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Original article

Bis-pyrrolyl-tetrazolyl derivatives as hybrid polar compounds: A case of lipophilic functional bioisosterism with bis-acetamides

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ABSTRACT

Based on previous studies on bis-acetamides that act as hybrid polar compounds to induce leukemia cell differentiation, an attempt was made to bioisosterically replace the amide moiety with the lipophilic non-classical bioisostere tetrazole. A pyrrole group was also included in the molecule in order to retain the hydrogen bond donor capability. Thus, by linking the two polar ring systems with a highly lipophilic methylene chain compounds **2**–**4** were synthesized and assessed for their anti-proliferative activity in combination with their ability to induce murine erythroleukemia (MEL) cell differentiation. Furthermore, an initial investigation of the structure–activity relation points for the active compound **3** was undertaken by synthesizing compound **5** (a p-xylene analog) and compound **8** (a methylamidopyrrolyl analog). All compounds caused a dose-dependent inhibition of MEL cell growth but to a different extent. Compound **3** (1,6-bis[5-(1*H*-pyrrol-1-yl)-2*H*-tetrazol-2-yl]hexane) promoted erythroid differentiation in a fifty-fold lower concentration than hexamethylenebisacetamide (HMBA). Though induction of differentiation was to a lesser extent than HMBA, it caused accumulation of 80% Hb-producing cells as compared to that produced by HMBA, leading to differentiation-depended cell growth inhibition equal to that of HMBA after 96 h in culture. Compound **3** represents a potent inducer of hemoglobin gene activation in leukemic cells.

1. Introduction

Human leukemias are considered as clonal hematopoietic disorders that are characterized by uncontrolled cell growth, failure of differentiation and acquired loss of cell death. Evidence indicates that leukemias are likely to be initiated by transformed hematopoietic stem cells (HSCs) [so called leukemia initiating cells (LICs) or leukemia stem cells (LSCs)] or early uncommitted multipotent progenitors being genetically or epigenetically aberrant [1–5]. Although leukemias, to some extent, are treatable diseases with conventional chemotherapeutic agents (antimetabolites, mitotic blockers, antitumor antibodies, alkylating agents and others), quite often chemotherapy fails due to cellular heterogeneity and acquired drug resistance developed during the course of the treatment.

The discovery over the past several years that leukemic cells can be induced to differentiate into post-mitotic cells, unable to support malignant growth with the use of structurally diversified agents, led to the so called "*Differentiation Therapy of Cancer*" [6–11]. This approach has been shown promising [6], since promyelocytic leukemia (PML) patients nowadays are successfully treated with differentiating agents, like all-trans retinoic acid, As_2O_3, and histone deacetylase inhibitors (HDACIs) that promote granulocytic differentiation and cell growth inhibition [12–18].

Retrospectively, in the search for inducers of leukemia cells' differentiation a variety of different classes were explored and among them the class of hybrid polar compounds (HPCs, formerly known as polar apolar inducers) appears to be the most efficacious. In this class, DMSO and hexamethylenebisacetamide (HMBA) constitute the prototypes, which are strong inducers of differentiation in several transformed cell lines [7]. The key structural characteristics in HPCs are two highly polar groups that are connected by an apolar methylene chain whose optimal length was found to be 5–7 carbon atoms [8].

Most recently, we have proposed the development of novel agents, that is, agents to be able to reduce cell growth by inducing

Abbreviations: HDACIs, histone deacetylase inhibitors; HMBA, hexamethylenebisacetamide; HPCs, hybrid polar compounds; HSCs, hematopoietic stem cells; LICs, leukemia initiating cells; LSCs, leukemia stem cells; MEL, murine erythroleukemia; PML, promyelocytic leukemia.

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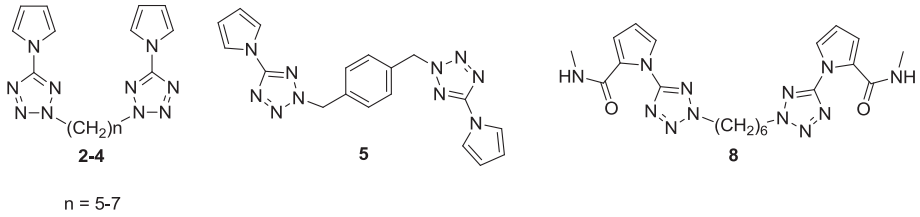


Fig. 1. Chemical structures of the synthesized compounds.

leukemic cell differentiation [19]. The central objective of our rationale was to develop agents that can be able to act at relatively low concentrations (<1 mM) and promote terminal maturation and growth arrest in the majority, if not all leukemic cells.

Under this framework, a series of 1*H*-pyrrol-1-yl-2*H*-tetrazol-5-yl-derivatives were synthesized as HPCs (compounds **2**–**5** and **8**, Fig. 1) and assessed for their biological activity as inducers of differentiation and inhibitors of cell growth. The design was based on a putative bioisosteric replacement of the acetamide moiety of HMBA, with that of the more lipophilic pyrrolyl-tetrazolyl structure. Tetrazoles can broadly act as non-classical bioisosteres of the amide moiety [20], while the C-2-H of the pyrrole ring is envisioned to participate in a hydrogen bond as a donor like the NH group of HMBA [21–23].

