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Synthesis of minoxidil conjugates and their evaluation as HL-60 differentiation agents

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ABSTRACT

Activation of minoxidil (MNX) with *N,N′*-carbonyldiimidazole and coupling with natural polyamines (PAs) and commercially available aliphatic or aromatic amines provided a series of new conjugates which were evaluated for their ability to induce differentiation to HL-60 acute myeloid leukemia cancer cells, using a modified NBTZ reduction test. Although neither MNX nor 4,4′-methylenedianiline (MDA) or 2,7-diaminofluorene (DAF), alone or in combination, had any effect, the MNX-spermine (SPM) conjugate (**11**) and the conjugates **7** and **8** of MNX with MDA and DAF exhibited a differentiation-inducing effect at a concentration of 10 μM without being toxic on proliferating human peripheral blood mononuclear cells.
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Cancer is a leading cause of death worldwide with projections estimating 13.1 million deaths in the year 2030.[1] Whilst several treatments do exist, many of them are relatively toxic to normal healthy cells. One goal of current research is to identify novel, safer and more effective treatments for a wider spectrum of neoplastic conditions in the hope of decreasing morbidity and mortality. This has resulted in more targeted therapies for cancer, including differentiation therapy.[2–7] Acute promyelotic leukemia (APL), an uncommon subtype of acute myeloid leukemia (AML), has been the main success story of differentiation therapy so far.[8] This disease of young adults was almost invariably fatal in the past, due to severe coagulopathy, until the revolutionary introduction of ATRA (*all-trans* retinoic acid) as a treatment.[9] The drug which is a ligand of the RARα receptor component of the fusion protein, when given in supra-physiological concentrations, is able to relieve the differentiation block posed by PML + RARα and effectively induce white cell maturation, managing to achieve a relapse-free survival of about 90%,[10] in combination with low doses of traditional chemotherapy. The serious side-effects of non-targeted standard chemotherapeutic drugs[11,12] are thus avoided.

Apart from suppressing the uncontrolled proliferation of leukemia cells themselves, usual chemotherapeutic drugs also result in suppression of the normal bone marrow, leading to anemia, immune-compromise and bleeding disorders, which may result in fatality. AML is the leukemia with the worst prognosis with average 10 year survival rates of around 10%.[2,13,14] Since ATRA works effectively only on the rare APL genetic subtype of AML, extensive research is being directed toward causing differentiation in other types of immature leukemic cells.[2,7,15,16] Initial testing makes use of cell lines derived from leukemic patients which are easily available and easily differentiated with positive controls chemicals (although these are usually too toxic to be used clinically) and therefore are used as common standards across many research laboratories.

Minoxidil (MNX) is a vasodilator primarily marketed as a drug for the reduction and slowing of androgenetic alopecia.[17] It exhibits its effect by a calcium blocking capacity and may also stimulate the production of vascular endothelial growth factor (VEGF).[18] It is not known to have any recognized cytotoxicity at micromolar concentrations although some non-toxic suppression of proliferation of fibroblasts and keratinocytes has been reported.[19–21] One of the advantages of using MNX as a target for chemical modification is its ease of entrance into cells without the need of a recognized receptor-dependent pathway.[22] On the other hand, natural polyamines (PAs) such as putrescine (PUT),

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spermidine (SPD) and spermine (SPM) are known to play an important role in the processes of differentiation and proliferation in leukemia.[23,24]

In connection with our studies toward the antioxidant activity of MNX-PA conjugates[25] and given that certain antioxidants exhibit also antiproliferative activity,[26] we tested the effect of two previously described MNX-PA conjugates, namely compounds **9** and **11**,[27] on the differentiation and proliferation of the HL-60 leukemia cell line. The initial evaluation of these compounds revealed induction of differentiation with compound **11**, which prompted us to further extend the study with eight new MNX conjugates (**2**–**8** and **10**, Fig. 1). With these compounds we wanted to establish the role of certain structural modifications on the differentiation-inducing activity of the resulting analogs. These modifications included: (a) the presence of an amino group in the chain (conjugates **3** and **9**), (b) the length of the aliphatic chain (conjugates **9** and **10**), (c) the nature of the side chain (aliphatic, aromatic or cyano- or amino-group incorporating aliphatic chain) (conjugates **2**–**4** and **9**), (d) additional substituted (with an amino or methoxy group) or unsubstituted phenyl rings and the anticipated increase in lipophilicity (conjugates **4** and **5**–**8**), (e) the site of conjugation (conjugates **4**–**6** and **7**–**8**), and (f) the increase of the rigidity of the amine counterpart (conjugates **7** and **8**).

