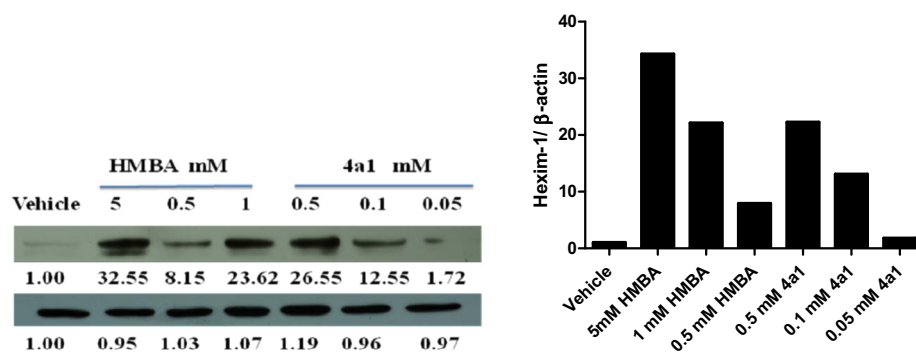


Figure 4. Dose-dependent study of compound **4a1** and HMBA on HEXIM1 expression. Level of HEXIM1 (upper panel) and β -actin (lower panel) was analyzed by Western blot of cell extracts with HEXIM1 antibody and β -actin, antibody respectively.

Compounds **3c2**, **2d1**, **3d1**, **2f2**, **3f2**, and **4a4** were not able to induce HEXIM1 expression, at 100 μ M. However, compounds **3a2** and **3d2** showed significant activity at 100 μ M that was not observed at 500 μ M. The variation in potency could be due to the precipitation of the two compounds at 500 μ M, which hampers cell penetration. Compounds **3b1** and **3b2** with the aromatic ring at B moiety showed good activity at 100 μ M, and induced HEXIM1 expression by 3.8 and 2 fold, respectively. Compounds **2a4**, **2a5**, and **4a1** exhibited reduced activity at lower concentrations, suggesting dose dependent stimulation of HEXIM1 expression by these compounds. Due to the unsymmetrical structural characteristics of **4a1**, a dose dependent study was performed and the results are presented in Figure 4. The compound dose dependently induced HEXIM1 expression suggesting that the unsymmetrical structure and increased hydrophobicity contribute to improved biological activity. Considering the aromatic moiety of compounds **3b1** and **3b2** and the bulky terminal moiety of compounds **2a4** and **2a5**, more potent analogs could be developed if several of these structural units are combined in the new drug design.

In brief, our findings have provided unique molecular scaffolds that significantly induced HEXIM1 expression in prostate cancer cells, and have opened a new lead optimization direction of HMBA. To the best of our knowledge, this is the first study focusing on the lead optimization of HMBA to generate more potent HEXIM1 inducers. The results support the potential of unsymmetrical HMBA derivatives as lead compounds. More potent HEXIM1 inducers have potential application in cancer, AIDS, and heart disease treatment. Further lead optimization of HMBA to generate more potent HEXIM1 inducers is currently underway in our laboratory.

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