In compounds **2**–**4** the lipophilic methylene chain varied between 5 and 7 carbon atoms [8], while in the most active template **3** further modifications were made by either drastically changing the spatial disposition of the pyrrolyl-tetrazolyl groups with the inclusion of a p-xylene chain (i.e. compound **5**) or by introducing functional residues which could participate in additional hydrogen-bond interactions such as the methylamido side-groups (i.e. compound **8**).

2. Results and discussion

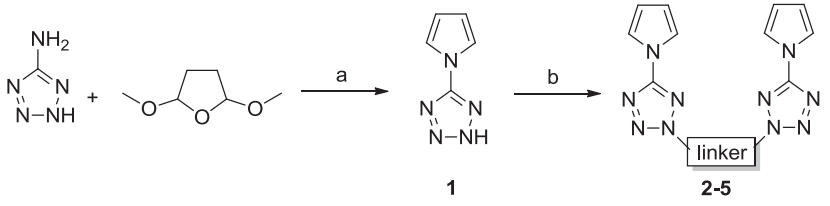
2.1. Chemistry

Compound **1** (Scheme 1) was synthesized by adapting a modified Clauson–Kaas reaction [24,25]. Phase transfer catalyzed condensation between the anion of **1** and α,ω-dibromoalkanes or 1,4-bis(bromomethyl)benzene provided the bis-pyrrolyl-tetrazolyl derivatives **2**–**5** in good yields (Scheme 1). Tetrazole can exist in two tautomeric forms, 2*H*-tetrazole being the most stable [26]. The majority of the nucleophilic alkylation reactions result in mixtures of *N*1- and *N*2-alkyl isomers [27], but the regio-selectivity observed in the present work, is presumably due to the introduced stereo-requirements of the utilized phase transfer catalyst [28]. The proposed reaction's regio-selectivity was established by ^{13}C NMR analysis. The chemical shifts of the tetrazolyl C-5 in compounds **2**–**5** ranged from 160.85 to 161.55 and compares favorably to previously reported values for *N*2-substituted tetrazoles [29].

Compound **8** was synthesized from **3** in three steps (Scheme 2). It involved an initial Friedel–Crafts acylation, followed by a haloform reaction [25] and a dehydrative amidation of the formed carboxylic acid [30,31].

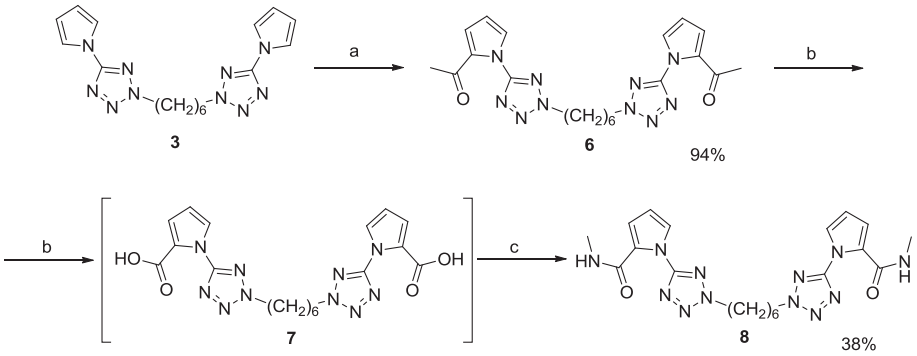
2.2. Evaluation of biological activity

The widely used model system of cultured murine erythroleukemia (MEL or Friend) cells [32,33] was employed to assess the biological activity of the synthesized compounds. Such an approach was based on observations over the past several years that chemical inducers were able to selectively restrict proliferation of leukemic cells *in vitro* (committed cells along the erythrocytic pathway) through a highly coordinated process regulating gene expression. In particular, cells committed to erythroid differentiation produce vast amounts of hemoglobin (Hb) (an authentic erythroid biomarker) and undergo a programmed loss of proliferative capacity restricted to only a few divisions, due to their irreversible growth arrest in G1/G0 phase of cell cycle (differentiation-dependent growth arrest) [10]. For the evaluation, each agent was dissolved in DMSO and tested in varying concentrations in a way to estimate the growth inhibition potential, as well as to determine the optimum inducing concentration for each compound being



cmpd	linker	yield
2	-$(CH_2)_5$-	64%
3	-$(CH_2)_6$-	72%
4	-$(CH_2)_7$-	68%
5	$^{-}H_2C$—C$_6$H$_4$—CH_2^{-}	44%

Scheme 1. Reagents and reaction conditions: (a) 4-chloropyridine hydrochloride, 1,4-dioxane, reflux 3 h; (b) α,ω-dibromoalkane or 1,4-bis(bromomethyl)benzene, benzyltributylammonium chloride, THF, reflux 22 h.



Scheme 2. Reagents and conditions: (a) $AlCl_3$, acetic anhydride, rt 2 h; (b) NaOBr, 1,4-dioxane/H_2O, rt 2 h; (c) CDI, CH_3NH_2, THF, rt overnight.

able to promote erythroid differentiation (Figs. 2 and 3). In all studies reported, however, the concentration of the solvent DMSO kept way below the 0.5% v/v that does not affect cell growth and differentiation of MEL cells in culture. HMBA, a well known potent inducer of MEL cell differentiation and growth inhibitor, was used as a positive control. Just to mention and in order to facilitate data comparison between synthesized agents with that of HMBA, only the optimum inducing concentration (5 mM) of HMBA was used in these studies. Indeed, at very low concentrations (<0.1 mM), HMBA exhibited no substantial effect on growth and differentiation of MEL cells as previously shown [34].

