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Antiproliferative and Apoptotic Effects of Two New Pd(II) Methylsarcosinedithiocarbamate Derivatives on Human Acute Myeloid Leukemia Cells In Vitro

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[Pd(MSDT)Cl]$_n$ palladium, chloro[methyl *N*-(dithiocarboxy-kS,kS′)-*N*-methylglycinate], and [Pd(MSDT)Br]$_n$ palladium, bromo[methyl *N*-(dithiocarboxy-kS,kS′)-*N*-methylglycinate], palladium (Pd)(II) derivatives are two newly synthesized Pd(II) derivatives of methylsarcosinedithiocarbamate (MSDT), containing a sulfur chelating ligand that is able to strongly bind the metal center, so preventing interactions with sulfur-containing enzymes. In fact, these reactions are believed to be responsible for the nephrotoxicity induced by platinum (II)-based drugs. Their activity has been evaluated in a panel of acute myeloid leukemia (AML) cell lines representing different French–American–British (FAB) subtypes and in the Philadelphia (Ph)-positive cell line K-562 and compared to cisplatin. Both compounds suppressed, in a dose-dependent manner, colony formation in methylcellulose with ID_{50} values comparable to those of the reference drug cisplatin, excluding the ML-3 cell line (ID_{50} 10-fold lower than cisplatin). Exposure of HL-60, ML-3, NB-4, and THP-1 cell lines to a cytotoxic concentration of [Pd(MSDT)Br]$_n$ (5 μM) determined: downregulation of the antiapoptotic molecule Bcl-2, upregulation of the proapoptotic molecule Bax; apoptosis induction, as evaluated by APO2.7 and annexin V staining; mitochondrial membrane permeabilization; and DNA fragmentation. In ML-3 cells the Pd(II) complexes were more active than cisplatin in apoptosis induction. Finally, [Pd(MSDT)Br]$_n$ showed an inhibitory effect on clonogenic growth of hematopoietic progenitors (CFU-GM, CFU-GEMM, and BFU-E) with both ID_{50} and ID_{90} comparable to those of cisplatin. Remarkably, the Pd(II) complex was more potent in inhibiting the clonogenic growth of the less differentiated AML cell lines KG-1a, HL-60, NB-4, ML-3, and THP-1 (ID_{50} ranging from 0.02 ± 0.001 to 0.52 ± 0.04 μM), compared to normal hematopoietic progenitors (ID_{50} of 2.1 ± 0.1, 3.8 ± 0.4, and 2.5 ± 0.2 μM) for CFU-GEMM, BFU-E, and CFU-GM, respectively). These data suggest that leukemic cells of myelomonoblast lineage might represent a preferential target for its cytotoxic activity compared to normal committed hemopoietic progenitor cells. Altogether, our results indicate that these new Pd(II) dithiocarbamate derivatives might represent novel potentially active drugs for the management of some selected myeloid leukemia strains, able to conjugate cytostatic and apoptotic activity with reduced toxicity.

Key words: Medicinal inorganic chemistry; Palladium compounds; Antineoplastic agents; Acute myeloid leukemia; Therapeutic index

INTRODUCTION

Acute myelogenous leukemia (AML) is usually treated with chemotherapeutic regimens that may include cytosine-arabinoside and anthracycline analogues. Although these standard treatments induce remission in most patients, the likelihood of relapse and development of resistant disease remains (1). Therefore, the discovery of more effective treatments for AML, particularly the ones that exploit apoptotic pathways, is essential because apoptotic resistance is a mechanism that can contribute to leukemogenesis and drug resistance (1,2).

Recent advances in medicinal inorganic chemistry demonstrate significant prospects for the utilization of metal complexes as drugs, which are presently used to treat different types of disorders, and new areas of application are rapidly emerging (3,4). Significant progress in platinum-based anticancer agents has been achieved,

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dependent in part on an understanding of the DNA binding mechanisms and pharmacological effects of cisplatin. Therefore, much attention is focused on designing new platinum compounds with improved pharmacological properties, a broad range of antitumor activities, and lower toxic side effects (nephrotoxicity, myelosuppression, and neurotoxicity) (5). In addition to cisplatin, three other compounds have been approved for clinical use (carboplatin, nedaplatin, and ixaliplatin) and a number of interesting platinum compounds including JM216, ZD0473, BBR3464, and lipoplatin are under clinical evaluation (5–7). Ruthenium complexes with antitumor activity are also emerging rapidly (8,9) and, besides their established use to treat arthritis, gold complexes (10–17).

Recently, with the aim of modulating the activity and toxicity of potential drugs, some platinum (II) [Pt(II)] and palladium (II) [Pd(II)] dithiocarbamate derivatives have been synthesized (18,19). In vitro studies and in vivo nephrotoxicity tests have confirmed that these compounds are highly cytotoxic towards HL-60 and HeLa cell lines and exhibit low renal toxicity compared to cisplatin itself (20). The choice of dithiocarbamate ligands is not accidental; in fact, dithiocarbamates are chemical substances that are able to bind many metals in different oxidation states, and they protect against cisplatin-induced nephrotoxicity in several animal models (19,21). Dithiocarbamates are also called blocking agents, as they prevent the formation of carcinogens from precursor substances (22). Moreover, in order to obtain compounds with higher cytotoxicity and lower nephrotoxicity than cisplatin, we have also synthesized and compared as chemotherapeutic agents new Pt(II), Pd(II), and Au(III) methylsarcosinedithiocarbamate derivatives. These new compounds are sufficiently stable under physiological conditions and represent promising candidates for pharmacological testing as antitumor agents because they are highly cytotoxic towards cultured human tumor cell lines (12–15).

The aim of the present investigation was to examine the in vitro cytotoxicity of two new Pd(II) methylsarcosinedithiocarbamate derivatives on a panel of AML cell lines. We compared the antiproliferative activity of Pd(II) complexes with the reference drug cisplatin and found that they are as effective as cisplatin, excluding the ML-3 cells in which Pd(II) complexes were more active than cisplatin. One of the main purposes of cancer chemotherapy is to commit tumor cells to apoptosis (23). These new compounds strongly induced apoptosis, and their in vitro myelotoxic activity on CD34+ hemopoietic progenitor cells was much less pronounced than the inhibition of colony formation of less differentiated AML cells.

