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Effect of substitution at N″-position of *N*′-hydroxy-*N*-amino guanidines on tumor cell growth

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ABSTRACT

Structural modification of one of our earlier reported lead molecule (ABNM13) has been carried out to study the effect of different substituents at the N″-position of *N*-hydroxy-*N*′-amino guanidines (HAGs) on their anticancer activity. Compounds with electron donating substituents were found to be less active. In contrast, those with electron withdrawing groups were found favorable for anticancer activity. The obtained results provide significant SAR information that may be useful for further drug designing with HAGs.

Hydroxyaminoguanidines (HAGs),[1–5] have been reported as anticancer agents, primarily against L1210 murine, and HL60 human leukemia cell lines. They were found to inhibit DNA synthesis by inhibiting the R2 subunit of the enzyme Ribonucleotide reductase (RR).[6,7] Earlier, we have identified[5,8] an anticancer lead molecule (ABNM13) with IC_{50} (HL60 cell line) of 11 μM, CC_{50} >100 μM that is a selectivity index of more than ten. It is a potent inhibitor of RR, and an arabinofuranosylcytosine (Ara-C) synergist. In our earlier studies we have also emphasized the need for its further structural modifications. ABNM13 has been identified through a virtual screening (VS) experiment, by screening an in-house ligand library, on a developed pharmacophore based QSAR model. To further expand these studies, we have explored different structural modifications of ABNM13, on anticancer activity.

Lead modification process often involves the application of structure or/and ligand based drug designing. We could not apply either, due to the following reasons.

(a) *Reasons for precluding the already developed QSAR model for further lead design*: ABNM13 has been identified by pharmacophore based virtual screening of an in-house library. The training set for developing such a model consisted of different HAGs with aromatic substitutions at the N′-position. Therefore, the developed model revealed designing information pertaining only to this position, and nowhere else. Therefore, our earlier developed model will be out of scope for predicting newer compounds with substitution at different positions (Fig. 1).

(b) *Reasons for precluding structure based designing*:

HAGs inhibit the M2 subunit of the enzyme RR. Mode of action of most of these analogs have been deduced by cell line assays, different spectroscopic studies, and enzyme assays. No crystallographic information of the ligand bound M2 subunit has been reported till date. In absence of these information, structure based drug designing on this target has been a serious concern, and therefore cannot be undertaken for the current work.

We have resorted to a classical drug designing approach. A few analogs with both electron donating and withdrawing substituents were synthesized, and tested for their anticancer activity. For the current work, we have used a synthetic modification strategy on our earlier developed lead molecule (Fig. 1). The synthesis of the compounds are presented in Schemes 1 and 2.

We report a method for synthesizing N″ substituted HAGs. The first step involved the synthesis of methyl dithiocarbazinate (**1**), which was prepared as per our earlier reported methodology.[9–11] Synthesis of compound **2** is straight forward nucleophilic addition between the amine and the aldehyde, with good yield of around 85%. The third step in the synthesis involved nucleophilic attack by different amines on –C=S, carbon of compound **2**. We used excess amine to drive the reaction in forward direction. Excess amine, and the use of high temperature (>120 °C) favors the hydrolysis of compound **2** to corresponding aldehyde, which was monitored by TLC co-spotting with anthraldehyde. This unavoidable side reaction makes the purification difficult for compounds **3–10**, and leads to poor yield. We managed to purify the crude product by using automated flash chromatography (BUCHI Sepacore, Switzerland), and employing a gradient elution technique.

