Antiproliferative and Erythroid Differentiation of Piperazine and Triphenyl Derivatives Against K-562 Human Chronic Myelogenous Leukemia

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Abstract. *Five piperazine derivatives (S)-4-benzyl-1-(4-bromo-3-methylphenyl)-2 methylpiperazine (A), (S)-1-benzyl-3-isobutylpiperazine-2,5-dione (B), (S)-1-benzyl-3 methyl-piperazine-2,5-dione (C), (S)-1,3-dibenzylpiperazine-2,5-dione (D), (E)-1-(3-methyl 4-((E)-3-(2-methylpropylidene) piperazin-1-yl) phenyl)-2-(2 methylpropylidene) piperazine (E) and triphenyl derivative ammonium 2-((2,3',3''-trimethyl-[1,1':4',1''-terphenyl]-4 yl)oxy)acetate (F) were tested for inhibition of K-562 cell proliferation and for induction of erythroid differentiation. Among them, two piperazine and one triphenyl derivatives, compounds A, E, and F inhibited the proliferation of the K562 cell lines exhibiting inhibition concentration 50 (IC_{50}) (IC_{50}) of values 30.10±1.6, 4.60±0.4 and 25.70±1.10 μg ml^{-1}, respectively. If compound A and F were added to suboptimal concentrations of the established anticancer drugs cytosine arabinoside or mithramycin, pronounced synergic effects were observed.*

Cancer is one of the world's most common diseases and a major cause of death. It is expected that cancer incidence will continue to rise and that cancer prevalence will increase in the coming years (1). Among different types of cancer, leukemia is one of the major causes of cancer-related deaths (2). Chronic myelogenous leukemia (CML) is characterized by increased growth of the myeloid line of cells in blood and bone marrow. Genetically, CML is characterized by the Philadelphia (Ph1) chromosome (22q-) which results from a reciprocal translocation between chromosomes 9 and 22 (3). Differentiation of K562 cells is associated with an increase of expression of embryo-fetal globins genes, such as ζ- ε and γ-globin (4). New research strategies aiming at effective prevention and improved treatment are therefore urgently needed. Many laboratories worldwide are searching for new agents.

Piperazine derivatives reveal interesting pharmacological activities. Most piperazine derivatives have been used as antihelminthics or antibiotics (5). Recently, it was shown that 1,4-*bis*-(4-(1*H*-benzo[d]imidazol-2-yl-phenyl)) piperazine (BIPP) induced apoptotic cell death of U937 leukemia cells indicating that BIPP might be of therapeutic value for human myeloid leukemia (6). In a previous investigation, the *in vitro* effects of novel piperazine derivatives of butyric acid on the induction of differentiation and growth inhibition of K-562 cells and HL60 myeloid leukemia cells were shown (7). Moreover, the inhibition of cancer cell proliferation of pentacyclic piperazines was assessed in three human cancer cell lines (K562 myelogenous leukemia, A549 lung carcinoma, MCF-7 breast adenocarcinoma) and two murine tumor cell lines (P388 and L1210 leukemia) (8). Furthermore, piperazine hydroxamate compounds were found to be cytotoxic towards three cancer cell lines of solid tumors (NCIH460, HCT116, U251) and HL60 promyelocytic leukemia cells (9). In a panel of synthetic chloroalkyl piperazines, most compounds revealed cytotoxic activity towards bel-7404 liver cancer cells (10). Several piperazine derivatives have reached the stage of clinical application. Interestingly, trans,trans-[{PtCl2(NH3)}2(piperazine)] was more cytotoxic than cisplatin against human tumor ovarian cell lines (11).