MATERIALS AND METHODS

Drugs

$[Pd(MSDT)Cl]_n$ and $[Pd(MSDT)Br]_n$ (Fig. 1A) were prepared as previously reported (13). Compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Milan, Italy), divided into aliquots, and stored at −80°C. Compounds and cisplatin (Pharmacia & Upjohn, Milano, Italy) were dissolved in Iscove's-modified Dulbecco's medium (IMDM; Biochrome KG, Berlin, Germany) and filter sterilized (0.2 μM) immediately before use. The final DMSO concentration had no effect on cell killing or apoptosis.

Cell Lines and Culture Conditions

K-562 (human chronic myeloid leukemia in blast crisis, Ph+, carrying the BCR-ABL fusion gene product), HEL (myeloblastic-erythroblastic), KG-1a (early myeloblasts, CD34+), HL-60 (intermediate myeloid promyelocytes), ML-3 (myelo-monoblast leukemia, following T-non-Hodgkin's lymphoma and T-ALL), THP-1 (monoblasts), and NB-4 (leukemia promyelocytes), harboring the t(15,17) translocation, were maintained in IMDM medium (Cambrex Bio Science, Milano) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 0.2 mg/ml penicillin/streptomycin, and 0.1% (w/v) L-glutamine (Cambrex) at 37°C in a 5% CO_2 fully humidified atmosphere. Sources and phenotypic characterization of all of the above cell lines have been reported in detail elsewhere (24).

Colony Assay

Clonogenic growth of AML cell lines was assayed as previously described (25). Briefly, 5×10^3/ml cells were suspended in 1 ml of IMDM medium containing 0.8% methylcellulose and 15% FBS (Gibco), in the presence of increasing drug concentrations (0.25–15 μM). After 14 days of incubation, plates were observed under phase-contrast microscopy and aggregates with ≥ 40 cells were scored as colonies. ID_{50} and ID_{90} represent the concentration of drug required to cause 50% and 90% clonogenic growth inhibition of treated cells compared to control cells. Alternatively, AML cell lines in exponential growth phase were incubated at 2×10^5 cells/ml in six-well flat-bottomed microplates in IMDM supplemented with 10% FBS in the presence of the drugs (5 μM). After 24 h, cells were harvested, washed in PBS, and then 4×10^3 AML cells were analyzed for clonogenic growth.

Measurement of Cell Cycle and Apoptosis

AML cell lines in exponential growth phase were incubated at 2×10^5 cells/ml in six-well flat-bottomed mi-

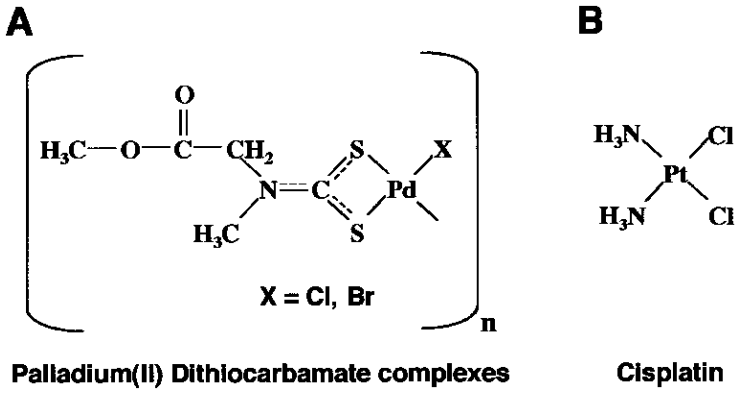


Figure 1. Structures of Pd(II) dithiocarbamate derivatives (A) and cisplatin (B).

croplates in IMDM supplemented with 10% FBS in the presence of the drugs (5 μM). After 72 h, cells were counted and cell viability assessed by trypan blue dye exclusion. Then AML cells were harvested, and their DNA fragmentation and cell cycle were determined by propidium iodide (PI) staining. Briefly, cells were washed twice with PBS, resuspended in a solution containing 50 μg/ml of PI, 0.1% sodium citrate, 0.1% Nonidet P-40, and 6.2 μg/ml of RNase for 30 min at room temperature. Samples were then analyzed by flow cytometry, gating out cell debris and fixation artifacts, to score the number of apoptotic cells represented as the percentage of events falling in an area immediately preceding the G_0/G_1 peak of DNA content histograms. The percentage of cells in each phase of the cell cycle was obtained using the Mod-FIT LT 3.0 program (Becton-Dickinson, Immunocytometry System, San Jose, CA, USA).

APO2.7 expression and annexin V binding were detected by flow cytometry as described previously (26). Briefly, cells were fixed with 1% paraformaldehyde at 4°C, permeabilized for 20 min on ice with digitonin (100 μg/ml; Sigma), washed once in cold PBS containing 2.5% FBS and 0.01% NaN_3 (PBSA), and incubated with 10 μl of phycoerythrin (PE)-conjugated anti-APO2.7 mAbs (Coulter-Immunotech, Fullerton, CA, USA) for 15 min at room temperature in the dark. Cells were then washed twice in PBSA and analyzed. For annexin V binding, cells were resuspended in 100 μl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with 10 μl of annexin V–fluorescein isothiocyanate (Pharmingen, Immunocytometry System, San Jose, CA, USA), and 10 μl of PI (10 μg/ml in binding buffer) in the dark for 15 min, and assayed after the addition of 300 μl of binding buffer to each sample. To detect the involvement of mitochondrial perturbation, CMXros (200 nM) (Molecular Probes, Eugene, OR, USA) was added to the AML cell culture for the last 30 min, washed twice with PBS, resuspended in PBS, and analyzed by flow cytometry (27).