From data illustrated in Figs. 2 and 3, it is apparent that all compounds caused a dose-dependent inhibition of MEL cell growth after 48 h in culture, but to a different extent varying from 30% to 80% at concentrations from 0.01 μM to 100 μM. Compounds **2**, **4**, **5** and **8** were found to induce erythroid differentiation in 5%–12% of cells at 100 μM after 96 h in culture. Interestingly, compound **3** exhibited a minimal effect as growth inhibitor (Fig. 2), but attained the ability to promote differentiation close to 30% of MEL cells (Fig. 3). Such behavior was similar to that of the known inducer HMBA and this effect on cell proliferation was more prominent after 96 h when differentiating cells accumulate in increasing numbers (Fig. 4B). A more detailed analysis shown in Fig. 4A indicated that compound **3** inhibited cell growth in a time-dependent manner by more than 75% inhibition after 96 h in culture. To this end, its inducing capacity has led to Hb accumulation (an internal marker of erythroid maturation as previously mentioned) at a level close to that observed by HMBA (Fig. 4C). The latter, suggests that the observed growth arrest of MEL cells is attributed to the induction of differentiation, instead of being just a direct cytotoxic effect exhibited by that compound (differentiation-dependent growth inhibition).

The data presented thus far, revealed that compound **3** exerts concentration- and time-dependent growth inhibitory effect, while it exhibited substantial differentiation inducing capacity (Figs. 3 and 4B and C). For this reason, this compound was further evaluated for its potential to activate β^{major} *globin* gene expression in comparison to β-actin gene. RT-PCR analysis indicated that compound **3** induced indeed transcription of β^{major} *globin* gene in levels comparable to that seen with HMBA (Fig. 5) and again in a fifty-fold lower concentration. It is known that β^{major} *globin* gene is developmentally regulated upon erythropoiesis and used as a marker for the assessment of MEL cell differentiation, whereas *β-actin* gene is a housekeeping gene, encoding a stable gene product, the β-actin, and it is used as control in gene expression studies. It should be noted that β-actin gene expression levels exhibited a similar pattern in all three MEL cell cultures employed, as expected. These data are consistent with the results illustrated in Fig. 4B and C where a substantial accumulation of benzidine-positive (Bz+) cells and production of Hb were observed.

3. Conclusion

In this work a bioisosteric replacement of the acetamide moiety of hybrid polar compounds that act as differentiation inducers with the more lipophilic pyrrollyl-tetrazolyl scaffold was shown. Among the synthesized compounds, **3** was shown to inhibit cell growth in

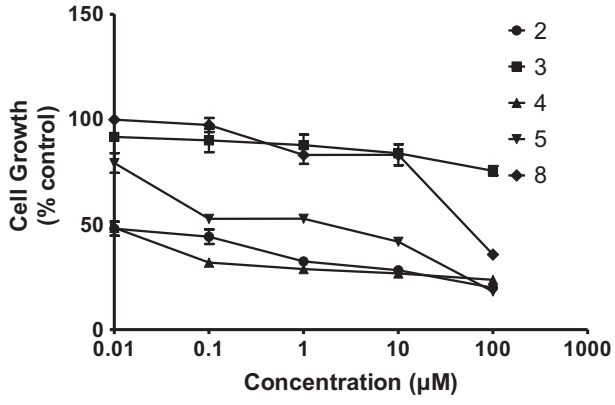


Fig. 2. Dose-dependent changes in cell growth of MEL cells after treatment with varying concentrations of the synthesized compounds for 48 h. Cell growth is expressed as percentage of that exhibited by untreated (control) MEL cells.

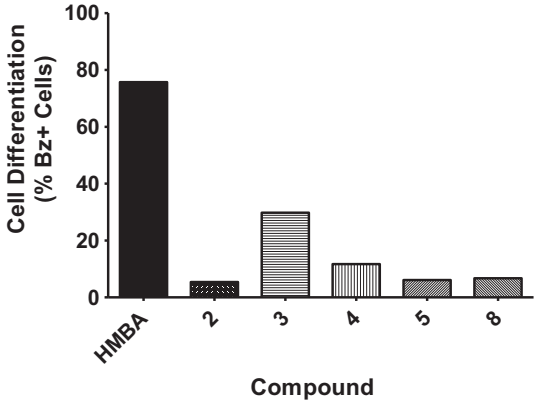


Fig. 3. Percentage of differentiated MEL cells (benzidine-positive cells) after treatment with 100 μM of the synthesized compounds for 96 h. It should be noted that the data of the optimum differentiation concentration (5 mM) of HMBA is included as a positive control.

