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Synthesis, characterization and antineoplastic activity of bis-aziridinyl dimeric naphthoquinone - a novel class of compounds with potent activity against acute myeloid leukemia cells

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Abstract

The synthesis, characterization and antileukemic activity of rationally designed amino dimeric naphthoquinone (BiQ) possessing aziridine as alkylating moiety is described. Bis-aziridinyl BiQ decreased proliferation of acute myeloid leukemia (AML) cell lines and primary cells from patients, and exhibited potent (nanomolar) inhibition of colony formation and overall cell survival in AML cells. Effective production of reactive oxygen species (ROS) and double stranded DNA breaks (DSB) induced by bis-aziridinyl BiQ is reported. Bis-dimethylamine BiQ, as the isostere of bis-aziridinyl BiQ but without the alkylating moiety did not show as potent anti-AML activity. Systemic administration of bis-aziridinyl BiQ was well tolerated in NSG mice.

The clinical outcomes of patients with acute myeloid leukemia (AML) treated with available cytotoxic, targeted, and hematopoietic stem cell transplant therapy remains unsatisfactory with 3–8% survival at 5 years in patients aged 60 years and older and up to 50% in patients younger than 60 years of age.^{1–3} This underscores an urgent need for development of novel, efficacious and well-tolerated therapeutic agents and strategies for treatment of AML. AML therapy is challenging because it is an extremely heterogeneous disease with various

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No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: AE and RGL

Development of methodology: AE, SF, DF

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Analysis and interpretation of data: AE, RGL, SF, DF

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leukemogenic mutations and cytogenetic abnormalities with poorly understood interplay among them in each patient.^{4, 5} One solution to this issue may be to target a broader characteristic that is common among all AML cells but is sufficiently different from normal tissues. Growing evidence indicates that AML cells, compared to normal cells and irrespective of their genetic heterogeneity, have an increased susceptibility to the disruption of balance between pro- and anti-oxidant forces.⁶

Quinone moieties such as mitomycin-C and RH1⁷ (benzoquinone),⁸ daunorubicin, doxorubicin, idarubicin, epirubicin, aclarubicin and mitoxantrone (anthraquinone),⁹ and atovaquone,¹⁰ 2,2'-binaphthoquinones¹¹ and β -lapachone¹² (naphthoquinone¹³) are the most preclinically tested and clinically investigated anticancer agents frequently used for treatment of solid and hematologic neoplasms. The cytotoxic mechanisms of action of these compounds mainly include (i) formation of semiquinone radicals and quinone redox cycling resulting in initiation and propagation of intracellular free oxygen radical chain reactions¹⁴, (ii) nicotinamide adenine dinucleotide phosphate (NADPH) depletion via interference with the enzyme NAD(P)H:Quinone Oxidoreductase 1 (NQO1)¹⁵, (iii) nicotinamide adenine dinucleotide (NAD) depletion via hyperactivation of poly(ADP ribose) polymerase (PARP)¹², and (iv) inhibition of DNA topoisomerase-II.¹⁶ Nevertheless, the tendency of neoplastic cells to become resistant to these agents has led to a constant effort for rational design of novel quinone-based anticancer agents that can overcome drug resistance in malignant cells.

We previously synthesized several dimeric naphthoquinone (BiQ) analogues with potent anti-integrase activity against human immunodeficiency virus (HIV),¹⁷ and later reported their cytotoxic activity against prostate¹⁸ and breast¹⁹ cancer cells. Recently, we reported that hydroxylated dimeric, but not monomeric, naphthoquinones could inhibit clonogenicity and induce apoptosis in AML cell lines and primary cells from patients (IC₅₀ 3–5 μ M) with favorable therapeutic index compared to normal hematopoietic cells.²⁰ To improve the potency and bioavailability of this class of compounds, we decided to incorporate both alkylating agents and amine groups into the quinone cores of each naphthoquinone unit. The quinone moiety can perturb cellular redox balance and its oxidation state would modulate the activity of the alkylating moiety that can form covalent bonds with a different cellular components. To this end, we elected to synthesize a BiQ moiety substituted with aziridine(s) as a bifunctional alkylating compound that can be activated after bioreduction either by one- or two-electron reducing enzymes to form the corresponding aziridinyl hydroBiQ.²¹ The electron rich hydroquinone moiety in hydroBiQ increases the electron density at nitrogen and changes the pK of the aziridine ring(s),²² such that the nitrogen is protonated and is more susceptible to nucleophilic attack under physiological pH. The result is a naphthoquinone-based aziridinium ion that may alkylate DNA and other biomolecules. Here we report the synthesis, characterization and anti-AML activity of rationally designed bis-aziridine BiQ and compare it with bis-dimethylamine BiQ.