The synthesis of the new compounds is depicted in Scheme 1. MNX was activated with *N*,*N*′-carbonyldiimidazole in DMF to give MNX-derived acylating agent **1**, according to published procedure.[27] This in turn was reacted with commercially available amines and N^1-tritylhexane-1,6-diamine to afford conjugates **2**–**8** and **12** in 40–60% yield. Conjugate **12**, upon trifluoroacetic acid (TFA)-mediated trityl (Trt) group removal, afforded conjugate **10** in 95% yield.

The synthesized compounds were tested for their ability to induce differentiation using a high passage HL-60 sub-line, which is very poorly responsive to ATRA-induced differentiation, at the concentrations of 0.1, 1 and 10 μM for 3 and 5 days of exposure. Conjugates **5**, **7**, **8** and **11** showed an increased NBT/MTT differentiation index on HL-60 cells at 10 μM, similar to that shown by a positive control both after 3 and 5 days of exposure (Fig. 2).

Compound **10** was active at day 3, but not so at day 5 of differentiation, suggesting it may induce monocytoid differentiation. ATRA does not have any effect on this late HL-60 passage and neither does MNX itself elevate the NBT/MTT differentiation index, suggesting that the unmodified MNX does not have any differentiation-inducing activity. MTT activity for all conjugates showed approximately a four-fold drop both at day 3 and 5 of differentiation, as it is often expected in differentiating cells, due to the reduction of cell proliferation associated with differentiation (Table 1; IC_{50} values).

The potent compounds **5**, **7**, **8**, **10** and **11** were also tested on peripheral blood mononuclear cells (PBMCs) to see if they would show any cytotoxic activity at the concentrations effective on HL-60 leukemia cells. In contrast to the clearly toxic effect of 10% DMSO, no cytotoxic effects for these compounds were detectable on human PBMCs from healthy donors (Fig. 3), at the doses noted to increase the differentiation index.

Regarding structure–activity relationships based on the biological evaluation results, we note that conjugates incorporating the amines β-aminonitrile (conjugate **2**), *n*-butylamine (conjugate **3**), benzylamine (conjugate **4**) and PUT (conjugate **9**) exhibited very low activity. When PUT was replaced by 1,6-diaminohexane (conjugate **10**), or SPM (conjugate **11**) the activity of the conjugate was much improved. In addition, when benzylamine was replaced by benzhydrylamine (conjugate **5**) the conjugate showed comparable activity with the conjugate **11** of SPM. However, when the replacement involved a 4,4′-dimethoxybenzhydrylamine moiety (conjugate **6**) the activity was dramatically decreased, suggesting that methoxy substituents on the aromatic rings should be avoided. Slightly better results were obtained when benzhydrylamine was replaced by the aromatic amine MDA (conjugate **7**) or its conformationally restricted analog, namely the aromatic amine