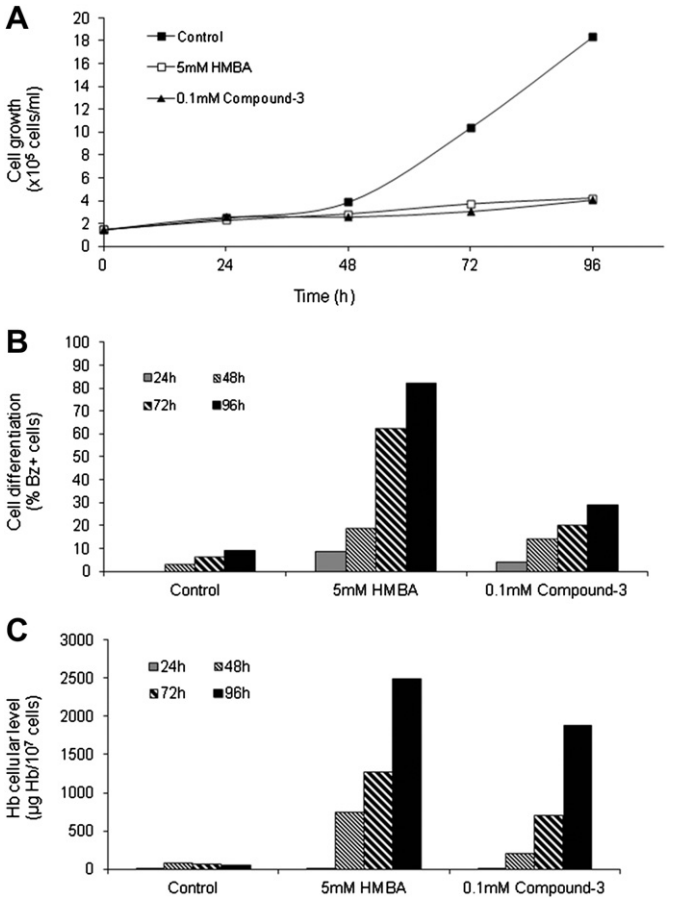


Fig. 4. Kinetic analysis of time-dependent effect of compound **3** (0.1 mM) on cell growth (**A**), erythroid differentiation (**B**) and Hb production (**C**) in MEL cells as compared to untreated (control) and HMBA-treated (5 mM) cultures.

a time- and concentration-dependent manner and to promote erythroid differentiation. Though promotion of differentiation was to a lesser extent than that promoted by HMBA, **3** caused Hb accumulation to a level close to that observed by HMBA, thus leading to inhibition of cell growth equal to that of HMBA and in a fifty-fold lower concentration.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

All reagents were purchased from Sigma–Aldrich and used without further purification. UV–vis spectra were obtained on a Shimadzu UV-1700 PharmSpec (UVprobe Ver. 2.21). IR spectra were taken with a Hitachi U-2001 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AW80 at 80 MHz or a Bruker AM 300 at 300 MHz with internal TMS standard. ^{13}C NMR spectra were recorded on a Bruker AM 300 at 75.5 MHz. Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. Coupling values (J) are given in hertz (Hz). Spin multiplicities are given as s (singlet), d (doublet), t (triplet), m (multiplet). Elemental analyses were performed with a Perkin–Elmer 2400 CHN analyzer in the Department of Organic Chemistry, School of Chemistry, Aristotle University of Thessaloniki, Greece, or for compound **6** at Galbraith Laboratories, Inc., Knoxville, TN. LC-MS analysis was performed on a Shimadzu LC-MS 2010EV system with SPD-20A UV/Vis Detector (pro-ACTINA S.A., Athens, Greece). Analysis using Electron Spray Ionization (ESI) interface was performed in a reverse phase C-18 column (Thermo Scientific BDS Hypersil, 250 mm × 4.6 mm, particle size 5 μM). The mobile phase consisted of water: methanol (4:6, isocratic mode) at a flow rate of 0.5 mL min^{-1}. Injection volume was 10 μL and product detected at 222, 254 nm. The mass detector was operated in the positive and negative mode with nitrogen as the nebulizer gas at a flow rate of 1.5 L min^{-1}. Mass spectra were recorded from 50 to 1000 Da. Melting points are uncorrected and were determined in open glass capillaries using a Mel-Temp II apparatus. Flash column chromatography was carried out with Merck silica gel 60 (230–400 Mesh ASTM). TLC was run with Fluka Silica gel/TLC-cards. All solvents used for column chromatography were routinely distilled prior to use. Petroleum ether refers to the fraction with bp 40–60 °C unless stated otherwise.

4.1.2. 5-(1H-Pyrrol-1-yl)-1H-tetrazole (**1**)

Prepared as described previously by Pegklidou et al. [35].

4.1.3. General method for the preparation of dimers (**2**–**5**)

The appropriate α,ω-dibromoalkane or 1,4-bis(bromomethyl) benzene (1 mmol) and a catalytic amount of benzyltributylammonium chloride were added to a stirred and under nitrogen atmosphere solution of **1** (3 mmol) in dry THF (30 mL). The mixture was cooled to 0 °C and 3 mmol of NaH (60% dispersion in mineral oil) were added. The resulting mixture was refluxed for 22 h. After this period, it was poured into a stirred ice cold mixture of dichloromethane (50 mL) and 5% HCl (50 mL). The two phases were separated and the aqueous phase was extracted with dichloromethane (2 × 50 mL). The combined organic extracts were washed with 10% $NaHCO_3$ (2 × 50 mL), brine and dried over anhydrous Na_2SO_4. The solvents were evaporated under reduced pressure and the residue was flash chromatographed with a suitable mixture of petroleum ether/ethyl acetate [10:1–5:1], followed by recrystallization from dichloromethane/petroleum ether or petroleum ether (bp. 80–110 °C).