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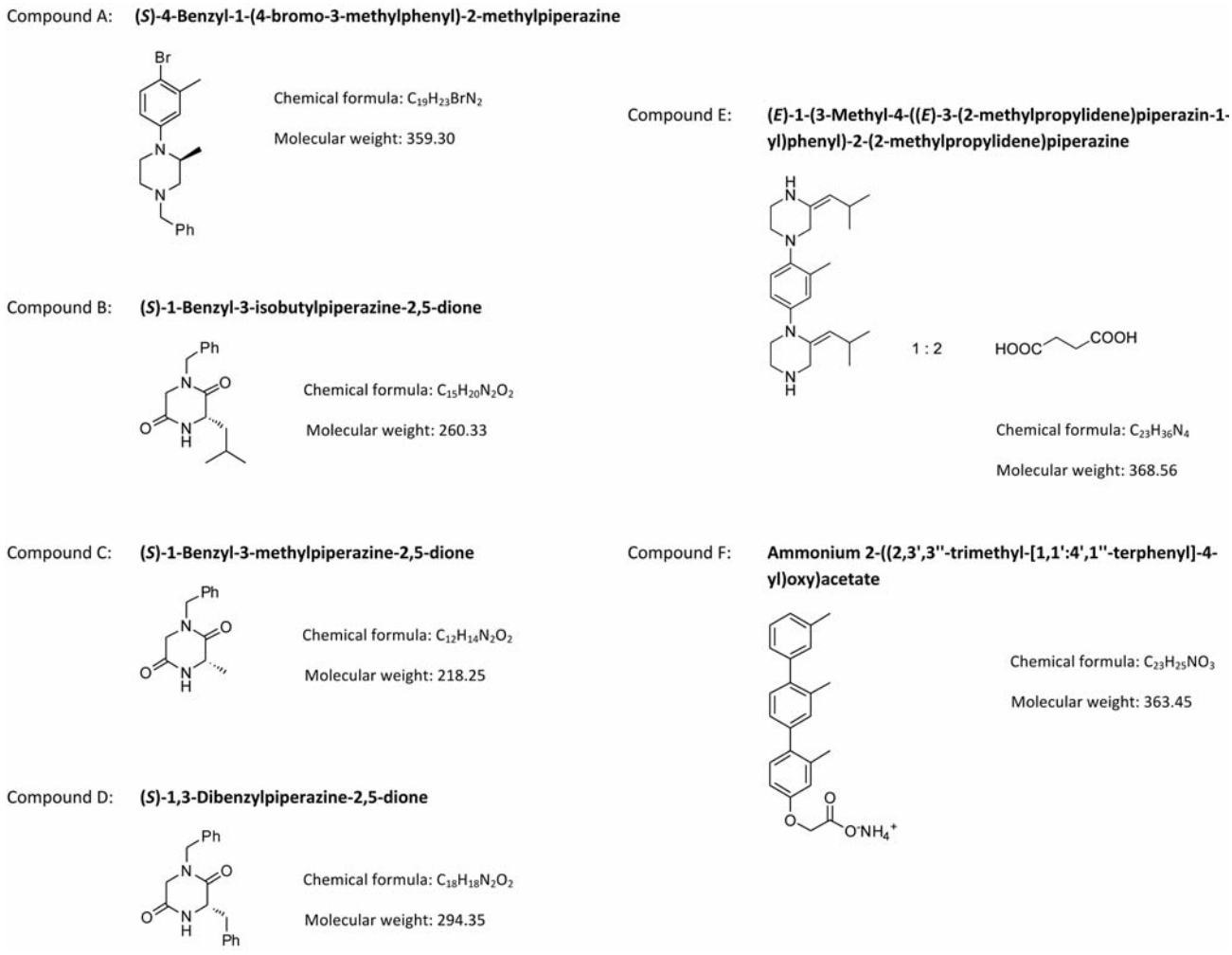

Figure 1. *Molecular structures of piperazine and triphenyl derivatives.*

The triphenyl derivative tamoxifen is administered to women who have breast cancer and can be a preventive therapy for women who have high risk for breast cancer (12). Tamoxifen reduces the incidence of invasive and non-invasive breast cancer. Its use as a breast cancer treatment agent is appropriate in many women at increased risk for the disease (13). The effect of anastrozole and tamoxifen as adjuvant treatment for postmenopausal with hormone-sensitive early breast cancer confirm the long-term superior efficacy and safety (14). A new class of triphenylmethyl-containing compounds has been described as potent cytotoxic anticancer agents against the human melanoma cell lines SK-MEL-5 and UACC-62 in cell culture (15). The first objective of this work was to study the antiproliferative activity of the piperazine derivatives on human CML cells (16). A second objective was to verify the ability of the piperazine derivatives to induce terminal erythroid differentiation using the human K-562 CML cell line as a model system (17).