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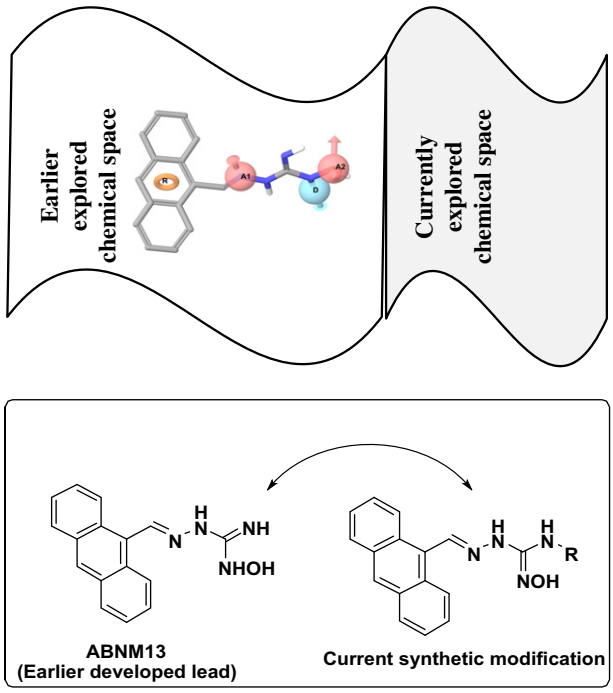


Figure 1. Lead modification strategy for the current work.

The intermediate step to synthesis of compounds **11–18** was achieved by the reaction of excess methyl iodide with compounds **3–10**. Finally, S-Me group was substituted with –NHOH group by reaction with hydroxylamine to obtain compounds **11–18**. Hydrolysis to corresponding aldehyde was observed, but not as drastic as observed during synthesis of compounds **3–10**, possibly due to low reaction temperature. Physicochemical and spectral data for these compounds are given in Table 1.

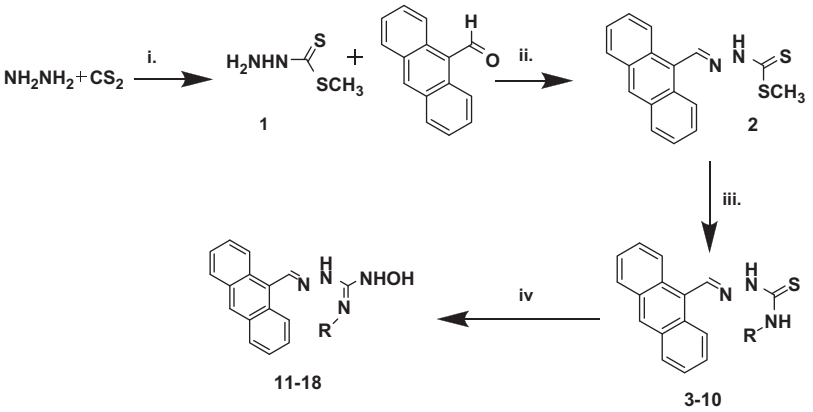
The synthesized compounds **11–18**, were screened against HL-60 human promyelocytic leukemia cell line.[12,13] Biological activities of the compounds are presented in Table 2.

We explored a range of substitutions, primarily aliphatic side chains, starting from smaller methyl group to bulkier phenyl ethyl group. The SAR analysis reveals, presence of aromatic ring in the N″ (compounds **16** and **17**) position is favorable for biological activity as compared with only aliphatic side chains. If we compare **11** & **16**, compound **11** with IC_{50} of 32 μM is twice less active than compound **16** with benzyl substitution. Similar correlation can also be observed if we compare compounds **12** & **17**. It indicates a clear influence of aromatic substituent towards biological activity. It might be an interesting strategy in future, to explore different aromatic substitutions at this position. All the other compounds with aliphatic side chain were found to be much less active.