*4.1.3.1. 1,5-Bis[5-(1H-pyrrol-1-yl)-2H-tetrazol-2-yl]pentane (**2**).* White solid (64%); mp 85 °C; ^{1}H NMR ($CDCl_3$): δ 1.20–2.33 (m, 6H, $CH_2CH_2CH_2CH_2CH_2$), 4.56 (t, 4H, tetrazolyl-CH_2, J = 4.0 Hz), 6.24–6.52 (m, 4H, pyrrolyl-3,4*H*), 7.32–7.60 (m, 4H, pyrrolyl-2,5*H*); ^{13}C NMR ($CDCl_3$): δ 22.4, 27.5, 52.2, 111.0, 118.3, 160.9; Anal. Calcd

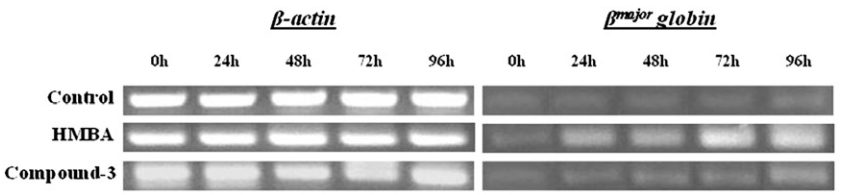


Fig. 5. Assessment of β^{major} *globin* and β-*actin* gene levels in control and agent-treated MEL cells by RT-PCR. Concentrations of compounds were as shown under Fig. 4.

for $C_{15}H_{18}N_{10}$: C, 53.24; H, 5.36; N, 41.39. Found: C, 53.29; H, 5.24; N, 41.52.

*4.1.3.2. 1,6-Bis[5-(1H-pyrrol-1-yl)-2H-tetrazol-2-yl]hexane (**3**).* White solid (72%); mp 108 °C; UV (EtOH absolute) λ_{max}: 230.6 ε_{max}: 11 120; ^{1}H NMR ($CDCl_3$): δ 1.21–1.75(m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.84–2.36 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 4.55 (t, 4H tetrazolyl-CH_2, J = 6.6 Hz), 6.27–6.52 (m, 4H, pyrrolyl-3,4*H*), 7.34–7.60 (m, 4H, pyrrolyl-2,5*H*); ^{13}C NMR ($CDCl_3$): δ 25.5, 28.7, 53.1, 111.7, 118.9, 161.5; *m/z* [ESI positive] 353 $(M+H)^+$; Anal. Calcd for $C_{16}H_{20}N_{10}$: C, 54.53; H, 5.72; N, 39.75. Found: C, 54.38; H, 5.45; N, 39.73.

*4.1.3.3. 1,7-Bis[5-(1H-pyrrol-1-yl)-2H-tetrazol-2-yl]heptane (**4**).* White solid (68%); mp 60 °C; ^{1}H NMR ($CDCl_3$): δ 1.11–1.55 (m, 6H, $CH_2CH_2CH_2CH_2CH_2CH_2CH_2$), 1.79–2.25 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2CH_2$), 4.55 (t, 4H, 4H tetrazolyl-CH_2, J = 8.0 Hz), 6.27–6.49 (m, 4H, pyrrolyl-3,4*H*), 7.35–7.58 (m, 4H, pyrrolyl-2,5*H*); ^{13}C NMR ($CDCl_3$): δ 25.9, 28.0, 28.8, 53.2, 111.7, 118.9, 161.5; Anal. Calcd for $C_{17}H_{22}N_{10}$0.06 hexane: C, 56.11; H, 6.20; N, 37.69. Found: C, 56.47; H, 6.06; N, 37.82.

*4.1.3.4. 1,4-Bis((5-(1H-pyrrol-1-yl)-2H-tetrazol-2-yl)methyl)benzene (**5**).* White solid (44%). mp 192–193 °C; UV (EtOH absolute) λ_{max}: 232.9 ε_{max}: 17 760. ^{1}H NMR ($CDCl_3$-DMSO-d_6): δ 5.95 (s, 4H, CH_2), 6.34–6.40 (m, 4H, pyrrolyl-3,4*H*), 7.40–7.48 (m, 8H, pyrrolyl-2,5*H* and phenyl-*H*); ^{13}C NMR (DMSO-d_6): δ 56.6, 112.5, 119.5, 129.3, 134.7, 161.6; Anal. Calcd for $C_{18}H_{16}N_{10}$: C, 58.06; H, 4.33; N, 37.61. Found: C, 57.96; H, 4.18; N, 37.60.

*4.1.4. 1,1′-(1,1′-(2,2′-(Hexane-1,6-diyl)bis(2H-tetrazole-5,2-diyl)) bis(1H-pyrrole-2,1-diyl))diethanone (**6**)*

To a stirred and under a nitrogen atmosphere mixture of 2728 mg (20.46 mmol) anhydrous $AlCl_3$ in 1,2-dichloroethane (30 mL), 1048 mg (10.26 mmol) of acetic anhydride were added slowly and over a 15 min period. Subsequently, 609 mg (1.73 mmol) of **3** were added and the resulting mixture was stirred for 2 h under a nitrogen atmosphere. The reaction mixture was poured into ice and water and the product was extracted with CH_2Cl_2 (2 × 20 mL). The combined organic extracts were washed with an aqueous solution of 10% $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. The solvents were evaporated under reduced pressure and the residue was flash chromatographed with petroleum ether/EtOAc 9:4 to provide an analytical sample of the title compound (709 mg, 94%); mp 63–65 °C; IR (nujol): 1652 cm^{-1} (C═O); ^{1}H NMR ($CDCl_3$): δ 1.18–1.61 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.85–2.32, 2.42 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), (s, 6H, $COCH_3$), 4.64 (t, 4H tetrazolyl-CH_2, J = 4.8 Hz), 6.28–6.53 (m, 2H, pyrrolyl-4*H*), 7.00–7.31 (m, 4H, pyrrolyl-3,5*H*); Anal. Calcd for $C_{20}H_{24}N_{10}O_2$: C, 55.04; H, 5.54; N, 32.09. Found: C, 55.24; H, 5.63; N, 32.39.

