Lead optimization of HMBA to develop potent HEXIM1 inducers

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Abstract

The potency of a series of Hexamethylene bis-acetamide (HMBA) derivatives inducing Hexamethylene bis-acetamide inducible protein 1 (HEXIM1) was determined in LNCaP prostate cancer cells. Several compounds with unsymmetrical structures showed significantly improved activity. Distinct from HMBA, these analogs have increased hydrophobicity and can improve the short half-life of HMBA, which is one of the factors that have limited the application of HMBA in clinics. The unsymmetrical scaffolds of the new analogs provide the basis for further lead optimization of the compounds using combinatorial chemistry strategy.

Keywords

HMBA; HEXIM1; derivatives; drug development

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

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pathological hypertrophy in the adult heart[12] 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, (Figure 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, i.e., 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.

The synthesis of the symmetrical and unsymmetrical derivatives is described in Scheme 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 μM, 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 (Figure 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 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 (Figure 2).

Some compounds were further evaluated at a lower concentration of 100 μM (Figure 3). The compounds did not precipitate in cell culture, suggesting they were water soluble at 100 μM. Compounds 3c2, 2d1, 3d1, 2f2, 3f2, and 4a4 were not able to induce HEXIM1 expression, at 100 μM. However, compounds 3a2 and 3d2 showed significant activity at 100 μM that was not observed at 500 μM. The variation in potency could be due to the precipitation of the two compounds at 500 μM, which hampers cell penetration. Compounds 3b1 and 3b2 with the aromatic ring at B moiety showed good activity at 100 μM, 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.
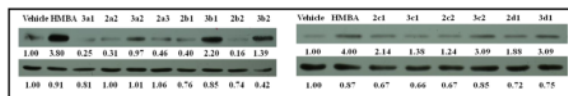
In brief, our findings have provided unique molecular scaffolds that significantly induced HEXIM1 expression in prostate cancer cells, and have opened a new lead optimization direction of HMBA. To the best of our knowledge, this is the first study focusing on the lead optimization of HMBA to generate 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.

Acknowledgments

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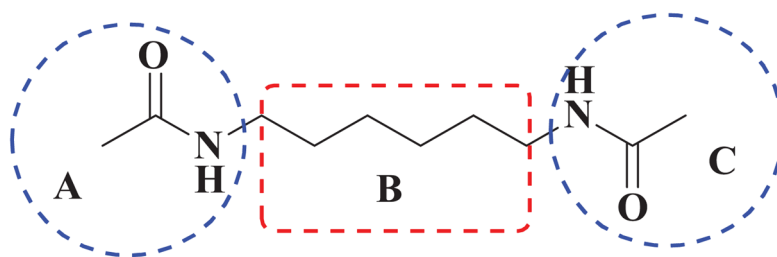
Figure 1.
HMBA analog design

	Vehicle	HMBA	3a1	2a2	3a2	2a3	2b1	3b1	2b2	3b2
	1.00	3.80	0.25	0.31	0.97	0.46	0.40	2.20	0.16	1.39
	1.00	0.91	0.81	1.00	1.01	1.06	0.76	0.85	0.74	0.42

	Vehicle	HMBA	2c1	3c1	2c2	3c2	2d1	3d1
	1.00	4.00	2.14	1.38	1.24	3.09	1.88	3.09
	1.00	0.87	0.67	0.66	0.67	0.85	0.72	0.75

	Vehicle	HMBA	2d2	3d2	2e1	3e1	2e2	3e2	2f1	3f1
	1.00	2.23	0.67	1.27	0.12	0.05	0.22	0.05	0.68	0.44
	1.00	0.93	0.80	1.33	1.01	1.09	1.40	0.77	1.25	1.40

	HMBA	Vehicle	2f2	3f2	2a4	2a5	4a1	4a3	4a4	4a5
	7.18	1.00	4.45	2.86	4.45	4.89	2.80	1.93	1.07	0.08
	0.39	1.00	1.37	1.57	1.40	1.76	1.45	0.75	0.80	0.62