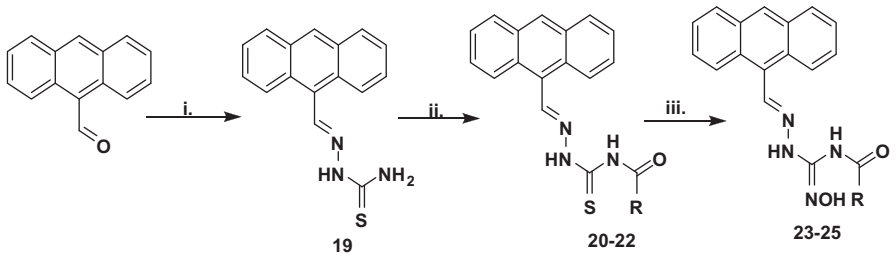
We also report the development of another series of compounds with electron withdrawing substituents at N″-position. Three compounds **23–25** (Table1) with the desired properties were synthesized. The synthetic procedure is outlined in Scheme 2. The method of synthesis is different from those for compounds **11–18**. The first step involves the formation of Schiff's base of thiosemicarbazide, and 9-anthraldehyde (**19**). The next step involves the amide coupling reaction of different carboxylic acids with compound **19**, in the presence of DCC/DMAP, to synthesize compounds **23–25**. Final two steps involve S-methylation followed by nucleophilic substitution with hydroxyl amine. These two steps are similar to that for compounds **11–18**. Compounds of the second series, **23–25** were found similar in activity to our earlier reported lead molecule ABNM13. It indicated that we are moving in right direction.

We pointed out that the pK_a values[14] of synthesized compounds have an influence on activity. Lower pK_a value (increase in acidity due to –NHOH group) favors the biological activity (Fig. 2).

We tried to explain this correlation by reviewing a few earlier studies,[15–17] and subsequently framing a plausible hypothesis. The M2 subunit of RR generates the free radical that reduces ribonucleotides to their corresponding deoxyribonucleotides. This reduction occurs via a single electron transfer from TYR176. The free radical on TYR176 is initiated by the radical-generating, diiron(III/IV) state via a single electron transfer, through a water molecule forming a bridge between the hydroxyl oxygen atom of TYR176, and the iron center. The radical scavengers interact with TYR176, replacing/disturbing the water bridge. Therefore, more acidic (lesser pK_a) –NHOH containing compounds may disrupt the water bridge in a better manner than their lesser acidic analogs. However, we need to support this hypothesis with further experimental support before we come down to any concrete conclusion(s). All our synthesized compounds in the first series (**11–18**) are alkyl derivatives, which are electron donating groups. Therefore, when these compounds dissociates (–NHOH/=NOH dissociates to corresponding anion $–NHO^-$/$=NO^-$) the anion is less stable than that of ABNM13 with unsubstituted N″-position. More stable anion means more ionization, and consequently more acidity (lower pK_a).With aromatic substitutions the anion produced is more stable than their aliphatic counterparts, which counts for lower pK_a values for compounds **15** & **16**. On the other hand, the

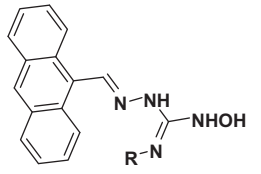


Scheme 1. Reagents and conditions: (i) KOH, <10 °C, stirring for 1–1.5 h; CH_3I, <10 °C, stirring for 2 h; (ii) dry EtOH, reflux, 2 h; (iii) DMF, K_2CO_3, aliphatic amines; (iv) in two steps (a) CH_3I, MeOH, reflux, 6 h, (b) hydroxyl amine, MeOH, rt, stirring, 12 h.



Scheme 2. Reagents and conditions: (i) thiosemicarbazide, EtOH, reflux 2 h; (ii) RCOOH, DCC, DMAP, stirring rt for 12–14 h; (iii) in two steps (a) CH_3I, DMF, reflux, 2 h, (b) hydroxyl amine, DMF/MeOH, rt, stirring, 12 h.