*4.1.5. 1,1′-(2,2′-(Hexane-1,6-diyl)bis(2H-tetrazole-5,2-diyl))bis(N-methyl-1H-pyrrole-2-carboxamide) (**8**)*

To a cold (ice bath) solution of **6** (628 mg, 1.44 mmol) in 1,4-dioxane (18 mL) and H_2O (13 mL) a freshly prepared cold solution of NaOBr [prepared by the addition of (1076 mg, 6.73 mmol) Br_2 to a stirred mixture of 25 mL 12% NaOH and 6.2 mL 1,4-dioxane] was added dropwise. After 2 h of stirring at rt, acetone (10 mL) was added and the reaction mixture was acidified with HCl, extracted with CH_2Cl_2 (2 × 50 mL), washed with brine, dried over anhydrous Na_2SO_4 and the solvents were removed under reduced pressure. The residue was dissolved in EtOH, H_2O was added and the precipitated was collected and dried to afford **7** (393 mg) which was used in the next step without further purification. IR (nujol): 3121 (O–H), 1673 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.22–1.64 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.82–2.34 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 4.67 (t, 4H, tetrazolyl-CH_2, J = 3.04 Hz), 6.23–6.81 (m, 4H, pyrrolyl-3,4*H*), 6.99–7.29 (m, 4H, pyrrolyl-2,5*H*).

Crude **7** and 295 mg (1.81 mmol) 1,1′-carbonyldiimidazole (CDI) were refluxed in 4.2 mL dry THF under a nitrogen atmosphere for 2 h. The mixture was cooled at rt, 1.7 mL of a solution of 2 M CH_3NH_2 in THF was added and the resulting mixture was stirred overnight at rt and under a nitrogen atmosphere. The reaction mixture was poured into water, and extracted with AcOEt (2 × 40 mL). The organic extracts were washed with 1 M HCl, brine, dried over anhydrous Na_2SO_4, were concentrated under reduced pressure and recrystallized from EtOH/H_2O (177 mg, 38%); mp 173–174 °C; IR (nujol): 3313 (N–H), 1643 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.44–1.51 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 2.03–2.14 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 2.89 (s, 6H, CH_3), 4.64 (t, 4H, tetrazolyl-CH_2, J = 7.0 Hz), 6.30–6.35 (m, 2H, pyrrolyl-4*H*), 6.47 (br s, 2H, N*H*), 6.74–6.80 (m, 2H, pyrrolyl-3*H*), 7.15–7.28 (m, 2H, pyrrolyl-5*H*); LC-MS purity 98.1% (222 nm) and 97.1% (254 nm); *m/z* [ESI positive] 467 $(M+H)^+$, 489 $(M+Na)^+$ *m/z* [ESI negative] 465 $(M-H)^-$.

4.2. Growth inhibition and induction of differentiation of MEL cells

4.2.1. Chemicals and cell cultures

All agents were dissolved in DMSO (Sigma–Aldrich) and used at different concentrations in cell cultures. Attention was paid to keep the concentration of solvent DMSO below the 0.5% v/v so that it does not affect cell growth and differentiation. MEL cells were used throughout this study. These cells were seeded at 1.0×10^5 cells/mL and grown in DMEM medium (Gibco) containing 10% v/v FBS (Fetal Bovine Serum, Gibco) and 1% v/v PS (Penicillin-Streptomycin, Gibco) at 37 °C with 5% CO_2 and humidified atmosphere (~95%).

4.2.2. Assessment of cell growth, erythroid differentiation and Hb production

Exponentially growing MEL cells (1.0×10^5 cells/mL) in culture with DMEM medium supplemented with 10% v/v FBS and 1% v/v PS, were incubated with compound **3** (10^{-4} M). Also, cell cultures with no addition (untreated-control) and/or the known inducer HMBA (5 mM) were served as negative and positive control, respectively. Cell growth in control and agent-treated MEL cell cultures was determined at various time intervals by removing samples of cells and measuring their number using a Neubauer hematocytometer. Cell growth of agent-treated cells was expressed as absolute values or a percentage of that observed for the control untreated cultures. Erythroid differentiation was assessed by staining benzidine-positive cells (Bz^+, Hb producing cells) with the use of benzidine-H_2O_2 dilution, as earlier described [36,37]. The content of total cellular Hb was determined spectrophotometrically, as previously described [38].

4.2.3. RNA extraction and RT-PCR analysis

MEL cells grown in culture, were treated separately with HMBA (5 mM), compound **3** (10^{-4} M) for 0 h, 24 h, 48 h, 72 h and 96 h, as described under Fig. 4. Untreated parental cells were served as control. At the end of time intervals, cells were harvested from culture, washed twice with PBS before the isolation of total cytoplasmic RNA and processed for RT-PCR analysis. 1 μg of cytoplasmic RNA from each sample was then used to assess the steady-state level of RNA transcripts encoded by β^{major} *globin* and/or *β-actin* genes. The generated DNA products were analyzed electrophoretically in 1% w/v agarose gel, stained with EtBr, visualized under UV light and the data obtained are shown in Fig. 5.