For Bcl-2 and Bax analyses, cells were fixed with 2% paraformaldehyde in PBS for 15 min at 4°C, then permeabilized with 1% Tween 20 for 30 min at 4°C, and finally incubated with FITC-conjugated mouse anti-human Bcl-2 (clone 124) (DAKO Citomation) or with 1 μg/ml of mouse anti-Bax alpha (BD-Pharmingen), followed by PE-conjugated goat anti-mouse IgG (Becton-Dickinson, Immunocytometry System). Irrelevant isotype-matched antibodies (Jackson's Immunoresearch Laboratories, WestGrove, PA, USA) were used to determine background fluorescence. Viable, antibody labeled cells were identified according to their forward and right angle scattering, electronically gated, and analyzed on a FACScalibur flow cytometer (Becton-Dickinson), by means of the CellQuest software (Becton-Dickinson).

Purification and Clonogenic Assay of CD34+ Cells

Mononuclear cells from cord blood (CBMC) were purified by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient separation and subsequently incubated with ammonium chloride for 5 min at 4°C to minimize the contamination with red blood cells. CD34+ cells were purified with microbead-conjugated anti-CD34 antibodies (Miltenyi Biotec, Italy). Purified CD34+ cells (3×10^3) were plated in triplicate in a methylcellulose medium (Methocult GFH4434; Stem Cell Technology Inc, Vancouver, Canada), supplemented with human recombinant hematopoietic growth factors: erythropoietin (Epo: 3 U/ml), rh Stem Cell Factor (50 ng/ml), rh GM-CSF (10 ng/ml), and rhIL-3 (10 ng/ml). Colony-forming unit GM (CFU-GM), colony-forming unit GEMM (CFU-GEMM), and burst-forming unit erythroid (BFU-E) were counted at day 14 according to published criteria.

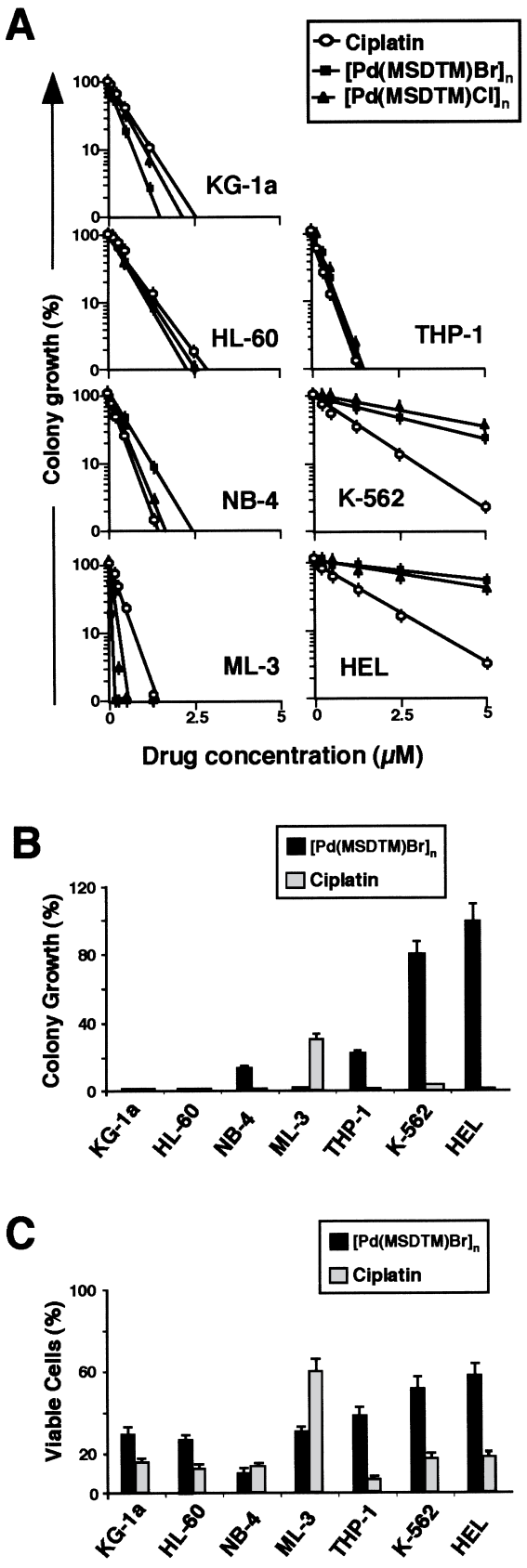
Statistical Analysis

Mean data values are presented with their standard deviation error (mean ± SD). Statistical comparisons were performed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of Pd(II) Methylsarcosinedithiocarbamate Derivatives on the Clonogenic Growth of AML Cell Lines

Traditional antineoplastic therapy is based on the use of chemotherapeutic compounds, which exert a cytotoxic effect on proliferating cells and promote the destruction of sensitive tumors. As a preliminary screening of their antiproliferative activity, the in vitro cytotoxic effects of Pd(II) complexes were evaluated on a panel of AML cell lines representing different FAB subtypes. For comparison purposes, the cytotoxic activity of cisplatin was evaluated under the same experimental conditions. Exposure of AML cells to increasing concentrations of both $[Pd(MSDT)Cl]_n$ and $[Pd(MSDT)Br]_n$ resulted in concentration-dependent clonogenic growth inhibition (Fig. 2A) with no significant differences occurring between the two Pd(II) complexes. ID_{50} values obtained from concentration–response curves showed that cisplatin was significantly ($p < 0.05$) more active on NB4, K562, and HEL cells, and comparable to Pd(II) complexes on KG-1a, HL-60, and THP-1 cells. Both Pd(II) complexes were almost ineffective on the more differentiated HEL and K562 cell lines (Fig. 2A). In contrast, these agents were very active on all of the less differentiated cell lines (KG-1a, HL-60, and THP-1).