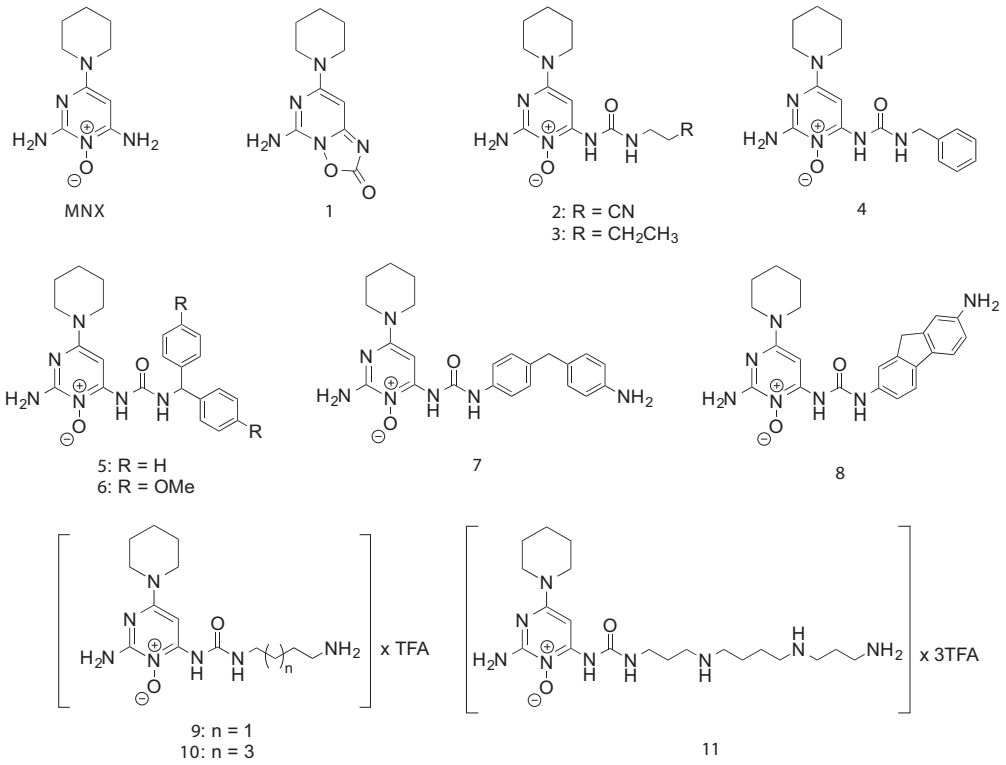
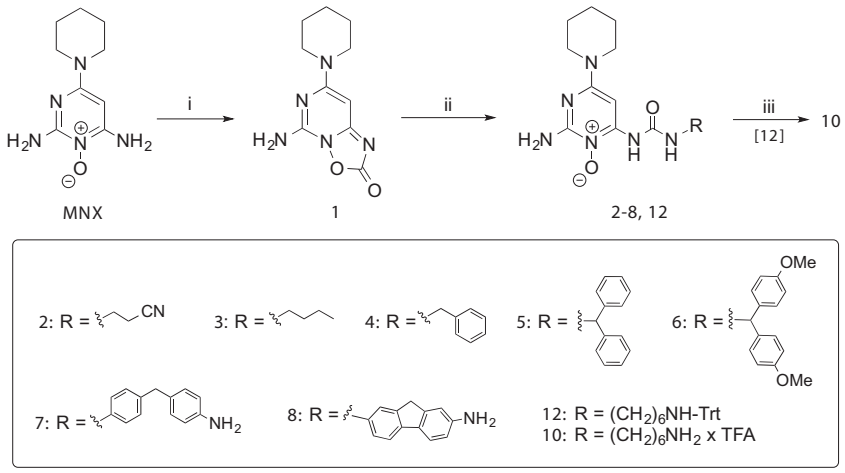


Figure 1. Structures of compounds related to the present work.



Scheme 1. Synthesis of MNX conjugates **2–8** and **10**. Reagents and conditions: (i) *N,N′*-carbonyldiimidazole, DMF, 60 °C, 3 h, 60%; (ii) R-NH_2, DMF, 60 °C, 2.5–16 h, 40–60%; (iii) 30% TFA in CH_2Cl_2, 2 h, RT, 95%.