One step RT-PCR was performed with the RobusT-I RT-PCR kit (Finnzymes) in 25 μL volume reactions. Total RNA extracted according to acid guanidinium thiocyanate-phenol-chloroform extraction method [39] from untreated (control) and agent-treated MEL cells and indicated in the text, was used as template

(1 μg/reaction). The RT-PCR running conditions were the following: 50 °C for 30 min, 94 °C for 2 min, (94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min) for 30 cycles and 72 °C for 10 min. *β-actin* gene was used as internal control. The following pairs of primer sequences were used: β^{major} *globin,* 5′-CTGCTGGTTGTCTACCCTTGG-3′ (sense) and 5′-CCTGAAGTTCTCAGGATCCAC-3′ (anti-sense); *β-actin,* 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (sense) and 5′-TAAAACG-CAGCTCAGTAACAGTCCG-3′ (anti-sense). RT-PCR products were analyzed by agarose gel (1% w/v) electrophoresis.

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References

[1] D. Bonnet, Cancer stem cells: AMLs show the way, Biochem. Soc. Trans. 33 (2005) 1531–1533.
[2] W.I. Chan, B.J. Huntly, Leukemia stem cells in acute myeloid leukemia, Semin. Oncol. 35 (2008) 326–335.
[3] T. Lapidot, C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, J.E. Dick, A cell initiating human acute myeloid leukaemia after transplantation into SCID mice, Nature 367 (1994) 645–648.
[4] A.S. Tsiftsoglou, I.D. Bonovolias, S.A. Tsiftsoglou, Multilevel targeting of hematopoietic stem cell self-renewal, differentiation and apoptosis for leukemia therapy, Pharmacol. Ther. 122 (2009) 264–280.
[5] J.C. Wang, J.E. Dick, Cancer stem cells: lessons from leukemia, Trends Cell Biol. 15 (2005) 494–501.
[6] M. Leszczyniecka, T. Roberts, P. Dent, S. Grant, P.B. Fisher, Differentiation therapy of human cancer: basic science and clinical applications, Pharmacol. Ther. 90 (2001) 105–156.
[7] P.A. Marks, R.A. Rifkind, Hexamethylene bisacetamide-induced differentiation of transformed cells: molecular and cellular effects and therapeutic application, Int. J. Cell Cloning 6 (1988) 230–240.
[8] R.C. Reuben, R.A. Rifkind, P.A. Marks, Chemically induced murine erythroleukemic differentiation, Biochim. Biophys. Acta 605 (1980) 325–346.
[9] A.C. Sartorelli, The 1985 walter hubert lecture. Malignant cell differentiation as a potential therapeutic approach, Br. J. Cancer 52 (1985) 293–302.
[10] A.S. Tsiftsoglou, I.S. Pappas, I.S. Vizirianakis, Mechanisms involved in the induced differentiation of leukemia cells, Pharmacol. Ther. 100 (2003) 257–290.
[11] A.S. Tsiftsoglou, I.S. Pappas, I.S. Vizirianakis, The developmental program of murine erythroleukemia cells, Oncol. Res. 13 (2003) 339–346.
[12] K. Drumea, Z.F. Yang, A. Rosmarin, Retinoic acid signaling in myelopoiesis, Curr. Opin. Hematol. 15 (2008) 37–41.
[13] F. Grignani, S. De Matteis, C. Nervi, L. Tomassoni, V. Gelmetti, M. Cioce, M. Fanelli, M. Ruthardt, F.F. Ferrara, I. Zamir, C. Seiser, F. Grignani, M.A. Lazar, S. Minucci, P.G. Pelicci, Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia, Nature 391 (1998) 815–818.
[14] V. Ibanez, A. Sharma, S. Buonamici, A. Verma, S. Kalakonda, J. Wang, S. Kadkol, Y. Sauntharajah, AML1-ETO decreases ETO-2 (MTG16) interactions with nuclear receptor corepressor, an effect that impairs granulocyte differentiation, Cancer Res. 64 (2004) 4547–4554.
[15] S. Minucci, M. Maccarana, M. Cioce, P. De Luca, V. Gelmetti, S. Segalla, L. Di Croce, S. Giavara, C. Matteucci, A. Gobbi, A. Bianchini, E. Colombo, I. Schiavoni, G. Badaracco, X. Hu, M.A. Lazar, N. Landsberger, C. Nervi, P.G. Pelicci, Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation, Mol. Cell 5 (2000) 811–820.
[16] T. Pabst, B.U. Mueller, N. Harakawa, C. Schoch, T. Haferlach, G. Behre, W. Hiddemann, D.E. Zhang, D.G. Tenen, AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia, Nat. Med. 7 (2001) 444–451.
[17] D. Passeri, A. Marcucci, G. Rizzo, M. Billi, M. Panigada, L. Leonardi, F. Tirone, F. Grignani, Btg2 enhances retinoic acid-induced differentiation by modulating histone H4 methylation and acetylation, Mol. Cell Biol. 26 (2006) 5023–5032.