FACING COLUMN

Figure 2. Growth inhibition by Pd(II) complexes. (A) Representative concentration survival curves of AML cells exposed to increasing concentrations of Pd(II) complexes. AML cells (5×10^3) were cultured in semisolid medium in the presence of increasing concentrations of $[Pd(MSDT)Br]_n$, $[Pd(MSDT)Cl]_n$, and cisplatin. After 14 days of incubation, plates were observed under phase-contrast microscopy and aggregates with ≥ 40 cells were scored as colonies. Results represent the mean ± SD of eight replicate wells. The negative exponential concentration-response survival curves were drawn by linear regression and may be described as a single parameter (the ID_{50} value). (B) AML cell lines were incubated at 2×10^5 cells/ml in six-well flat-bottomed microplates in IMDM supplemented with 10% FBS in the presence and the absence of the drugs (5 μM). After 24 h, cells were harvested, washed in PBS, and then 4×10^3 AML cells were analyzed for clonogenic growth. (C) AML cells were incubated at 2×10^5 cells/ml in the presence and absence of the drugs (5 μM). After 72 h, cells were counted and cell viability assessed by trypan blue exclusion dye.

However, both complexes were more cytotoxic than cisplatin against the myelomonoblastic cell line ML-3, with statistically ($p < 0.05$) different ID_{50} values of 0.05 ± 0.004 and 0.02 ± 0.001 for $[Pd(MSDT)Cl]_n$ and $[Pd(MSDT)Br]_n$, respectively, versus 0.40 ± 0.03 for cisplatin. In another series of experiments, AML cells were seeded in a methylcellulose medium after a relatively short exposure (24 h) to cisplatin and $[Pd(MSDT)Br]_n$. As shown in Figure 2B, $[Pd(MSDT)Br]_n$ strongly inhibited the clonogenic growth of AML cells, excluding K562 and HEL cells. In the ML-3 cell line, $[Pd(MSDT)Br]_n$ was significantly more active than cisplatin. Additional experiments were carried out with a continuous exposure to the drugs for 72 h in liquid cultures. As shown in Figure 2C, cisplatin exhibited a strong growth inhibition in all the AML cell lines tested (ranging from 7% to 18% of control), excluding ML-3 cells (60% of control). Also, in liquid culture, $[Pd(MSDT)Br]_n$ was more active in KG-1, HL-60, NB4, and THP-1 than in K-562 and HEL cells, and more active than cisplatin in the ML-3 cell line (Fig. 2C), although the differences in sensitivity were less evident using the clonogenic growth assay.

Taken together, our results suggest a comparable activity of cisplatin and Pd(II) compounds in the less differentiated AML cell lines, but a superior cisplatin activity in the more differentiated HEL and K-562 cell lines. In contrast, the Pd(II) complexes always were significantly more active than cisplatin in the ML-3 cell line.

Pd(II) Methylsarcosinedithiocarbamate Derivatives Induce Apoptosis of AML Cells

In view of the above growth inhibition effects, we next determined whether these compounds also induced cell death by apoptosis, the predominant mechanism by which cancer cells die in response to an immune attack or to most cytotoxic drugs (23). All of the AML cells were incubated with a single cytotoxic dose (5 μM) of $[Pd(MSDT)Br]_n$ and cisplatin, counted, and then early apoptosis was analyzed by means of both annexin V and Apo2.7 staining. As shown in Figure 3A and B, excluding K562 and HEL cells, exposure to both $[Pd(MSDT)Br]_n$ and cisplatin induced consistent exposure to phosphatidylserine together with PI staining, suggesting that cell membrane damage may be a consequence of this treatment. Similar results were obtained in the presence of $[Pd(MSDT)Cl]_n$ (data not shown). Moreover, ML-3 cells were more sensitive to the apoptotic effects of $[Pd(MSDT)Br]_n$ compared to cisplatin (Fig. 3C), after an incubation period of 24 h; this effect was also evident after 48 and 72 h. To further demonstrate the efficacy of $[Pd(MSDT)Br]_n$ in inducing apoptosis, we measured the expression APO2.7, a protein confined to the mito-

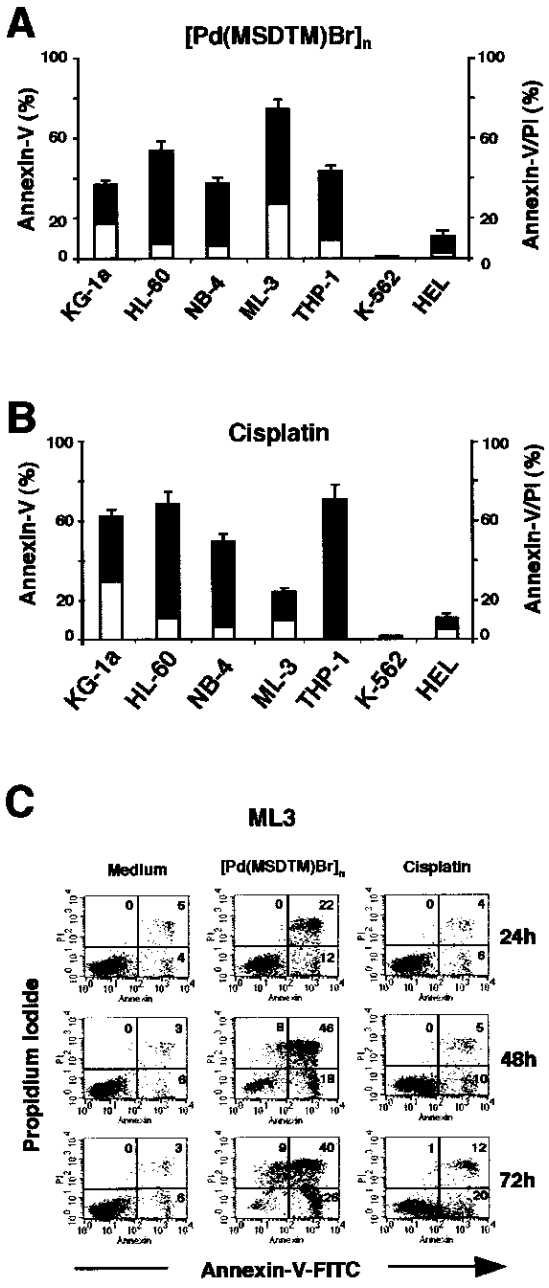


Figure 3. Induction of apoptosis of AML cell lines by $[Pd(MSDT)Br]_n$ and cisplatin. Exponentially growing AML cell lines were cultured in the presence and absence of (A) $[Pd(MSDT)Br]_n$ and (B) cisplatin (5 μM). After 72 h, AML cells were double stained with annexin V-FITC and PI. Results are expressed as the percentage of annexin V-FITC (white bars) and annexin V-FITC/PI (dark bars) stained cells ± SD and are representative of three independent experiments. (C) Representative dot plots of ML-3 cells analyzed after 24, 48, and 72 h. The percentages of stained cells are indicated in the quadrants.