Materials and Methods

Chemicals. In the present investigation, chemicals and reagents used for the study of antiproliferative activities were purchased from Sigma-Aldrich Co., while other chemicals, solvents and reagents were purchased from VWR (Milan, Italy). The fetal bovine serum was obtained from CELBIO (Milano, Italy). The five piperazine derivative compounds (*S*)-4-benzyl-1-(4-bromo-3-methylphenyl)-2-methylpiperazine (A), (*S*)-1-benzyl-3-isobutylpiperazine-2,5-dione (B), (*S*)-1-benzyl-3-methylpiperazine-2,5-dione (C), (*S*)-1,3-dibenzylpiperazine-2,5-dione (D) and (*E*)-1-(3-methyl-4-((*E*)-3-(2-methylpropylidene) piperazin-1-yl) phenyl)- 2-(2-methyl-

Table I. *Cytotoxicity of piperazine and triphenyl derivative compounds on K562 cell growth and effect on differentiation (% of benzidine-positive cells) after five days of culture.*

Compound	IC_{50} μM	Differentiation max (%)
A	30.10±1.60 μM	3±0.50 (30 μM)
B	174.8±3.40 μM	2±0.70 (175 μM)
C	140.50±2.60 μM	5±1 (150 μM)
D	149.7±2.30 μM	3±0.50 (150 μM)
E	4.6±0.40 μM	2±0.5 (5 μM)
F	25.70±1.10 μM	3±0.70 (25 μM)
AraC	250±5 nM	92±5 (0.5 μM)

IC_{50} values represent the average±SD of three independent experiments. Differences within and between groups were evaluated by one-way analysis of variance test ***p<0.0001 (F=187.6 r^2=0.98), followed by a multi-comparison Dunnett's test: **p<0.01 compared with the positive control Ara C.

Table II. *Level of K562 differentiation induced by known erythroid inducers, piperazine and triphenyl derivatives in synergic effect with suboptimal concentration of the commercial drug cytosine arabinose (AraC).*

Inducer	Differentiation max (%)
AraC (75 nM) + A(30 μM)	52±2.5
AraC (75 nM) +B(175 μM)	13±1.5
AraC (75 nM) + C(150 μM)	15±1.7
AraC (75 nM) + D (150 μM)	13±1.2
AraC (75 nM) + E (5 μM)	14±1.1
AraC (75 nM) +F (25 μM)	57±2
AraC (75 nM)	12±1.5
AraC (0.5 μM)	92±5

Differentiation max (%) values represent the average±SD of three independent experiments. Differences within and between groups were evaluated by one-way analysis of variance test ***p<0.0001 (F=187.6 r^2=0.98) followed by a multi comparison Dunnett's test: **p<0.01 compared with AraC.

propylidene)piperazine (E) and triphenyl derivative ammonium 2-((2,3'',3''-trimethyl-[1,1':4',1''-terphenyl]-4yl)oxy) acetate (F) were provided by the Institute of Organic Chemistry, University of Regensburg, Germany (18) (Figure 1).

Cell lines, culture conditions and in vitro antiproliferative activity assays. Human K562 CML cells were cultured in a humidified atmosphere at 5% CO_2 in RPMI-1640 (Flow Laboratories, Irvine, UK) supplemented with 10% fetal bovine serum: 100 units/ml penicillin and 100 mg/ml streptomycin. The *in vitro* antiproliferative activities of piperazine derivatives were assayed as follows: Cell number ml-1 was determined by using a model ZBI coulter Counter (Coulter Electronics, Hialeah, FL, USA). K562 cells were plated at an initial density of 3×10^4 cells ml^{-1} and the cell number ml^{-1} was determined after five days, when untreated cells were in the log phase of cell growth (19).

In vitro induction of erythroid differentiation. Erythroid differentiation induced by piperazine derivatives was evaluated by counting benzidine-positive cells after suspending the cells in a solution containing 0.2% benzidine in 0.5 M glacial acetic acid, 10% H_2O_2, as described elsewhere (14). Induction of differentiation was compared with that obtained using well- established inducers of differentiation of K562 cells, cytosine arabinose and mithramycin (17, 20).

Statistical analysis. All experiments were carried out in triplicate. Data were expressed as means±S.D. Differences were evaluated by one-way analysis of variance (ANOVA) test completed by Dunnett's test. Differences were considered significant at p<0.01. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression curve with the use of Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

In order to evaluate piperazine derivatives, K-562 cells were seeded at the initial cell density of 3×10^{-4} cells/ml and then cultured for five days in presence or absence of compounds at concentrations ranging from 0.1 $\mu g\times ml^{-1}$ to 100 $\mu g\times ml^{-1}$. The five piperazine derivative compounds A- E and the triphenyl derivative F were able to exert antiproliferative activity against K562 cells (Table I).