Figure 2. Effect of HMBA analogs on HEXIM1 expression

LNCaP cells were treated with vehicle, HMBA (5mM) and its analogs at 500 μM for 24h. 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.
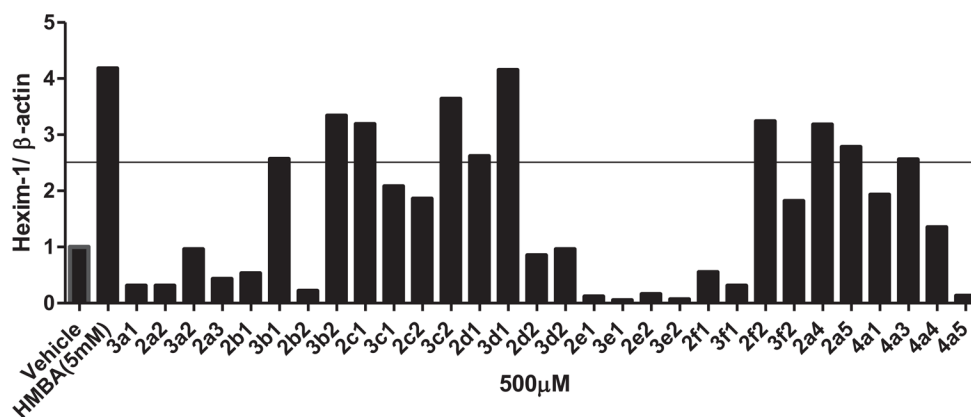
Figure 3. Lower concentration of HMBA analogs on HEXIM1 expression
LNCaP cells were treated with vehicle, HMBA (5mM) and identified analogs at 100 µM for 24h. Level of HEXIM1 (upper panel) and β-actin (lower panel) was analyzed by Western blot of cell