Amination of dichloro BiQ **1** was accomplished through treatment with limiting and excess amounts of aziridine to afford mono- and bis-aziridinyl (**2** and **3**) BiQ, or excess dimethylamine to afford bis-dimethylamino BiQ (**4**) (Scheme 1). Compound **2** (3-(Aziridin-1-yl)-3'-chloro-[2,2'-binaphthalene]-1,1',4,4'-tetraone) and **3** (3,3'-Di(aziridin-1-

yl)-[2,2'-binaphthalene]-1,1',4,4'-tetraone) were isolated in 49% and 44% yield, respectively, by dropwise addition of aziridine to a solution of 3,3'-dichloro-[2,2'-binaphthalene]-1,1',4,4'-tetraone (**1**) in anhydrous tetrahydrofuran (THF) at room temperature (RT) and stirring for 16–20 hours. Initial gentle warming (35 °C) was required to complete dissolution of **1**. The dichloro BiQ **1** was synthesized according to the literature.²³ Aziridine was used at 2 and 6 equivalents concentration of **1** for synthesis of mono- (**2**) and bis- (**3**) aziridinyl BiQ, respectively. The next day, the reaction mixture was partitioned between CH₂Cl₂ and water. The organic layer was collected, washed with water (x2) and brine, dried (Na₂SO₄), filtered and concentrated. The residue was adsorbed onto silica gel in the cold and chromatographed over silica gel using a Biotage Isolera running with a gradient of ethyl acetate (EtOAc) in hexane to provide the title compound as an orange red solid.

To demonstrate the importance of presence of aziridine moiety on the naphthoquinone core for antileukemic activity, we synthesized **4** (3,3'-bis(dimethylamino)-[2,2'-binaphthalene]-1,1',4,4'-tetraone) that, in theory, cannot effectively undergo a nucleophilic attack by DNA. Compound **4** is the isostere of bis-aziridine BiQ with similar electronics and size characteristics but without the alkylating moiety. As shown in Scheme 1, in a pressure tube and in presence of N,N-diisopropylethylamine, dimethylamine hydrochloride was added to **1**, and the solution was stirred in dichloromethane for 48 hours. After removing all volatile materials in the reaction solution in vacuo, the crude solid was preabsorbed onto silica gel and chromatographed in 75% EtOAc/hexanes to 100% EtOAc. The purified product was triturated with hexanes and ether (1:1, 5 mL), and the final dark red product was collected by filtration in 48% yield.

To investigate antiproliferative activity of amino-BiQs against AML cells, we first performed an MTT-like cell proliferation assay in the AML cell lines using a reagent called WST-1 (Promega, Wisconsin). We observed a concentration-dependent decrease in metabolic activity of all cell lines after exposure to compound **3**, with IC₅₀ values of 0.18±0.06 µM for MOLM-14, 1.05±0.05 µM for MV4-11, and 0.65±0.30 µM for THP-1 cells (See Supplementary Data Figure S1). Compared to **3**, compounds **2** and **4** demonstrated less potent anti-AML activities. IC₅₀ values of **2** were 3.9±1.0 µM for MOLM-14 and 7.7±1.3 µM for THP-1 cells. Compound **4** showed IC₅₀s of 2.9±0.9 and 2.4±0.3 against MOLM-14 and MV4-11 cells, respectively (See Supplementary Data, Methods and Materials).