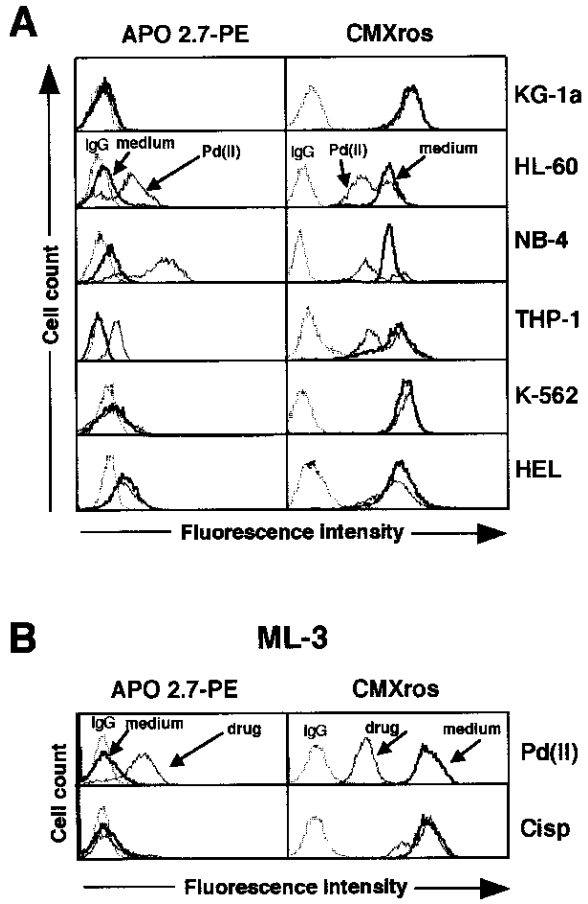


Figure 4. Induction of apoptosis of AML cell lines by [Pd(MSDT)Br]$_n$ (A) AML cell lines were cultured in the presence and absence of [Pd(MSDT)Br]$_n$ and cisplatin (5 μM). Representative fluorescence histograms show APO2.7 expression (left panel) and mitochondrial membrane depolarization (CM Xros) (right panel). Dotted lines indicate background fluorescence of cells, as determined by isotype-matched immunoglobulins. The *x* and *y* axes indicate the logarithm of the relative intensity of red fluorescence and relative cell number, respectively. Data shown refer to a representative experiment, repeated three times. (B) ML-3 cells were cultured in the presence and absence of [Pd(MSDT)Br]$_n$ or cisplatin (5 μM). Representative fluorescence histograms show APO2.7 expression (left panel) and mitochondrial membrane depolarization (CM Xros) (right panel).

chondrial membrane that can be detected during the early stages of apoptosis. As shown in Figure 4A, APO2.7 expression in the presence of [Pd(MSDT)Br]$_n$ was strongly upregulated in the most sensitive cell lines (HL-60, NB-4, and THP-1), thereby confirming the apoptotic potential of the Pd(II) derivatives. The dissipation of the inner mitochondrial transmembrane potential marks the point-of-no-return during the apoptotic program and occurs prior to DNA fragmentation (28–30). Thus, evaluation of mitochondrial membrane depolarization is of critical importance for the assessment of apoptosis. As shown in Figure 4A, and in accordance with the induction of the apoptotic markers, APO2.7 and annexin V, [Pd(MSDT)Br]$_n$ produced a consistent decrease in the mitochondrial membrane potential in HL-60, NB-4, and THP-1 cells, as evaluated by flow cytometry with CMXros, a mitocondrial-specific dye used to evaluate mitochondrial membrane potential changes during apoptosis. Almost no measurable effects were detected for K562 and HEL cells. As shown in Figure 4B, at the cytotoxic concentration of 5 μM [Pd(MSDT)Br]$_n$ but not cisplatin induced Apo2.7 expression and decreased the mitochondrial membrane potential changes in ML3 cells. Similar results were obtained in the presence of the [Pd(MSDT)Cl]$_n$ complex (data not shown).

The ability of both Pd(II) complexes and cisplatin to inhibit cell cycle progression and promote DNA fragmentation was also determined using PI staining and flow cytometric analyses. As shown in Figure 5, after a 72-h treatment, both Pd(II) complexes produced an increase in apoptotic nuclei in ML-3, HL-60, NB-4, and THP-1 cells, identified by flow cytometry as a subdiploid peak (sub-G_1) in DNA content histograms. Cisplatin caused significant DNA fragmentation in all cell lines, excluding HL-60, ML-3, and K-562 cells. Moreover, cell cycle analyses showed very different activities depending on the drug used and the cell line analyzed, while only in HL-60, NB-4, and ML-3 cells both Pd(II) compounds and cisplatin produced the same cell cycle phase modifications (Fig. 5).

Effects of Pd(II) Methylsarcosinedithiocarbamate Derivatives on Bcl-2 and Bax Levels

Bcl-2 family proteins are evolutionarily well-conserved apoptosis-regulating proteins (31). The balance between proapoptotic and antiapoptotic Bcl-2 proteins is crucial for determining cell fate. Both Bcl-2 and the integral membrane form of Bax (Bax-alpha) were analyzed by flow cytometry to test the possibility that changes in the levels of Bcl-2 family proteins were involved in the Pd(II) complexes-induced apoptosis. In accordance with phosphatidyl serine exposure, Apo2.7 induction, and the decrease in mitochondrial transmembrane potential, treatment with [Pd(MSDT)Br]$_n$ caused a pronounced decrease in the Bcl-2 antiapoptotic molecule in the HL-60, NB-4, and THP-1 cell lines (Fig. 6A). Conversely, the proapoptotic molecule Bax was always upregulated or induced in HL-60, NB-4, and THP-1 cells (Fig. 6A). Similar results were obtained in the presence of [Pd(MSDT)Cl]$_n$ (data not shown). As shown in Figure 6B, at the cytotoxic concentration of 5 μM, Pd(MSDT)Br]$_n$ but not cisplatin significantly induced Bax expression and strongly downregulated Bcl-2 in ML-3 cells.