Table 1
Structures, physicochemical and spectral data (^{1}H NMR and FAB-MS) for compounds **11**–**18** and **23**–**25**



Code	R	Yield (%)	Mp (°C)	Spectral data
11	$-CH_3$	65	195.1	MS-FAB (*m*/*z*): $[M+H]^+$ 294,^{1}H NMR (300 MHz, DMSO-d_6) δ 11.41 (s, 1H), δ 7.5–8.25 (m,9H), δ 6.31 (s, 1H), δ 2.6 (s, 3H).
12	$-C_2H_5$	65	196.8	MS-FAB (*m*/*z*): $[M+H]^+$ 308, ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.83 (s, 1H), δ 7.5–9.27 (m,10H), δ 6.48 (s, 1H), δ 6.21 (s, 1H), δ 5.76 (s, 1H), δ 2.13(m,2H), δ 1.27 (t, 3H).
13	$-C_3H_7$	63	179.2	MS-FAB (*m*/*z*): $[M+H]^+$ 306, ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.8 (s, 1H), δ 7.5–8.27 (m, 5H), δ 6.89 (s, 1H), δ 5.89 (s, 1H), δ 1.04 (s, 3H).
14	$-iC_3H_7$	70	181.9	MS-FAB (*m*/*z*): $[M+H]^+$, 306,^{1}HNMR (300 MHz, DMSO-d_6) 11.37 (s, 1H), δ 7.91–9.21 (m, 9H), δ 9.81 (s, 1H), δ 3.57–3.61 (m, 1H), δ 1.22 (s, 6H).
15	Cyclohexyl	62	202.6	MS-FAB (*m*/*z*): $[M+H]^+$,361, ^{1}HNMR (300 MHz, $CDCl_3$, DMSO-d_6) δ 11.76 (s, 1H) 7.54–9.21 (m, 10H), 3.54–3.56 (d, 3H), 1.63–1.44 (m, 1H), 1.30–1.33 (m, 2H), 1.57(t, 2H) 0.93 (t, *J* = 7.5 Hz, 1H).
16	$-CH_2$ Ph	69	223.9	MS-FAB (*m*/*z*): $[M+H]^+$ 319, ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.68 (s, 1H), 7.56–9.28 (m, 12H), 3.54–3.56 (d, 3H), 1.63–1.44 (m, 1H), 1.30–1.33 (m,2H),1.57(t,2H) 0.93 (t, *J* = 7.5 Hz, 1H).
17	$-CH_2CH_2Ph$	73	241.1	MS-FAB (*m*/*z*): $[M+H]^+$ 384,^{1}H NMR(300 MHz,DMSO-d_6, δ 9.22(s,1H), 6.99–8.68 (m,12H), δ 3.95 (t, 2H), 3.76 (t, 2H), 3.13 (s, 1H).
18	$-C_4H_{10}$	75	178.9	MS-FAB (*m*/*z*): $[M+H]^+$, 319, ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.68 (s, 1H),7.56–9.28 (m,12H) δ 3.62–3.51 (m, 2H), 3.08 (s, 1H), 1.62–1.49 (m, 2H), 1.45–1.34 (m, 2H), 1.07 (s, 3H).
23	$-COCH_3$	55	184.6	FAB-MS, 321 (M+1), ^{1}H NMR (300 MHz, DMSO) δ 8.49 (s, 1H), δ 7.55–8.02 (m,9H), δ 4.59 (s, 1H), δ 4.59 (s, 1H) δ 2.16 (s, 3H).
24	–CO(4-nitro)Ph	46	176.8	FAB-MS, 428(M+1), ^{1}H NMR (300 MHz, DMSO) δ 10.13 (s, 1H), 8.46–7.40 (m, 14H), 4.46 (s, 1H), 3.05 (s, 1H).
25	–CO(3,5-dinitro)Ph	50	194.5	FAB-MS, 473(M+1), ^{1}H NMR (300 MHz, DMSO) δ 9.76 (s, 1H), 8.98 (s, 1H), 8.91 (s, 1H), 8.59 (s, 1H), 8.33–7.17 (m, 10H), 4.55 (s, 1H), 3.03 (s, 1H).

second series compounds (**23**–**25**) with electron withdrawing substituents are significantly more acidic (lower pK_a).

We conclude, electron donating substituent at N″-position is unfavorable for anticancer activity, and must be avoided in future designing. On the other hand we found the effect of electron withdrawing substituents is not detrimental. This significant SAR information may serve as a guideline for future inhibitor design on this target.