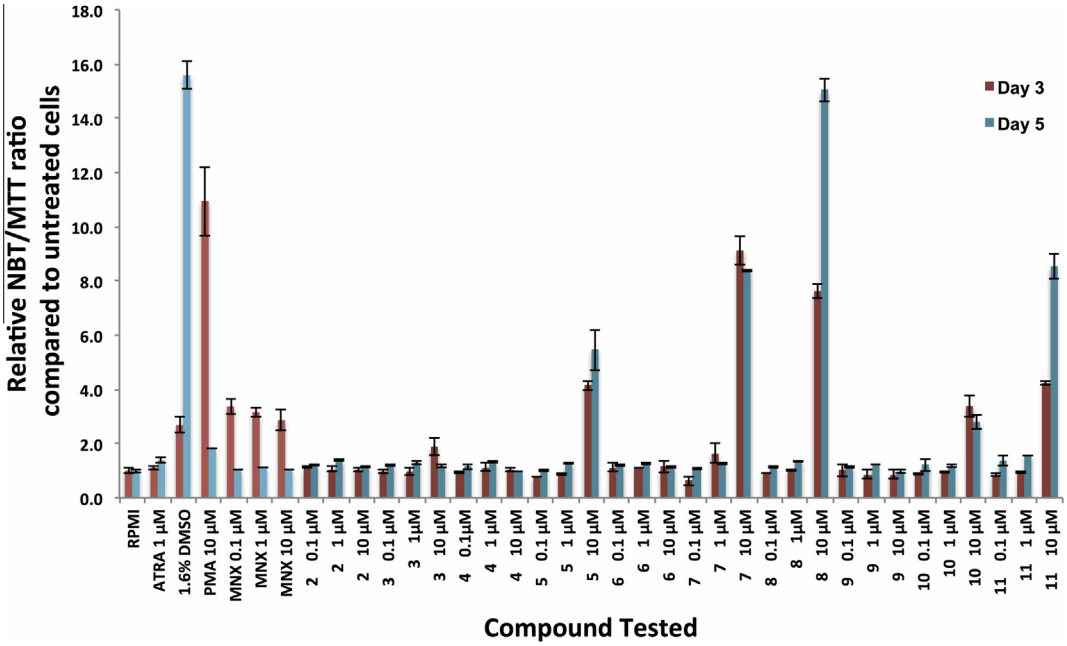


Figure 2. Graph depicting differentiation of HL-60 cells exposed to MNX and MNX conjugates **2–11**, as well as to positive and negative controls for 3 and 5 days. PMA: phorbol myristate acetate, DMSO: Dimethyl Sulfoxide. Each value is an average of three replicates for each of two independent experiments. Each data point is the measure of NBTZ reduction divided by MTT activity to give a proxy for respiratory burst activity (a differentiation marker) per cell and equalized to the value of the cells in unexposed media (RPMI). Error bars are calculated using standard error for each data point.

Table 1
IC_{50} and *C*log*P* values for MNX and MNX-conjugates **2–11** on HL-60 myeloid leukemia cells

Compound	Amine counterpart	IC_{50} µM (±SD)	*C*log*P*[a]
MNX	—	>10	0.02
2	3-Aminopropionitrile	>10	−0.02
3	*n*-Butylamine	>10	1.88
4	Benzylamine	>10	2.06
5	Benzhydrylamine	3.05 ± 0.06	3.41
6	4,4′-Dimethoxybenzhydrylamine	>10	3.25
7	4,4′-Methylenedianiline (MDA)	3.02 ± 0.08	3.04
8	2,7-Diaminofluorene (DAF)	3.04 ± 0.06	2.91
9	PUT	>10	−0.01
10	1,6-Diaminohexane	3.30 ± 0.10	1.05
11	SPM	3.02 ± 0.02	−0.26

[a] *C-QSAR Database*, Biobyte Corp., 201 West 4th Str., Suite 204, Claremont CA, California 91711, USA.

DAF (conjugate **8**). Although three of the most active (IC_{50} 3.02–3.05 µM) conjugates **5**, **7** and **8** are associated with high *C*log*P* values (2.91–3.41), the much less lipophilic (*C*log*P* = −0.26) MNX-SPM conjugate (**11**) presents equipotent differentiation activity to MNX-MDA conjugate (**7**) and the second most lipophilic conjugate **6** (*C*log*P* = 3.25) has very low activity (IC_{50} >10 µM). These results suggest that lipophilicity seems not to be a prerequisite for the differentiation induction potential.

In order to establish our initial observation that conjugation is indeed necessary for improving the biological profile of MNX and also to exclude the involvement of the amine counterpart, two of the most active compounds, conjugates **7** and **8**, were compared against co-administered unconjugated MNX and the corresponding amine counterparts MDA and DAF. Interestingly, such co-treatments as well as treatments with the amines MDA and DAF alone, did not show any particular differentiation or

