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NOVEL POLYAMIDE INDUCERS OF HL-60 CELLULAR DIFFERENTIATION

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Abstract Four polyamides based on the structure of spermine and spermidine were prepared and tested in vitro in an HL-60 cell differentiation assay. Their activity was compared with that of hexamethylene bisacetamide (HMBA) **1** whose optimal activity (ca. 65% differentiation) is at its IC_{50} of 4 mM. Triamides **7** and **13** were as good as or superior to **1** at concentrations (16 mM) which were below their IC_{50}s.

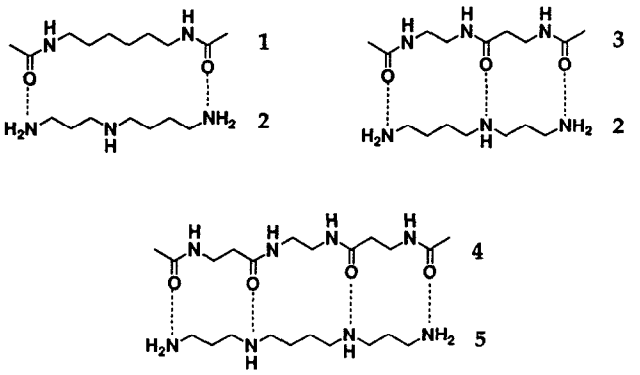
Differentiation therapy seeks to arrest the unrestrained growth of cancer cells using agents that induce the cells to adopt the more benign characteristics of their parental cell type.[1] Many classes of compounds have been found which will cause differentiation of varying proportions of numerous transformed cell lines, most notably leukemia cells. These agents include retinyl[2] and vitamin D derivatives,[3] nucleosides,[4] dimethylsulfoxide (DMSO),[5] N, N-dimethylformamide (DMF),[6] butyrates and other carboxylates,[7] hexamethylene bisacetamide (HMBA) **1**,[8] and lipids.[9] Marks and co-workers prepared and tested a number of compounds whose basis was the structure of **1**, one of the most successful differentiating agents.[10] They noted a discrete balance of polar and apolar constituents in the most successful of their compounds. HMBA **1** has shown promise in human clinical trials, but its effectiveness has been limited by toxicity at effective concentrations, and by fast metabolism which requires continuous infusion to maintain those concentrations.[11]

Polyamines are known to be critical messengers in the initiation and maintenance of cellular proliferation.[12] Quemener and co-workers have noted a decrease in nuclear spermine **5** levels as spermatocytes differentiate to spermatids.[13] Unnatural polyamines which down-regulate polyamine biosynthetic enzymes have been shown to be very active against certain cancers.[14] In addition, endogenous N^8- and N^1-acetyl spermidines are potent, though very toxic, inducers of HL60 cell differentiation.[15] Therefore, the modulation of polyamine biosynthesis might be one way in which terminal differentiation can be achieved.

We noticed that the distance between the carbonyl oxygens in **1** is roughly the same as that between the terminal nitrogens in the ubiquitous polyamine spermidine **2** (figure 1). If the activity of **1** lay in its ability to bind to spermidine receptors or to interact with **2** itself, then the addition of a third amide recognition element (to give **3**) in the place of the interior nitrogen of **2** might greatly increase potency by increasing binding strength. By the same reasoning, we thought that a tetraamide analogue **4** of spermine **5** might also be effective. In addition, we prepared per-acetylated derivatives of **2** and **5**.

In this report we describe the synthesis, HL60 differentiating activity, and in vitro cytotoxicity of a series of polyamides based on the structure of spermidine **2** and spermine **5**.

Figure 1



Differentiation Assay The samples were tested for their ability to induce differentiation of HL60 cells (American Type Culture Collection, Rockville, MD) in vitro as described previously.[16] The ability of the cells to reduce cytochrome c was monitored spectrophotometrically at 550 nm. The results are reported as optical densities (OD_{550}) of an average of four replicates for each of two assays. OD_{550} is proportional to degree of differentiation with OD_{550}=0.345 representing roughly 100%. HL60 cells (negative control) typically have OD_{550} values <0.05 while positive control cells have OD_{550} values >0.2.