Because compound **3** demonstrated a superior potency, it was selected for testing against primary leukemia cells from patients and the remaining experiments including mechanistic assays. Table 1 summarizes the genetic characteristics of primary leukemia cells as well as their sensitivity to compound **3**. To measure the selectivity of **3** against neoplastic cells, we treated normal hematopoietic bone marrow cells as well. Interestingly, IC₅₀ of bis-aziridinyl BiQ **3** for normal bone marrow cells was 3.37±1.27 µM, which was approximately five to eighteen times higher than those for AML cell lines and 1.5–2 times higher for primary leukemia cells, suggesting a favorable therapeutic index of this agent.

We next determined the effect of bis-aziridinyl-BiQ **3** on AML cell survival and viability, as well as on clonogenic activity, which is an *in vitro* assay to test the ability of every leukemic

cell in the population to produce a colony by demonstrating neoplasticity and undergoing unlimited division. After 24 hours exposure to **3**, AML cell lines MOLM-14 and THP-1 exhibited marked reduction in clonogenic activity (Figure 1, top). Importantly, reduction of clonogenic activity was achieved at concentrations relative to the respective IC₅₀ values of **3** for the AML cell lines (i.e. 1 μ M). Moreover, this activity was observed with only 24 hours exposure and subsequent removal of **3**, and the trend held true for both MOLM-14 and THP-1 with different genetic characteristics. Similarly, cell survival decreased significantly in AML cells treated with **3** for 72 hour, using trypan blue exclusion to detect dead cells (those that incorporate trypan blue) on the automated cell counter. Viable cell number decreased with increasing concentrations of **3** with MOLM-14 being more sensitive than THP-1 to the bis-aziridinyl BiQ (Figure 1, bottom).

In order to elucidate the mechanisms by which bis-aziridinyl BiQ exerts its anti-leukemic activity, we examined the amount of reactive oxygen species (ROS) that cells produced upon treatment with **3**. Both MOLM-14 and THP-1 cell lines were exposed to varying concentrations of **3** and observed over a period of 2–6 hours. Treatment with **3** resulted in a rapid (within 2 hours) production of ROS (Figure 2). This effect was also dose-dependent and observable in both the MOLM-14 and THP-1 cell lines. Production of ROS is consistent with the ability of dimeric naphthoquinone moiety to undergo futile redox cycling.¹¹

Taken into account the observed potent cytotoxicity towards AML cells and significant induction of ROS, we were interested in determining whether bis-aziridinyl BiQ **3** has any direct impact on DNA damage in AML cells. We decided to investigate the effect of treatment with **3** on MOLM-14 cells to induce double strand DNA breaks. The double strand DNA breaks are always followed by the phosphorylation of histone H2Ax (γ -H2Ax), a known biomarker for DNA damage.²⁴ After only 24 hours exposure to physiologically relevant concentrations (200 nM, 1.0 and 2.0 μ M) of **3**, MOLM-14 cells exhibited substantial γ -H2Ax phosphorylation.²⁵ Similar to what was observed with ROS, colony formation and survival assays, this effect was strongly dose-dependent.

Considering the significant antineoplastic activity of **3**, we aimed to test the safety and tolerability of this agent *in vivo*. We treated female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice with 5, 10 and 15 mg/kg of **3** delivered by intraperitoneal (IP) injection for 5 consecutive days. As shown in Figure 4, the mice tolerated the compound well with no weight loss observed during the 5 days of dosing and one week observation period.

One promising approach to developing safe and effective treatment for AML can lie in designing molecules that can relatively selectively augment oxidative stress and directly damage DNA in rapidly proliferating AML cells. To this end, we rationally designed a unique dimeric amino-naphthoquinone, which possesses aziridine groups (Azir-BiQ) and tested it against AML cells. Aziridinyl BiQ showed a potent anti-leukemic effect with favorable therapeutic index when compared with normal hematopoietic cells and was well tolerated in animal.