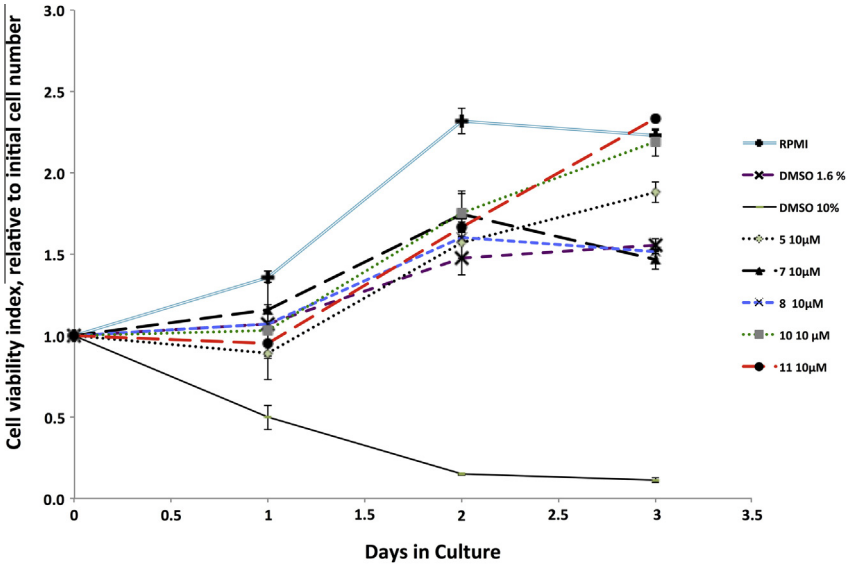


Figure 3. Graph depicting the cell growth curves of peripheral blood mononuclear cells from normal individual volunteers exposed to MNX-conjugates **5**, **7**, **8**, **10** and **11**. Each data point is an average of the MTT values of three separate replicates. 1.6% DMSO is a normal differentiation control, whilst 10% DMSO is a toxic control. Error bars are calculated using standard error for each data point.

cytotoxic activities at 10 μM, in contrast to compounds **7** and **8** (Fig. 4).

The HL-60 cells exposed to compounds **7**, **8** and **10** were also assessed for morphological evidence of differentiation after staining with Leishman's stain. Differentiation was also morphologically recognizable in addition to the NBT/MTT ratio. HL-60 cells exposed to the MNX-conjugates **7**, **8** and **10** can be compared to those differentiated with the positive controls DMSO and PMA as well as with undifferentiated HL-60 cells. Cells treated with the parent compound MNX are also shown (Fig. 5).

It is apparent from this figure that MNX alone, as well as MDA (counterpart amine of **7**), have very little effect on differentiation and the cells retain euchromatic nuclear material and large nuclei. On the contrary, conjugates **7**, **8** and **10** cause a number of changes characteristic of differentiation, namely the reduction in cell number, the condensation of nuclear chromatin and the irregularity of nuclear and cytoplasmic shape in some cells. Despite showing very high NBT/MTT ratios they do not show the complete differentiation induced by DMSO and PMA. This may be explained by the fact that oxidative burst enzymatic activity capable of reducing NBT is an earlier marker of differentiation, being already highly expressed in cells just beyond the promyelocyte stage of maturation and long before the final band cell and granulocyte stage.[28] It can be also noted that regarding HL-60, being leukemia cells and not classical myeloid blasts, even agents producing near full differentiation do not always produce classical looking granulocytes and macrophages.

Regarding the mechanism through which this differentiation induction is being caused is at present unclear. Natural PAs have been shown to be involved both in differentiation and in