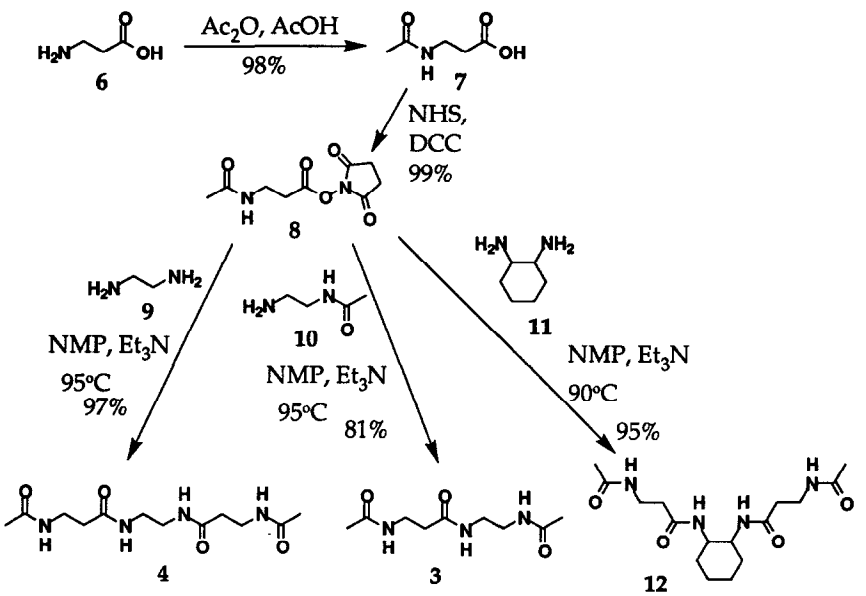
Cytotoxicity Assay The cytotoxic IC_{50} was assessed using the vital stain MTT in a modification of the procedure of Mosmann.[17] After exposure of the cells to the differentiating agents for 3 days 25μL of MTT (5 mg/mL in Hanks' balanced salt solution) was added to 100 μL of the cell suspension, and the plates were incubated for 30-60 minutes (37°C, 5% CO_2). The formazan was dissolved by addition of 100 μL of 2-propanol and ODs were determined at 570 nm.

Results and Discussion The syntheses of the linear polyamides **3**, **5**, and **12** were carried out in a straightforward manner (scheme 1). Acetylation of β-alanine **6** and formation of the NHS active ester **8** proceeded almost quantitatively. A number of attempts to couple **8** to ethylenediamine **9** at room temperature in various solvents resulted in poor yields of diacetylated product, probably because mono-acetylation significantly reduces the nucleophilicity of the second nitrogen. Reaction of **8** with **9**, **10**, and **11** could all be forced to completion by heating in N-methylpyrrolidinone (NMP) with an equivalent of triethylamine. In each case dilution of the cooled reaction mixture with methylene chloride precipitated the solid which, after washing and drying in vacuo, was analytically pure.[18]

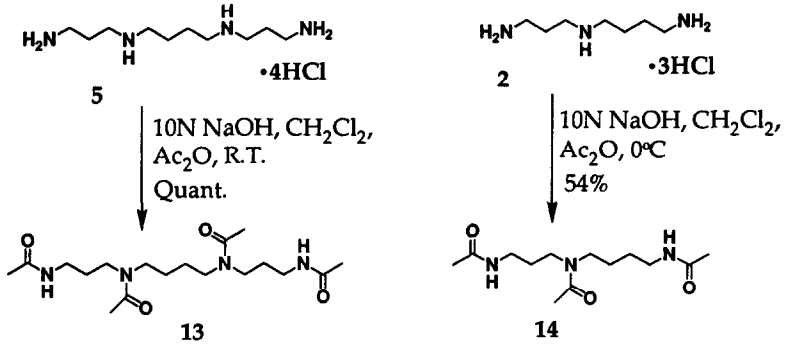
The great affinity of the free base polyamines spermine **5** and spermidine **2** for chlorocarbon solvents allowed efficient acetylation in a two-phase system as shown in scheme 2. Cooling the reaction to 0°C may

have slowed transfer of spermidine **2** to the organic phase resulting in a lower yield of **14**.[19] Compounds **3**, **4**, **13** and **14** were completely soluble in water to a concentration of >10 mg/mL. However, the conformationally restricted diaminocyclohexane analogue **12** was not soluble at the initial assay concentration and could not be tested.

Scheme 1



Scheme 2



While none of the polyamides synthesized demonstrated superior in vitro potency to **1** (figure 2), some interesting observations can be made. Tetraamide **4** exhibited activity which closely resembles that of **1** until

ca. 45% differentiation is reached at 2 mM and then drops off to almost nothing at 4 mM. A loss of activity at higher concentrations has been seen before for some differentiating agents.[10] Both the linear triamide **3** and spermidine triacetamide **14** possessed differentiating activity comparable to **1**, but at a four-fold higher concentration. However, optimal activity for **1** is at its IC_{50} (4 mM), while **3** and **14** exhibited activity which was as good or superior to **1** at concentrations below theirs. It is possible that, through intracellular metabolism, **14** serves as a less toxic prodrug of the potent differentiating agent N^8-acetylspermidine, since leukemia cells are known to be able to deacylate HMBA **1**.[15] Insertion of an amide group into **1** to give the three amide oxygens a spatial relationship closer to spermine **2** did not improve potency of **3**. This may argue in favor of Marks and co-workers' idea of a required balance between polar and hydrophobic regions on the