Our findings have two innovative aspects: the first novel aspect is conceptual, that is, exploitation of the cellular oxidative state and its aberrancy in AML cells with novel dimeric

naphthoquinones. Through cyclic voltammetry studies, we have demonstrated that BiQ can undergo four redox steps, of which the cathodic and anodic peak potentials can be tuned to specifically target cellular metabolic processes.¹⁹ For some of the naphthoquinones, cytotoxicity could be predicted by redox potentials. Participation in redox cycling, generates significant levels of ROS which may contribute to the cytotoxicity of the compounds.

The second innovative aspect of this research involves the specific BiQ derivative, i.e. bis-aziridinyl BiQ that was designed and synthesized by us to simultaneously carry unique properties, each of which targets different fragments of AML cells allowing, in theory, no alternative mechanism to escape cell death. Bis-aziridinyl BiQs are amino-naphthoquinones possessing a cyclic amino group, i.e. aziridine, in the 2-position of the 1,4-naphthoquinone moiety. The heterocyclic nitrogen atom in this position empowers geometric modification of the molecules and of their reduction intermediates and modulation of the substituent's effects on the electronic properties of the quinone system. In addition, the presence of the aziridine group provides a classical DNA alkylator causing inter- and intra-strand DNA cross link.²⁶

We continue to explore the chemistry of halogenated and hydroxylated nitro-BiQ in organic synthesis and are applying this chemistry to the systems that allow us to efficiently integrate *in vivo*, *in vitro*, structural, and biochemical properties to paint a comprehensive picture of the effects of this class of compounds on the oxidative state, genetic and epigenetic of AML cells. Depending on the oxidoreductases or other bases for altered electron donation, present in AML cells, the electron-accepting potential of quinones could in principle be tuned to yield selective cytotoxicity for cells with a particular redox environment, allowing initiation of a cascade of electron transport only in AML cells, with dysregulated redox state, and not in normal cells, with the consequent increase in ROS producing selective cell killing. Our ultimate goal is to identify the optimal BiQ for introduction into clinical trials in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

For colony formation assay, cell lines were treated with the indicated doses of aziridinyl BiQ **3** for 24 hours, and subsequently washed and plated in methylcellulose to observe clonogenic potential after 7–10 days. With just 24 hours treatment, **3** exhibited a statistically significant ($p<0.05$) inhibition of colony formation at 1, 10, and 100 μM (Top) in both MOLM-14 and THP-1 cells. Cell survival was determined using trypan blue exclusion, in which cells are plated and the following day treated similarly to the proliferation assay. After 72 hours, a statistically significant ($p<0.05$) reduction in viable cells was seen at 0.1, 1, 10, and 100 μM in MOLM-14, and at 1, 10, and 100 μM in THP-1 (Bottom).

Figure 2.

Treatment of AML cells with **3** induced ROS production in a dose-dependent manner. MOLM-14 and THP-1 cells were loaded with the ROS dye H₂DCFA and subsequently plated into 96 well plates. Cells were treated with vehicle (as negative control), **3** or hydrogen peroxide (as positive control), and the relative fluorescence units (RFU) were read at various time points. A dose-dependent increase in ROS production was observed approximately 2 hours post-treatment. *RFU went above the instrument detection limit.

Figure 3.

Treatment of MOLM-14 cells with bis-aziridinyI-BiQ **3** induces double strand DNA breaks as measured by immunofluorescence. Upon exposure to physiologically relevant concentrations of **3**, dsDNA breaks appeared as indicated by the present of the punctate pink foci (γ -H2Ax expression) observed in the nucleus of cells. DMSO (vehicle) and radiation (6 Gray) were used as negative and positive controls, respectively.

Figure 4.

The effect of bis-aziridinyI BiQ **3** on female NSG mice. Compound **3** was administered via intraperitoneal injection for 5 consecutive days and monitored for one additional week. * denotes days of dosing. Mean body weight loss did not exceed 10%. Three mice were included per group.

Scheme 1.