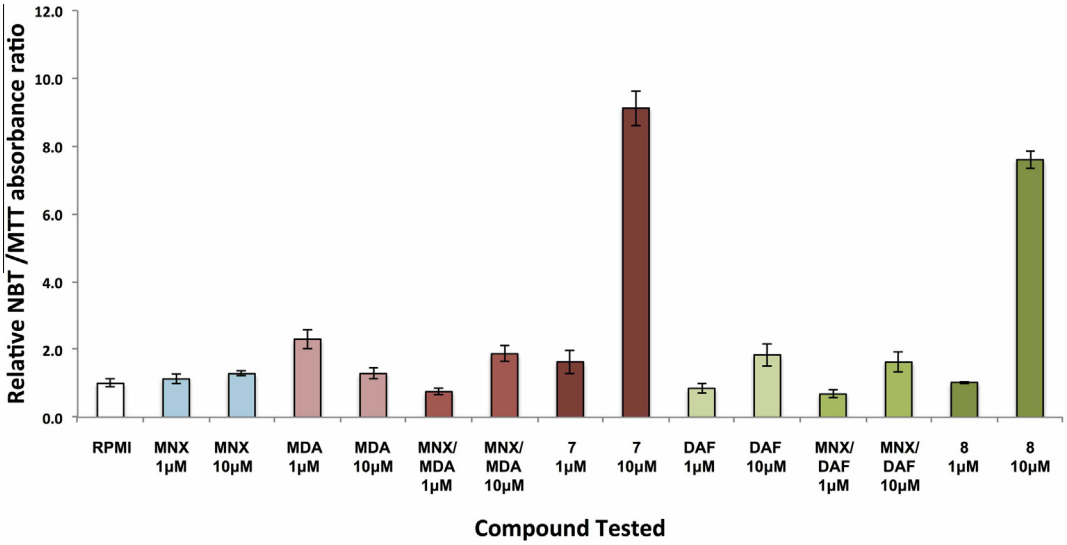


Figure 4. Graph depicting differentiation of HL-60 cells exposed to MNX conjugates **7** and **8** as well as the parent compound MNX and co-dosage with MNX and the conjugated amines for 3 days. Each value is an average of three replicates. Each data point represents the measure of NBTZ reduction divided by MTT activity to give a proxy for respiratory burst activity (a differentiation marker) per cell and equalized to the value of the cells in unexposed media (RPMI). Error bars are calculated using standard error for each data point. Negative and positive controls are also included.