The detailed procedure for synthesis, and biological evaluation of the compounds **11–18** are given as follows.

To an ice cooled solution (<10 °C) of 19.8 g (0.3 M) of potassium hydroxide in 24 mL of water and 20 mL isopropanol was added 17.1 mL of 80% pure hydrazine hydrate and constantly stirred. 18.2 mL (0.3 M) of ice cooled carbon disulfide was added drop wise to the stirred solution, maintained at <10 °C for about 1–1.5 h. The bright yellow mixture formed was stirred for an additional 1 h. Ice cooled iodomethane solution (18.7 mL, 0.3 M) was added dropwise to the bright yellow mixture formed over a period of 2 h with stirring. After addition, stirring was continued for an additional 1 h. The white precipitate obtained was filtered and washed with ice cooled water. The crude product was recrystallized from dichloromethane. Yield: 93%, mp 90.5 °C (lit.[9] mp 90–92 °C).

Methyl dithiocarbazinate (0.02 M) was dissolved in 20 mL of ethanol and an equimolar amount (0.02 M) of anthraldehyde was added to it. The mixture was refluxed for 2 h on steam bath and was monitored by TLC for the completion of reaction. The crude compound is purified by recrystallization from EtOH. Yield: 85 %, mp 198 °C.

To a solution of compound **2** (0.01 M) in DMF anhydrous potassium carbonate was added (250 mg/mL) and stirred for 15 min. Different aliphatic amines (0.1 M) were added; the reaction mixture was stirred, and gradually refluxed at 120 °C for 3–6 h, under nitrogen atmosphere. For amines of low boiling point, we employed, coiled condenser with ice cold water circulation ~4 °C. For compounds **3** & **4** reactions occurred at 65 °C, stirring for 6 h. The reactions were monitored using lead acetate paper, which

Table 2
Growth inhibition of human HL-60 promyelocytic leukemia cells after incubation with **11**–**18**, and **23**–**25** for 72 h

Code	IC_{50} (μM)
11	32.9 ± 1.30
12	22.6 ± 0.37
13	24.9 ± 2.17
14	29.8 ± 2.11
15	21.1 ± 0.51
16	19.3 ± 0.38
17	19.6 ± 1.79
18	21.1 ± 1.15
23	20.0 ± 2.74
24	10.0 ± 0.40
25	8.0 ± 1.00

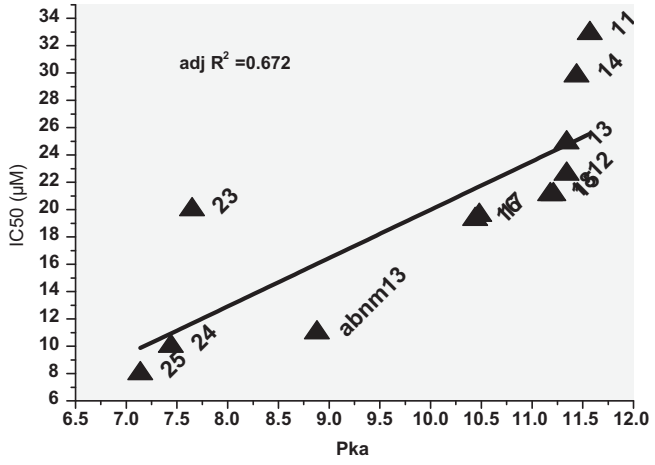


Figure 2. Plot of biological activity (IC_{50} in μM) of compounds **11**–**18** with predicted pK_a values.

darkened to brown/black color due to evolution of methyl mercaptan. The reaction was continued till the evolution of methyl mercaptan. Completion of the reactions were also confirmed by TLC (50% EtOAc:Hexane) till no spots for compound **2** were observed. The reactions were quenched with brine, extracted thrice with 10 mL EtOAc, and dried with anhydrous Na_2SO_4. For all the cases the crude product yielded in three spots on TLC, under UV light 254/365 nm and iodine vapors. We employed 10% H_2SO_4:MeOH charring to judge the relative percentage of all the three spots. We found the middle spot was most prominent amongst all, and targeted the same for further purification. Compounds **3**–**10** were purified by employing 100–200 silica gel as stationary phase and employing different gradient of EtOAc:Hexane starting from 5% to 20% to obtain the pure compounds. The pure compounds appeared as bright yellow to yellowish brown crystals. Physicochemical and spectral data are given in Supplementary data.