Synthesis of mono- and bis-aziridinyl (**2** and **3**) and bis-dimethylamino dimeric naphthoquinones. (**2**) Aziridine (21 μ L, 0.4 mmol, 2 equiv), **1** (76 mg, 0.2 mmol, 1 equiv), THF, RT, 12 h, 49%; ¹H-NMR (400 MHz, CDCl₃): δ 8.28-8.26 (m, 1H, Ar), 8.18-8.15 (m, 2H, Ar), 8.11-8.09 (m, 1H, Ar), 7.84-7.82 (m, 2H, Ar), 7.76-7.74 (m, 2H, Ar), 2.33, 2.26 (Aziridine, 4H, CH₂CH₂, J_{AB} = 6.2 Hz); ¹³C-NMR: (100 MHz, *d*₆-DMSO) 181.4, 181.2, 180.5, 177.1, 154.9, 144.9, 141.5, 135.4, 135.2, 135.1, 134.2, 131.9, 131.7, 131.6 (2), 127.6, 127.4, 126.8, 126.2, 121.5, 28.5 (2); MS (ESI) *m/z* calcd for C₂₂H₁₂ClNO₄ (M⁺): 389.1, found: 390.0 (M+H⁺). (**3**) Aziridine (63 μ L, 1.2 mmol, 6 equiv), **1** (76 mg, 0.2 mmol, 1 equiv), Et₃N (84 mL, 0.6 mmol, 3 equiv), THF, RT, 16 h, 44%; ¹H-NMR (400 MHz, CDCl₃): δ 8.16-8.11 (m, 4 H, Ar), 7.75-7.73 (m, 4H, Ar), 2.31-2.26 (m, 8H, 2 $\times$ CH₂CH₂); ¹³C-NMR (100 MHz, *d*₆-DMSO): 182.5, 180.9, 154.3, 134.8, 133.8, 132.5, 131.7, 126.5, 126.2, 122.9, 20.5; MS (ESI) *m/z* calcd for C₂₄H₁₆N₂O₄ (M⁺): 396.1, found: 397.0 (M+H⁺). (**4**) Dimethylamine hydrochloride (0.329 g, 4.03 mmol), **1** (0.218 g, 0.569 mmol), N,N-diisopropylethylamine (0.7 mL), CH₂Cl₂ (10 mL), pressure tube, RT, 48 h, 48%; ¹H-NMR (400 MHz, DMSO-*d*₆) δ 8.01-7.99 (d, 2H, Ar), 7.91 (d, 2H, Ar), 7.81-7.78 (m, 4H, Ar), 2.89 (s, 12H); MS (ESI) *m/z* calcd for C₂₄H₂₀N₂O₄ (M⁺): 400.1, found: 401.1 (M+H⁺).

Table 1

Characteristics of primary AML cells and their sensitivity to compound 3

Leukemia Cells	Leukemia Type	Karyotype	Mutations	IC ₅₀ values (μM)
A	relapsed AML post-BMT	46,XX,t(1;5)(q25;q13)[5]/46,XX[15]	FLT3-ITD with 84% allelic burden, NPM1-WT	2.84
B	chronic myelomonocytic leukemia (CMML)	46,XY[20]	FLT3-WT	2.08
C	AML	46-51,XY,+4,+8,+3mar[9] (trisomy 4, trisomy 8, and 3 marker chromosomes)	DNMT3A mutated (c.2902G>A - p.R882), FLT3-WT, NPM1-WT, IDH1/IDH2-WT, CEBPα-WT	2.25±1.34
D	AML	46,XX[20]	FLT3-ITD (p.T582insl6) with 8% allelic burden, FLT3 point mutation (p.D835Y) with mutation level 29%, NPM1 mutation (p.Trp288Cysfs*12) with mutation level 45%, IDH1 (p.Arg132His) with mutation level 59%	1.97

BMT = bone marrow transplant, CEBPα = CCAAT/enhancer binding protein, DNMT3A = DNA (cytosine-5-)methyltransferase 3 alpha, FLT3-ITD = fms-like tyrosine kinase 3 internal tandem duplication, IDH = isocitrate dehydrogenase, NPM1 = nucleophosmine 1, WT = wild type, the numbers in [] shows the total number of metaphase cells from two cultures that were analyzed by Giemsa Banding (GTG banding) at the 450 band level.