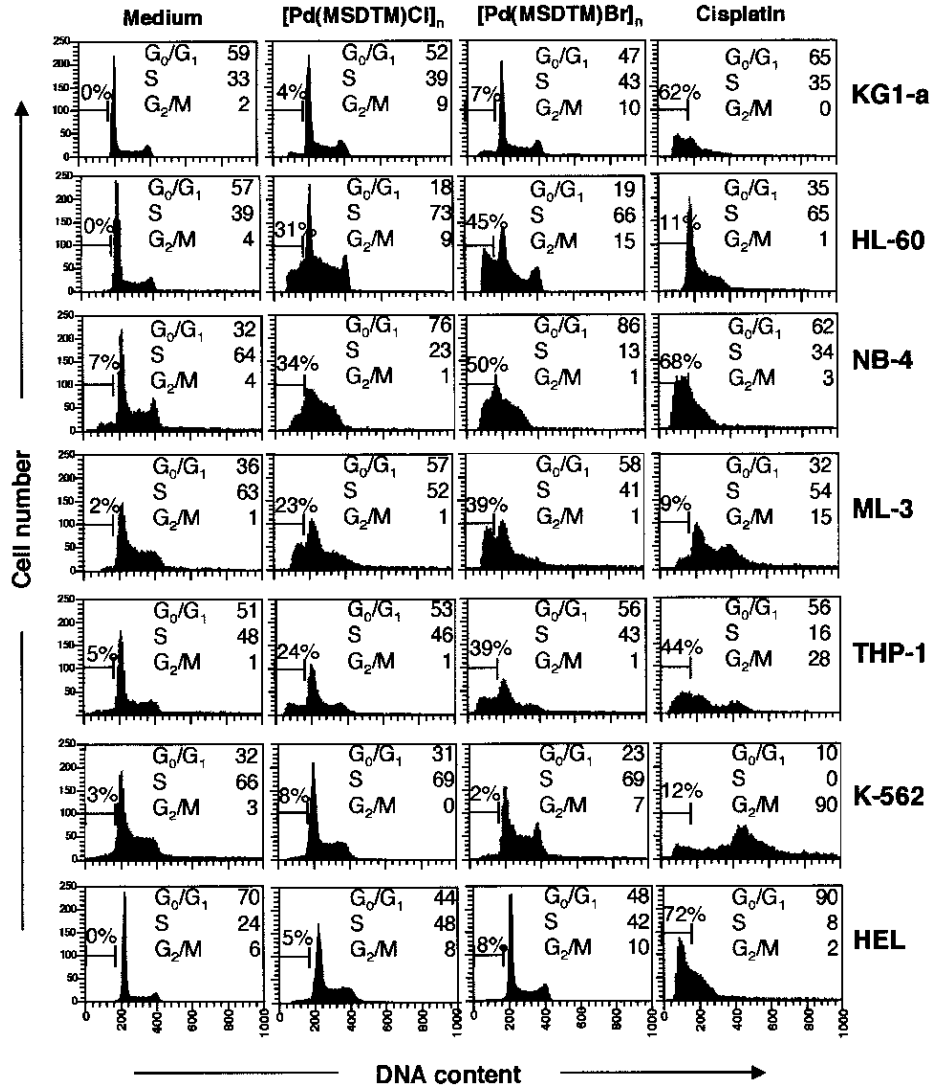


Figure 5. Cell cycle analysis after Pd(II) complexes and cisplatin treatment. AML cells were cultured in the presence of $[Pd(MSDT)Cl]_n$, $[Pd(MSDT)Br]_n$, or cisplatin (5 μM). After 72 h, cell cycle and DNA fragmentation were determined by propidium iodide staining. The percentages of the G_0/G_1 phase, S phase, and G_2M phase cells are shown; the percentages of sub-G_1 cells are also reported. The data shown refer to a representative experiment repeated twice.

Taken together, our results clearly demonstrate that apoptosis is a mechanism involved in the growth inhibition produced by the Pd(II) dithiocarbamate derivatives and that Pd(II) complexes were more active than cisplatin in ML-3 cells, as evaluated by growth inhibition and apoptosis induction.

Effects of $[Pd(MSDT)Br]_n$ on the Clonogenic Growth of Normal Hematopoietic Progenitor Cells

Because one of the negative consequences of chemotherapeutic regimens is myelosuppression, we evaluated the myelotoxicity of $[Pd(MSDT)Br]_n$ by means of an in vitro hematotoxicology predictive model (32). As shown in Figure 7, $[Pd(MSDT)Br]_n$ inhibited CFU-GM, BFU-E, and CFU-GEMM growth in a concentration-dependent manner with an ID_{50} similar to that of the reference drug cisplatin (Table 1). When also considering the ID_{90} values, which have been suggested to represent a better predictive parameter for the hematotoxicity of anticancer drugs (33), we obtained comparable results.