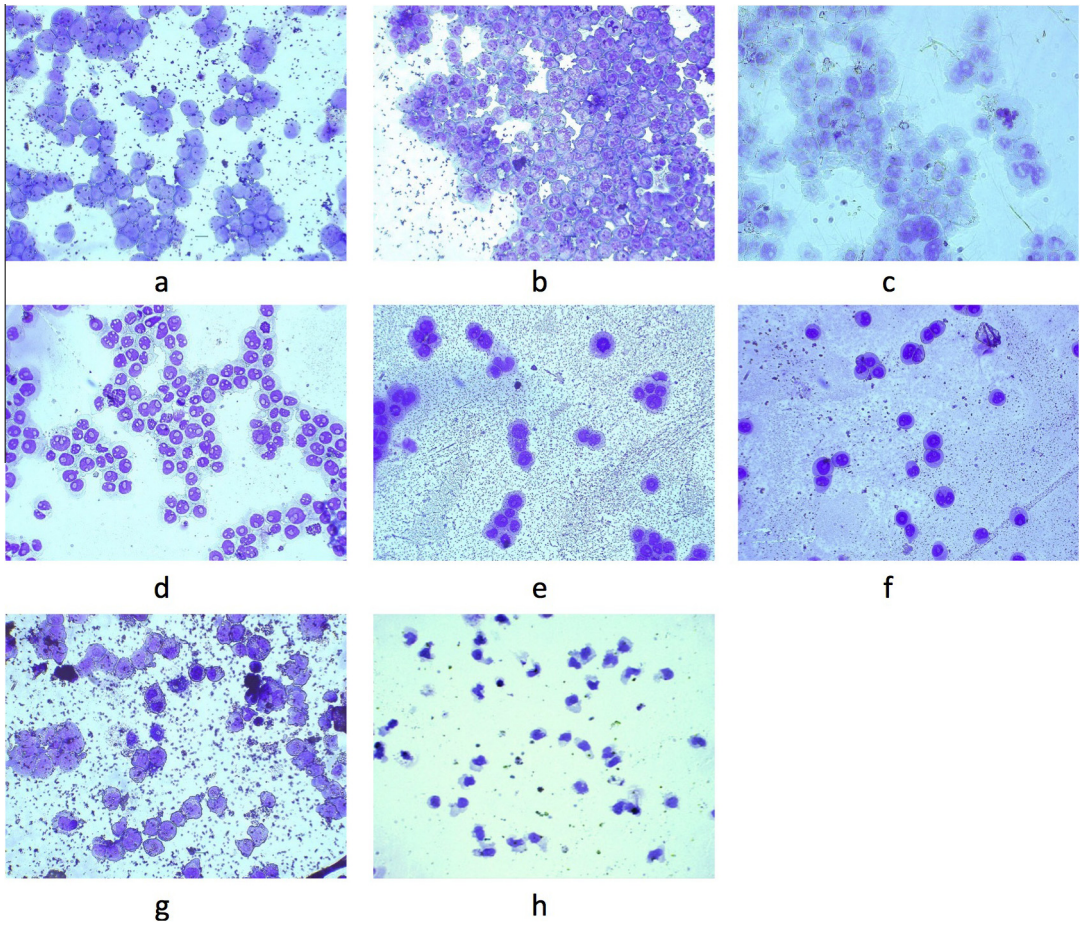


Figure 5. Images taken after cytocentrifugation of cells exposed to different compounds for 3 days onto slides. Each slide is then air dried for fixing and stained with Leishman's stain. Images are at 40× magnification. (a) RPMI medium alone. (b) MNX, 10 μM. (c) MDA, 10 μM. (d) Compound **7**, 10 μM. (e) Compound **8**, 10 μM. (f) Compound **10**, 10 μM. (g) PMA, 10 μM. (h) 1.6% DMSO.

differentiation-blocking, particularly where reduction of SPD and PUT as well as the accumulation of SPM block HL-60 differentiation by multiple inducers.[29–32] Analogs of SPM have actually been shown to be effective cytotoxic agents in the K562 Chronic Myeloid Leukemia cell line.[33]

One of the mechanisms through which many molecules can induce differentiation in myeloid leukemia cells is through the induction of DNA breaks.[34] This activity has already been confirmed for the amine counterpart of conjugate **7**, that is MDA.[35] In fact, MDA has been shown to be carcinogenic in some animal studies when added at 150 to 300 parts per million in drinking water for rodents.[36] However, whilst increasing the risk of certain tissue cancers, which could relate to their potential for DNA damage, the same study also showed that MDA produced reduction in leukemia risk, which agrees with our results. Despite this fact which can be a cause of concern, numerous other tissues exposed to MDA in other animal studies at lower dosages, including normal peripheral blood lymphocytes, do not show any marked toxicity or DNA adduct formation, despite adduct formation in blood proteins.[37] This correlates well with the lack of toxicity seen here on the peripheral blood mononuclear cells. However, changes in PUT and SPM levels (amine counterparts in MNX conjugates **9** and **11**, respectively) are also known to be involved in the differentiation of other cells types, including keratinocytes,[38] suggesting that the effect cannot be purely associated to the DNA damage in the present compounds.

It should be noted that the reduction of SPM in HL-60 cells, induced to differentiate with PMA, was not affected by inhibitors of the enzymes involved in the normal biosynthetic polyamine pathway (including ornithine decarboxylase). On the other hand, major changes in activity of these enzymes did not occur with PMA differentiation either, despite the changes in intracellular PA levels.[36] It is thus suspected that other pathways involving the PAs are being affected in this differentiation and in fact these pathways might well be where the current MNX conjugates are being involved. In complementary research, increasing SPM levels artificially in the cells, abrogates myeloid leukemia cell differentiation.[39]

Conjugate **11** might possibly act as a PA analog activating spermine oxidase which catabolizes SPM.[40] Upregulation of the catabolic oxidase has been seen both with PA analogs and other compounds.[41] Thus reduction of SPM levels due to the presence of conjugate **11** might result in differentiation induction. Since H_2O_2 is also produced by this reaction, this might play a role in causing DNA breaks, also resulting in differentiation.

Furthermore, it has been shown that the N^1-acetylated 1,6-diaminohexane is deacetylated upon cell entry.[42] 1,6-diaminohexane, which is the amine counterpart of conjugate **10**, is known to have a strong differentiation effect on erythroleukemia. Thus, conjugate **10** might act in a similar manner, resulting in this case in myeloid differentiation.

In conclusion, it is clear that conjugation of MNX to selected amines and PAs can result in strong differentiation-inducing

agents, which may offer methods of treatment for resistant forms of AML. It appears that the best candidates should contain as amine counterpart an aromatic amine connected through a methylene group to another aryl moiety or a long-chain PA. Further work along the line of preparing a more extended library of MNX-amine and -PA conjugates, taking into consideration our preliminary results, as well as studies toward the elucidation of the mechanism(s) of differentiation induction caused by these compounds, are now in progress.

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Supplementary data

Supplementary data (experimental procedures and characterization data for all compounds, as well as copies of ^{1}H and ^{13}C NMR spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.01.048. These data include MOL files and InChiKeys of the most important compounds described in this article.

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