extracts with HEXIM1 antibody and β-actin antibody 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 4. Dose-dependent study of compound 4a1 and HMBA on HEXIM1 expression
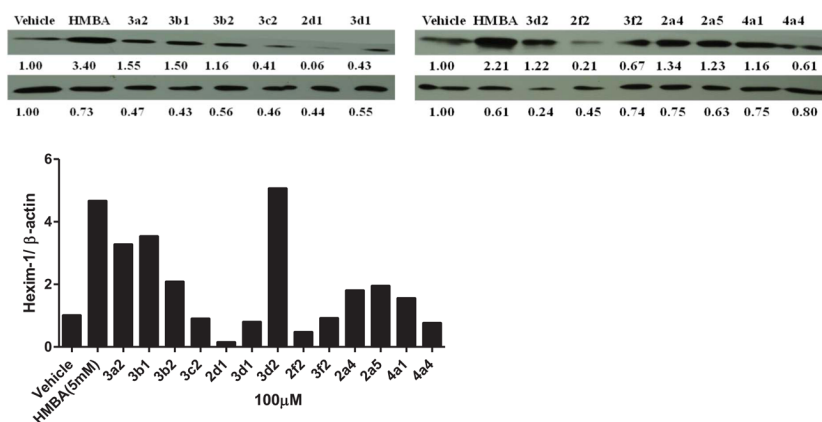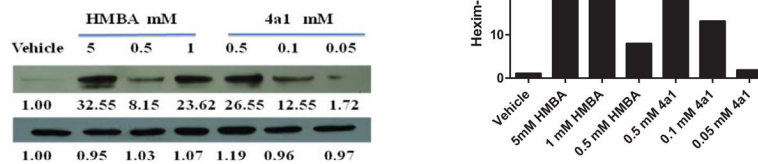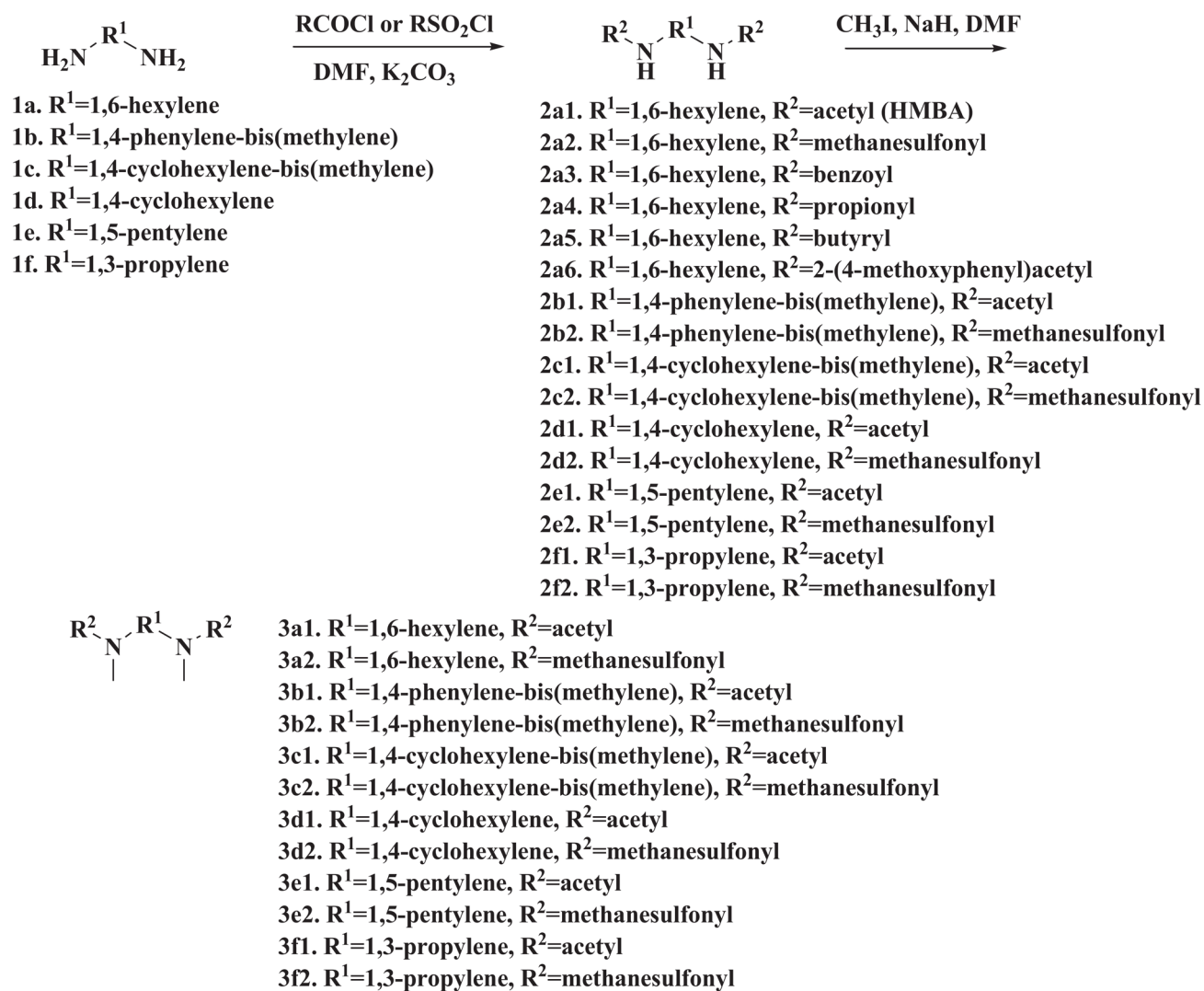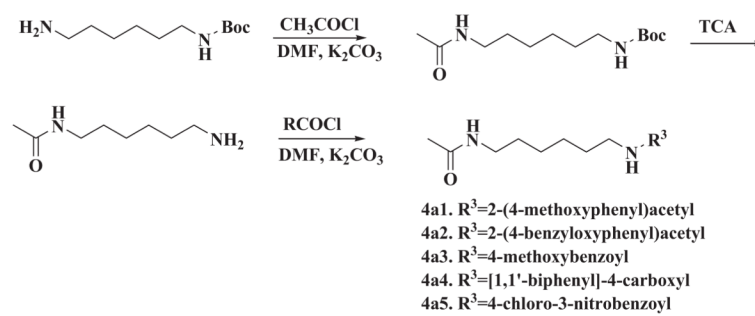Level of HEXIM1 (upper panel) and β-actin (lower panel) was analyzed by Western blot of cell extracts with HEXIM1 antibody and β-actin antibody respectively.