Compounds **3**–**10** (0.01 M) thus obtained were dissolved/suspended in MeOH, and stirred vigorously for 10 min, and gradually raising the temp to 50 °C. Excess iodomethane (10 M equiv) was added drop wise for a period of 30 min, while stirring vigorously. The reaction mixture was refluxed at 60 °C for 6 h using ice-cold water circulator ~4 °C, employing a coiled condenser. After 2–3 h, precipitate starts appearing. The reaction was monitored on TLC 50% EtOAc:Hexane. After completion the reaction mixture was cooled, the precipitate was filtered. Washed with hexane and finally with cold MeOH. These compounds were used without further purification in the next step.

Hydroxyl amine (0.1 M) solution in cold MeOH was added drop wise over a period of 30 min to a methanolic suspension of compounds from previous step while stirring. Stirring was further continued for 12 h. Completion of reaction was monitored by TLC using 50% EtOAc:Hexane as solvent system and evolution of methyl mercaptan on lead acetate paper.

Thiosemicarbazide (0.5 M), and different anthraldehyde (0.5 M) were refluxed for 3 h to obtain compound **19**. The resulting compound was re-crystallized from 95% ethanol, mp 235 °C, yield 90%.

DCC (0.6 M), DMAP (0.5 M), and different carboxylic acids (0.5 M) were dissolved in dry DCM. To it 0.5 M of compound **19** in dry DCM was added dropwise for 2 h through a pressure equalizing dropping funnel while stirring the reaction mixture. The reaction mixture was further stirred for 12–14 h. The progress of the reaction was monitored on TLC (5% MeOH:DCM). After completion of the reaction, it was quenched 10% citric acid solution (to remove dicyclohexyl urea formed). The resulting suspension was extracted thrice with sufficient amount DCM. The DCM layer was concentrated to dryness to get the crude product.

Compounds **20**–**22,** were further purified by flash chromatography using different gradients of DCM and methanol.

For synthesis of S-methyl derivatives, compounds **20**–**24** (0.01 M) were dissolved in sufficient dry DMF. Approximately, 10 M excess of iodomethane was added. The mixture was stirred, and gradually refluxed to 60 °C for 2 h. Coiled condenser with ice cold water circulation ~4 °C was employed. Completion of the reactions were confirmed by TLC (50% EtOAc:Hexane) till no spots for the starting compounds were observed. After completion of the reaction excess iodomethane was distilled off, and the reaction was quenched with brine solution, and extracted several times with ethyl acetate. The organic layer was evaporated to dryness, and the resulting solid was used in the next step without further purification.

The solid obtained was dissolved in DMF, to it excess hydroxyl amine hydrochloride (0.1 M), potassium hydroxide (0.1 M) and 5 mL MeOH was added, and stirred vigorously. Stirring was further continued for 12 h. Completion of reaction was monitored by TLC using 5% MeOH:DCM as solvent system. Compounds **23**–**25**, were purified by flash chromatography using different gradient of MeOH:DCM.

The human HL-60 promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria). All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). Cell counts were determined using a microcellcounter CC-110 (SYSMEX, Kobe, Japan). Cells growing in the logarithmic phase of growth were used for the experiment described below.

HL-60 cells (0.1 × 106/mL) were seeded in 25 cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of compounds at 37 °C under cell culture conditions. Cell counts and IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) were determined after 72 h using a microcellcounter CC-110. Viability of cells was determined by staining with trypan blue. Results were calculated as number of viable cells. Data are means ± SD of at least three experiments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.048.

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