[18] R. Villa, L. Morey, V.A. Raker, M. Buschbeck, A. Gutierrez, F. De Santis, M. Corsaro, F. Varas, D. Bossi, S. Minucci, P.G. Pelicci, L. Di Croce, The methyl-CpG binding protein MBD1 is required for PML-RARalpha function, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 1400–1405.
[19] I.S. Vizirianakis, M. Chatzopoulou, I.D. Bonovolias, I. Nicolaou, V.J. Demopoulos, A.S. Tsiftsoglou, Toward the development of innovative bifunctional agents to induce differentiation and to promote apoptosis in leukemia: clinical candidates and perspectives, J. Med. Chem. 53 (2010) 6779–6810.
[20] M. Manturewicz, Z. Grzonka, L. Borovickova, J. Slaninova, Oxytocin analogues with amide groups substituted by tetrazole groups in position 4, 5 or 9, Acta Biochim. Pol. 54 (2007) 805–811.
[21] R.J. Herr, 5-Substituted-1*H*-tetrazoles as carboxylic acid isosteres: medicinal chemistry and synthetic methods, Bioorg. Med. Chem. 10 (2002) 3379–3393.
[22] N.A. Meanwell, Synopsis of some recent tactical application of bioisosteres in drug design, J. Med. Chem. 54 (2011) 2529–2591.
[23] A.C. Pierce, K.L. Sandretto, G.W. Bemis, Kinase inhibitors and the case for CH.O hydrogen bonds in protein-ligand binding, Proteins 49 (2002) 567–576.
[24] I. Cardinaud, A. Gueiffier, J.C. Debouzy, J.C. Milhavet, J.P. Chapat, Synthesis of pyrroloquinoline and pyrrolonaphthyridine by an intramolecular cyclisation reaction, Heterocycles 36 (1993) 2513–2522.
[25] I. Nicolaou, V.J. Demopoulos, Substituted pyrrol-1-ylacetic acids that combine aldose reductase enzyme inhibitory activity and ability to prevent the nonenzymatic irreversible modification of proteins from monosaccharides, J. Med. Chem. 46 (2003) 417–426.
[26] F. Lenda, F. Guenoun, B. Tazi, N.B. Iarbi, H. Allouchi, J. Martinez, F. Lamaty, Synthesis of new tetrazole-substituted pyroaminoadipic and pipecolic acid derivatives, Eur. J. Org. Chem. (2005) 326–333.
[27] G.I. Koldobskii, R.B. Kharbash, 2-Substituted and 2,5-Disubstituted tetrazoles, Russ. J. Org. Chem. 39 (2003) 453–470.
[28] L.V. Myznikov, T.V. Artamonova, V.K. Bel'skii, A.I. Stash, N.K. Skvortsov, G.I. Koldobskii, Tetrazoles: XLIV. Synthesis and chemical properties of 5-substituted 2-triphenylmethyltetrazoles, Russ. J. Org. Chem. 38 (2002) 1360–1369.
[29] S.J. Byard, J.M. Herbert, Preparation and NMR spectroscopic studies of the glucuronides of Irbesartan, Tetrahedron 55 (1999) 5931–5936.
[30] B. Li, B. Andresen, M.F. Brown, R.A. Buzon, C.K.F. Chiu, M. Couturier, E. Dias, F.J. Urban, V.J. Jasys, J.C. Kath, W. Kissel, T. Le, Z.J. Li, J. Negri, C.S. Poss, J. Tucker, D. Whritenour, K. Zandi, Process development of CP-481715, a novel CCR1 antagonist, Org. Process Res. Dev. 9 (2005) 466–471.
[31] I. Ukrainets, N. Bereznyakova, A. Turov, S. Shishkina, 4-Hydroxy-2-quinolones 123. Amidation of 2-bromomethyl-5-oxo-1,2-dihydro-5*H*-oxazolo[3,2-α]-quinoline-4-carboxylic acid, Chem. Heterocycl. Comp. 43 (2007) 871–878.
[32] C. Friend, W. Scher, J.G. Holland, T. Sato, Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 378–382.
[33] P.A. Marks, R.A. Rifkind, Erythroleukemic differentiation, Annu. Rev. Biochem. 47 (1978) 419–448.
[34] R.C. Reuben, R.I. Wife, R. Breslow, A new group of potent inducers of differentiation in murine erythroleukemia cells, Proc. Natl. Acad. Sci. U.S.A. 73 (1976) 862–866.
[35] K. Pegklidou, C. Koukoulitsa, I. Nicolaou, V.J. Demopoulos, Design and synthesis of novel series of pyrrole based chemotypes and their evaluation as selective aldose reductase inhibitors. A case of bioisosterism between a carboxylic acid moiety and that of a tetrazole, Bioorg. Med. Chem. 18 (2010) 2107–2114.
[36] I.D. Bonovolias, A.S. Tsiftsoglou, Hemin counteracts the repression of Bcl-2 and NrF2 genes and the cell killing induced by imatinib in human Bcr-Abl(+) CML cells, Oncol. Res. 17 (2009) 535–547.
[37] A.S. Tsiftsoglou, W. Wong, C. Wheeler, H.N. Steinberg, S.H. Robinson, Prevention of anthracycline-induced cytotoxicity in hemopoietic cells by hemin, Cancer Res. 46 (1986) 3436–3440.
[38] I.S. Vizirianakis, W. Wong, A.S. Tsiftsoglou, Analysis of the inhibition of commitment of murine erythroleukemia (MEL) cells to terminal maturation by N6-methyladenosine, Biochem. Pharmacol. 44 (1992) 927–936.
[39] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.