In general, the efficacy of a drug depends on its therapeutic index, which represents the ratio of its efficacy to toxicity. Remarkably, $[Pd(MSDT)Br]_n$ was more potent in inhibiting the growth of AML-derived colonies (ID_{50} ranging from 0.02 to 0.52 μM) in the less differentiated KG-1a, HL-60, NB-4, ML-3, and THP-1 cell lines

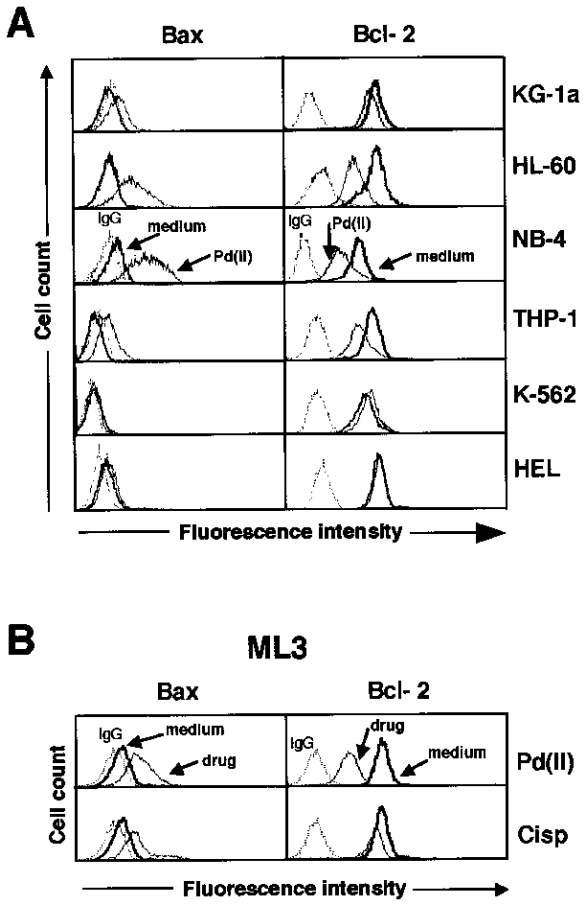


Figure 6. Modulation of Bcl-2 and Bax molecules by the [Pd(MSDT)Br]$_n$ complex. (A) Exponentially growing AML cells were cultured in the absence (medium) or presence of [Pd(MSDT)Br]$_n$ (5 μM) for 72 h. Dotted lines indicate background fluorescence of cells, as determined by isotype-matched immunoglobulins. The *x* and *y* axes indicate the logarithm of the relative intensity of red fluorescence and relative cell number, respectively. The data shown refer to a representative experiment, repeated three times. (B) ML-3 cells were cultured in the presence and absence of [Pd(MSDT)Br]$_n$ or cisplatin (5 μM). Representative fluorescence histograms show Bax (left panel) and Bcl-2 expression (right panel).

than that of normal-derived colonies, with ID_{50} values of 2.1 ± 0.1 (μM) for CFU-GEMM, 3.8 ± 0.4 (μM) for BFU-E, and 2.5 ± 0.2 (μM) for CFU-GM.

DISCUSSION

Platinating agents, including cisplatin, carboplatin, and oxaliplatin, have been used clinically for nearly 30 years as part of the treatment of many types of cancers, including lymphomas and leukemias. Despite their relatively great effectiveness, there are clinical problems related to the use of platinum compounds in curative therapy, such as severe normal tissue toxicity and the frequent occurrence of initial and acquired resistance to treatment. The most important adverse side effect of cisplatin is nephrotoxicity, which is related to platinum binding and inactivation of thiol-containing enzymes (6,21). Several new compounds with reduced toxicity and high specificity have been developed, such as ruthenium complexes (8,9), and the second-generation platinum drug carboplatin that is active in treating AML (34,35)

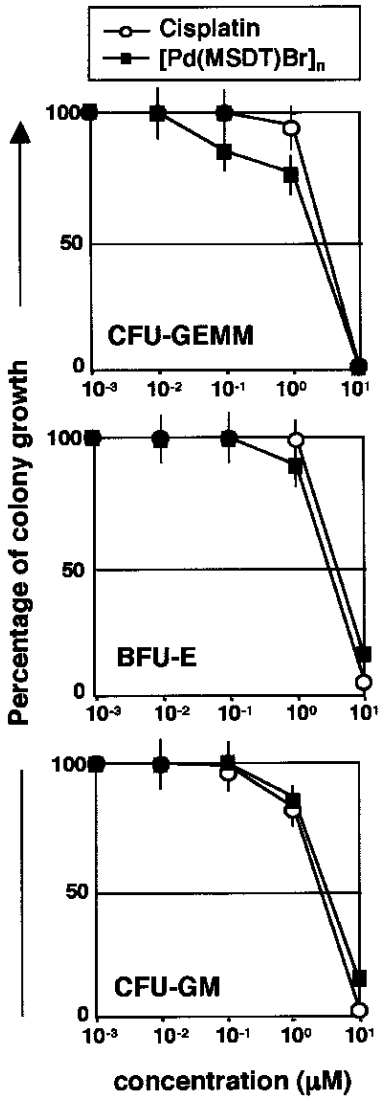


Figure 7. Clonogenic growth inhibition of hematopoietic progenitors by [Pd(MSDT)Br]$_n$. CD34+ purified cells were cultured in semisolid medium in the presence of increasing concentrations of [Pd(MSDT)Br]$_n$ or cisplatin. After 14 days of incubation, plates were observed under phase contrast microscopy and CFU-GM, CFU-GEMM, and BFU-E were counted. Results represent the mean ± SD of three replicate plates.

Table 1. Response to Cisplatin and $[Pd(MSDT)Br]_n$ of CD34+ Hemopoietic Progenitors

	ID_{50} (μM)			ID_{90} (μM)		
Drugs	CFU-GM	BFU-E	CFU-GEMM	CFU-GM	BFU-E	CFU-GEMM
$[Pd(MSDT)Br]_n$	2.5 ± 0.2	3.8 ± 0.4	2.1 ± 0.1	6.0 ± 0.5	8.4 ± 0.7	7.2 ± 0.6
Cisplatin	3.0 ± 0.3	2.9 ± 0.3	3.0 ± 0.2	8.1 ± 0.7	9.0 ± 0.8	8.0 ± 0.7

ID_{50} and ID_{90} values were obtained from the inhibition curves showed in Figure 7. They represents the mean ± SD of three experiments. CFU-GM, colony-forming unit, granulocyte-macrophage; BFU-E, burst-forming unit, erythroid; CFU-GEMM, colony-forming unit, granulocyte-erythroid-megakaryocytic-macrophage.

or blast crisis in chronic myeloid leukemia (CML) (36). Finally, the potential application of gold compounds as a new class of anticancer drugs has recently been explored (8–17).