Table III. *Level of K562 differentiation induced by known erythroid inducers, piperazine and triphenyl derivatives in synergic effect with suboptimal concentration of commercial drug mithramycine (Mit).*

Inducer	Differentiation max (%)
Mit (12 nM) + A (30 μM)	98±2
Mit (12 nM) + B (175 μM)	37±4
Mit (12 nM) + C (150 μM)	42±4.5
Mit (12 nM) +D (150 μM)	38±3.7
Mit (12 nM) + E (5 μM)	39±4.3
Mit (12 nM) + F (25 μM)	92±2
Mit (25 nM)	87±4
Mit (12 nM)	35±3.4

Differentiation max (%) values represent the average±SD of three independent experiments. Differences within and between groups were evaluated by one-way analysis of variance test ***p<0.0001 (F=187.6 r^2=0.98) followed by a multi-comparison Dunnett's test: **p<0.01 compared with Mithramycin.

We used the anti-cancer drugs mithramycin and cytosine arabinoside as potent inducers of erythroid differentiation of K562 cells. When the piperazine derivative A was added to a suboptimal concentration of mithramycin (12 μM) or cytosine arabinoside (75 nM) synergistic effects were observed. The treatment of cell culture with 500 nM of cytosine arabinoside led to 92±5% benzidine-positive cells,

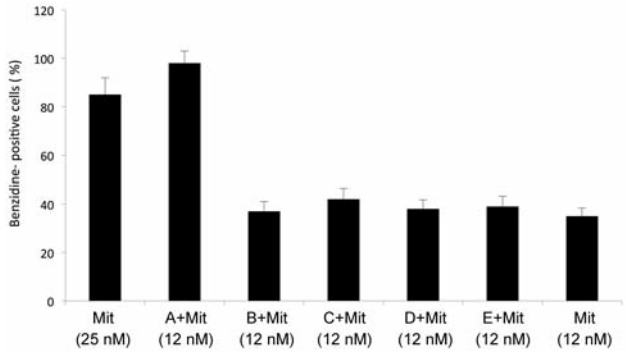

Figure 2. *Concentration-dependent effects of five piperazine derivatives with sub-optimal concentration of mithramycin (12 nM) after five days of culture. Data are 87±4, 98±2, 37±4, 42±4.5, 38±3.7, 39±4.3, 35±3.4.*

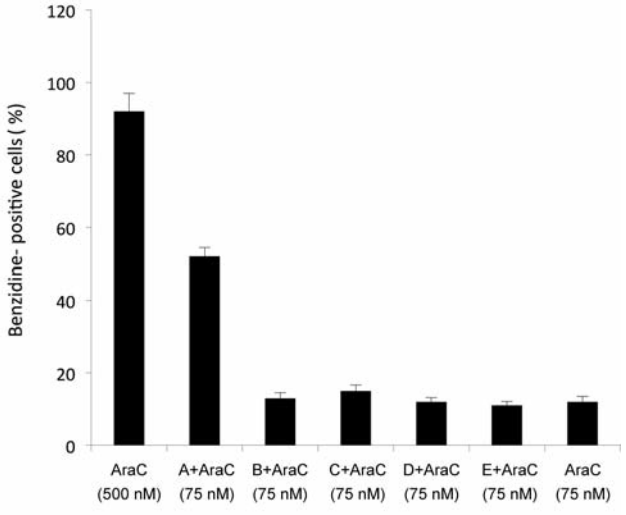

Figure 3. *Concentration-dependent effects of five piperazine derivatives with sub-optimal concentration of cytosine arabinoside (75 nM) after five days of culture. Data are 92±5, 52±2.5, 13±1.5, 15±1.7, 13±1.2, 14±1.1, 12±1.5.*

while treatment with 25 nM mithramycin led to 87±4% benzidine-positive cells. When 12 nM of mithramycin were added to 30 μM piperazine A, the combination caused a significant increase of benzidine-positive cells from 3±0.50% to 98±2%. In fact, the proportion of erythroid-differentiated cells rose from 3±0.5% to 98±2% indicating a synergistic effect (Table III, Figure 2). Moreover, a combination of 75 nM cytosine arabinoside and 30 μM piperazine A caused a significant increase of benzidine-positive cells from 3±0.50% to 52±2.5%. The proportion of erythroid differentiation increased from 3±0.5% to 52±2.5% indicating a synergistic effect (Table II, Figure 3). Furthermore, if 12 nM mithramycin were added to 175 μM piperazine B, 150 μM