$$H_2N \overset{R^1}{\diagup} NH_2 \quad \xrightarrow[\text{DMF, K}_2\text{CO}_3]{\text{RCOCl or RSO}_2\text{Cl}} \quad R^2 \underset{H}{\overset{}{N}} \overset{R^1}{\diagup} \underset{H}{\overset{}{N}} R^2 \quad \xrightarrow{\text{CH}_3\text{I, NaH, DMF}}$$

1a. R^1=1,6-hexylene
1b. R^1=1,4-phenylene-bis(methylene)
1c. R^1=1,4-cyclohexylene-bis(methylene)
1d. R^1=1,4-cyclohexylene
1e. R^1=1,5-pentylene
1f. R^1=1,3-propylene

2a1. R^1=1,6-hexylene, R^2=acetyl (HMBA)
2a2. R^1=1,6-hexylene, R^2=methanesulfonyl
2a3. R^1=1,6-hexylene, R^2=benzoyl
2a4. R^1=1,6-hexylene, R^2=propionyl
2a5. R^1=1,6-hexylene, R^2=butyryl
2a6. R^1=1,6-hexylene, R^2=2-(4-methoxyphenyl)acetyl
2b1. R^1=1,4-phenylene-bis(methylene), R^2=acetyl
2b2. R^1=1,4-phenylene-bis(methylene), R^2=methanesulfonyl
2c1. R^1=1,4-cyclohexylene-bis(methylene), R^2=acetyl
2c2. R^1=1,4-cyclohexylene-bis(methylene), R^2=methanesulfonyl
2d1. R^1=1,4-cyclohexylene, R^2=acetyl
2d2. R^1=1,4-cyclohexylene, R^2=methanesulfonyl
2e1. R^1=1,5-pentylene, R^2=acetyl
2e2. R^1=1,5-pentylene, R^2=methanesulfonyl
2f1. R^1=1,3-propylene, R^2=acetyl
2f2. R^1=1,3-propylene, R^2=methanesulfonyl

$$R^2 \underset{|}{\overset{}{N}} \overset{R^1}{\diagup} \underset{|}{\overset{}{N}} R^2$$

3a1. R^1=1,6-hexylene, R^2=acetyl
3a2. R^1=1,6-hexylene, R^2=methanesulfonyl
3b1. R^1=1,4-phenylene-bis(methylene), R^2=acetyl
3b2. R^1=1,4-phenylene-bis(methylene), R^2=methanesulfonyl
3c1. R^1=1,4-cyclohexylene-bis(methylene), R^2=acetyl
3c2. R^1=1,4-cyclohexylene-bis(methylene), R^2=methanesulfonyl
3d1. R^1=1,4-cyclohexylene, R^2=acetyl
3d2. R^1=1,4-cyclohexylene, R^2=methanesulfonyl
3e1. R^1=1,5-pentylene, R^2=acetyl
3e2. R^1=1,5-pentylene, R^2=methanesulfonyl
3f1. R^1=1,3-propylene, R^2=acetyl
3f2. R^1=1,3-propylene, R^2=methanesulfonyl

Scheme 1.
Synthesis of symmetrical HMBA analogs

Scheme 2.
Synthesis of unsymmetrical HMBA analogs

4a1. R^3=2-(4-methoxyphenyl)acetyl
4a2. R^3=2-(4-benzyloxyphenyl)acetyl
4a3. R^3=4-methoxybenzoyl
4a4. R^3=[1,1'-biphenyl]-4-carboxyl
4a5. R^3=4-chloro-3-nitrobenzoyl