AML is a malignant disease characterized by an aberrant accumulation of immature myeloid hematopoietic cells. Remission can be achieved in most patients, but a relapse is common and the long-term survival is poor for most cases (1). For this reason, the discovery of better antileukemic drugs is essential. In order to obtain less toxic compounds that are more active than cisplatin, new Pd(II) methylsarcosine-dithiocarbamate derivatives have been synthesized, purified, and fully characterized chemically (12,13).

In the present study, we performed an initial screening to evaluate the antitumor activity and the mechanism of action of two new Pd(II) complexes on a panel of 6 AML cell lines representing different FAB subtypes and in the Ph+ cell line K562 (human chronic myeloid leukemia in blast crisis). These new Pd(II) complexes produced a concentration-dependent clonogenic growth inhibition on most myeloid leukemic cell lines tested, but exhibited a comparable or lesser activity with respect to the control molecule cisplatin in all but the ML-3 cell line. This cell line was found to be very sensitive to the antiproliferative and apoptotic action of both Pd(II) complexes. The ML-3 cell line, derived from a patient with acute myelocytic leukemia preceded by a T-non-Hodgkin's lymphoma, has a biphenotypic (myelomonocytic/T-cell) marker expression. It is tempting to speculate that it might represent a new model for testing the antiproliferative activity of the Pd(II) dithiocarbamates or other new derivatives.

Comparable results were obtained when clonogenic growth of AML cells was evaluated after a relatively short incubation time of 24 h with cisplatin or $[Pd(MSDT)Br]_n$ (the same drug concentration used for the apoptosis assay), and when viable cells were counted after treatment for 72 h in liquid culture.

It is now well established that most anticancer agents induce apoptosis or programmed cell death, a genetic program that allows the control of cellular homeostasis (37). Cytotoxic concentrations of Pd(II) dithiocarbamate derivatives induced apoptotic death in the sensitive AML cell lines, as documented by APO2.7 and annexin V staining, mitochondrial membrane depolarization, and DNA fragmentation. After 72 h of incubation, we found a strong induction of APO2.7, a mitochondrial membrane protein that appears to be exposed on cells undergoing apoptosis (38). The annexin V protein associated with cytoplasmic membrane damage and DNA fragmentation was also increased, suggesting that Pd(II) complexes inhibit leukemia cell growth through apoptosis induction.

The primary cause of treatment failure in AML is the emergence of both resistant disease and early relapse, and one of the most frequent causes of these phenomena are the defects in the mitochondrial-mediated apoptotic pathway (2). This pathway is regulated by the Bcl-2 family of antiapoptotic (Bcl-2, Bcl-xl, mcl-1) and proapoptotic proteins (Bax, Bad, Bak). Bcl-2 functions as an inhibitor of mitochondrial permeabilization by changing its conformation in the mitochondrial membrane in order to bind membrane-inserted Bax monomers and to prevent productive oligomerization of Bax (1,39). The Bax/Bcl-2 ratio, as determined by flow cytometric analysis, represents a crucial clinical event in AML: in fact, a lower Bax/Bcl-2 ratio confers a very poor prognosis, with decreased rates of complete remission and overall survival (39–42). Therefore, Bcl-2 and Bax both represent sensitive indicators of clinical outcome and potential targets of novel proapoptotic molecules designed to circumvent chemoresistance (2,31).

$[Pd(MSDT)Br]_n$ produced a significant downregulation of the antiapoptotic molecule Bcl-2 and an upregulation of Bax in AML cell lines, decreasing the Bcl-2/Bax ratio, thereby suggesting that our Pd(II) complexes might be included in new therapeutic regimens capable of inducing apoptosis. One of the major events during apoptosis is the permeabilization of the mitochondrial outer membrane that is facilitated by the Bcl-2 family of proteins. Consistently with the downregulation of Bcl-2

and upregulation of Bax by the Pd(II) complexes, we observed a significant mitochondrial membrane depolarization.

Classical Pt(II) complexes are known to induce characteristic cell cycle alterations resulting in an increase in the G_2M cell fraction (43). Cell cycle analysis showed a very different activity depending on the cell line and drug analyzed, except for HL-60, NB-4, and ML-3 cells, in which both Pd(II) compounds and cisplatin caused the same cell cycle phase modification. Finally, the superior sensitivity of ML-3 cells to apoptosis induction by Pd(II) complexes compared to cisplatin was clearly demonstrated.

Taken together, the data presented in this report demonstrate that Pd(II) complexes are antitumor agents for AML cells, able to trigger apoptosis and characterized by superior activity compared to cisplatin in the ML-3 cell line, and suggest that these new compounds might represent potentially active new agents in selected primary and secondary AML.

Most anticancer and many anti-HIV compounds produce severe myelotoxicity that limits their clinical utility (32). Recent reports demonstrated that the in vitro colony forming unit granulocyte/macrophage assay represents a predictive model of human myelotoxicity (32). $[Pd(MSDT)Br]_n$ exhibited low myelotoxicity in vitro, and inhibited the clonogenic growth of the hemopoietic progenitors with an ID_{50} comparable to that of cisplatin. Our data clearly showed that the $[Pd(MSDT)Br]_n$ complex has a good therapeutic index, because it is more effective in inhibiting clonogenic growth of the less differentiated AML cells relative to normal hemopoietic progenitors.

Tumor cell resistance to chemotherapeutic agents is a central problem in medical oncology (1). It is well known that an increased rate of drug inactivation in some cases is due to the high affinity of platinum and other soft heavy metals for sulfured ligands such as glutathione, metallotionine, and other sulfur-containing biomolecules. Considering that our compounds contain a dithiocarbamate group capable of preventing a reaction with other sulfur-containing proteins (12,19), we can hypothesize that a higher in vivo stability occurs, with decreased chemoresistence and toxicity. However, further preclinical studies will be required to evaluate the efficacy of Pd(II) dithiocarbamate derivatives as single agents in multiple preclinical tumor models, as well as assessing their effectiveness in combination with existing standard therapy for AML. It will also be of interest to assess the efficacy of these compounds against other tumor types.

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