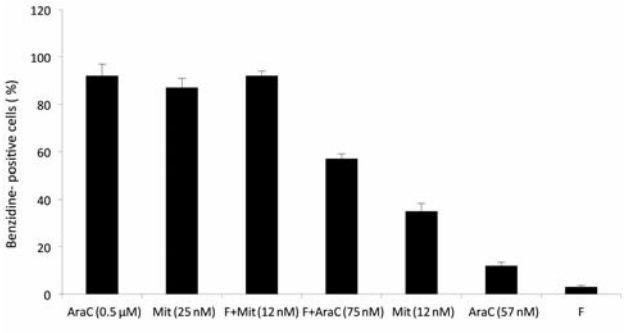

Figure 4. *Concentration-dependent effects of triphenyl derivative with sub-optimal concentration of mithramycin (12 nM) and cytosine arabinoside (75 nM) after five days of culture. Data are 92±5, 87±4, 92±2, 57±2, 35±3.4, 12±1.5, 3±0.7.*

pirazine C, 150 μM of piperazine D or 25 μM piperazine E, the combinations revealed significant increases of benzidine-positive cells from 2±0,70%, 5±1%, 3±05% and 2±0,5% to 37±4%, 42±4.5%, 38±3.7% and 39±4.3% (Table III, Figure 2), respectively. Moreover, 75 nM of cytosine arabinoside in combination with 175 μM piperazine B, 150 μM piperazine C, 150 μM piperazine D and 25 μM piperazine E, resulted in significant increases of benzidine-positive cells from 2±0,70%, 5±1%, 3±05% and 2±0.5% to 13±1.5%, 15±1.7%, 13±1.2% and 14±1.0%, respectively (Table II, Figure 3). In fact, an induction of benzidine-positive cells was observed by a combination of positive control 12 nM mithramycin and 75 nM cytosine arabinoside as control drugs without pronounced synergic effect. In other side, When 12 nM of mithramycin were added to 25 μM triphenyl F, the combination caused a significant increase of benzidine-positive cells from 3±0.70% to 92±7%. In fact, the proportion of erythroid-differentiated cells rose from 3±0.5% to 92±7% indicating a synergistic effect (Table III). Moreover, a combination of 75 nM cytosine arabinoside and 25 μM triphenyl derivative F caused a significant increase of benzidine-positive cells from 3±0.50% to 57±2.0%. The proportion of erythroid differentiation increased from 3±0.5% to 57±2.0% indicating a synergistic effect (Table II, Figure 4).

Discussion

It has been recently demonstrated that some piperazine derivatives are cytotoxic to cancer cells and induce their apoptosis (21). In particular, the piperazine nucleus was found in a broad range of biologically-active compounds with anti-fungal (22), anti-malarial and anti-psychotic properties (23). Several piperazine derivatives have reached the stage of clinical application. Among the known important

drugs, imatinib mesylate-resistant human CML cell lines can be treated with erythroid inducers, as recently demonstrated using the phytoalexin resveratrol (24). The triphenyl-alkene derivative panomifene (EGIS-5660) proved to be the most active antiestrogenic compound and binds to specific estrogen receptors, exhibits inhibitory effects on experimental mammary tumors both *in vitro* and in vivo and is currently used as a therapeutic agent against breast cancer (25). Several hydroxylated derivatives of tamoxifen were tested for their true activities and effects on the growth of T47D human breast cancer cells *in vitro* (26). The complexes of triphenyltin(IV) derivatives of malonic acid, succinic acid, glutaric acid, and adipic acid were successfully synthesized and obtained in solid form and The cytotoxicity of the complexes was tested against promyelocytic leukemic cells, HL-60. The results showed that the four complexes synthesized gave IC_{50} values lower than etoposide (27).

In conclusion, we report on the inhibitory activity of piperazine and triphenyl compounds A, E and F on cell growth and erythroid differentiation of K562 human CML cells. Interestingly, the combination of both A and F with the established anticancer drugs cytosine arabinoside, and mithramycin provoked a significant increase of dead cells, indicating synergistic drug interactions. These data suggest that piperazine and triphenyl compounds A, E and F could be considered potential candidates for cancer drug development.

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