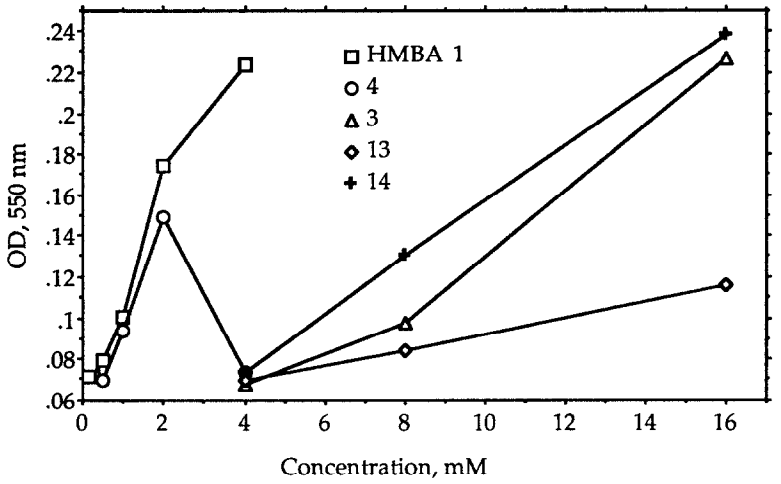


Figure 2. Differentiating Activity of Various Polyamides on HL60 Cells in vitro.

differentiating agent,[10] whether the mechanism of action involves discrete binding to a receptor or a bulk physical effect on a cellular component (i.e. the plasma membrane). Recently, comparative molecular field analysis by Callery and co-workers has been carried out on **1**, its metabolites, and other known amide-containing differentiating agents.[20] This study found almost equal contributions to differentiating activity from steric interactions, electrostatic potential and molecular weight. Based on the resulting contour maps a series of unsymmetric N-alkylamides based on **3** and **4** might be more effective. These changes might add back to our compounds the hydrophobic properties of **1** lost by adding the amide bond(s) while preserving the spatial interactions. Intracellular metabolism of the compounds tested has not been investigated but may be facile. As with **1** activity of metabolites might be superior to the parent in which case knowledge of their identity and rate of formation is important.

Although none of these compounds proved more potent than HMBA **1** in the HL60 cell differentiation assay, two of them, **3** and **14**, were as good as, or superior to, **1** at concentrations below their IC_{50}s.

Modifications to these structures, taking into account empirical polar/apolar structure-activity relationships may result in an increase in potency with no concomitant gain in toxicity.

Table 1. Cytotoxicity of Polyamides toward HL60 Cells in vitro.

Compound.	IC_{50}, mM
HMBA **1**	4
3	>16
4	6
13	>16
14	>16

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18. For **3**: (white solid) mp 274-275°C; MS (DCI) 216 $(MH)^+$, 174 $(MH_2\text{-}C_2H_3O)^+$, 157 $(M\text{-}C_2H_4NO)^+$; IR (KBr) ν_{max} 3290, 1642, 1558, 1438, 1370; Anal. calc. for $C_9H_{17}N_3O_3$: C-50.22, H-7.96, N-19.52; found: C-49.98, H-7.89, N-19.65; For **4**: (white solid) mp 205-206°C; MS (DCI) 287 $(MH)^+$, 172 $(MH_2\text{-}C_5H_8NO_2)^+$; IR (KBr) ν_{max} 3292, 1641, 1550, 1436, 1372; Anal. calc. for $C_{12}H_{22}N_4O_4\text{-}1/2H_2O$: C-48.80, H-7.84, N-18.97; found: C-48.41, H-7.49, N-19.40; For **12**: (white solid) mp 281-282°C; MS (DCI) 341 $(MH)^+$, 282 $(M\text{-}C_2H_4NO)^+$, 254 $(M\text{-}C_4H_8NO_2)^+$; IR (KBr) ν_{max} 3284, 1648, 1550, 1436, 1372; Anal. calc. for $C_{16}H_{28}N_4O_4$: C-56.45, H-8.29, N-16.46; found: C-56.18, H-8.21, N-16.66.
19 For **13**: (thick oil) IR (film) ν_{max} 3290, 1626, 1558, 1486, 1428, 1374; Accurate mass calc. for $C_{18}H_{35}N_4O_4$: 371.2658; found: 371.2644; Anal. calc. for $C_{18}H_{34}N_4O_4\text{-}1.5H_2O$: C-54.39, H-9.38, N-14.09. Found: C-54.29, H-9.47, N-13.71; For **14**: (thick oil) IR (film) ν_{max} 3290, 1632, 1558, 1486, 1434, 1372; Accurate mass calc. for $C_{13}H_{26}N_3O_3$: 272.1974; found: 272.1982; Anal. calc. for $C_{13}H_{25}N_3O_3\text{-}H_2O$: C-53.96, H-9.40, N-14.52. Found: C-53.50, H-9.27, N-15.22.
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