

# N3-Methyladenine Induces Early Poly(ADP-Ribosylation), Reduction of Nuclear Factor- $\kappa$ B DNA Binding Ability, and Nuclear Up-Regulation of Telomerase Activity

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## ABSTRACT

Methylation of N3-adenine represents a novel pharmacological strategy for the treatment of resistant tumors. However, little is known about the biochemical pathways involved in cell death induced by N3-methyladenine. In the present study, we show that MeOSO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>-lexitropsin (Me-Lex), a compound generating almost exclusively N3-methyladenine (>99%), provoked a burst of poly(ADP-ribosylation) and loss of mitochondrial membrane potential in leukemia cells. These events were followed by a marked decrease in nuclear poly(ADP-ribose) polymerase-1 (PARP-1) expression and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity. Moreover, DNA damage generated by N3-methyladenine induced a marked decrease in telomerase in the cytosol that was accompanied by a transient up-regulation of activity in the nucleus, as a consequence of nuclear translocation of

telomerase in response to genotoxic damage. PARP-1 inhibition blocked ADP-ribose polymer formation, preserved mitochondrial membrane integrity, and counteracted the reduction of NF- $\kappa$ B activity, thus preventing the appearance of necrosis. On the other hand, because PARP-1 is a component of the base excision repair (BER), the combination of Me-Lex + PARP-1 inhibitor triggered apoptosis as a result of disruption of BER process. In conclusion, the present study provides new insight into the cellular response to N3-adenine-selective methylating agents that can be exploited for the treatment of tumors unresponsive to classical wide-spectrum methylating agents. Moreover, the results underline the central and paradoxical role of PARP-1 in cell death induced by N3-methyladenine: effector of necrosis and coordinator of methylpurine repair.

Monofunctional methylating agents include antitumor drugs that interact with DNA, generating a wide spectrum of base adducts endowed with cytotoxic and/or mutagenic activity. The three major modified bases are represented by N7-methylguanine, N3-methyladenine (N3-MeA), and O<sup>6</sup>-methylguanine (O<sup>6</sup>-MeG), and the extent to which each type of

methyl adduct is generated depends on the nature of the methylating compound.

Although N7-methylguanine is quantitatively the prevalent lesion (~65–80%), this adduct seems to have little or no lethal effects, whereas unrepaired N3-MeA is a highly toxic lesion (Fronza and Gold, 2004). Because the percentage of N3-MeA generated by methylating agents of clinical interest [i.e., temozolomide (TMZ) or dacabazine], or by compounds of exclusive experimental use [e.g., *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methyl nitrosourea (MNU)] is low (~8–12%), this modified base is unlikely to contribute to toxicity (Kyrtopoulos et al., 1997; Newlands et al., 1997). In fact, together with N7-methylguanine, N3-MeA is promptly

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**ABBREVIATIONS:** N3-MeA, N3-methyladenine; O<sup>6</sup>-MeG, O<sup>6</sup>-methylguanine; TMZ, temozolomide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, methyl nitrosourea; BER, base excision repair; AGT, O<sup>6</sup>-alkylguanine DNA alkyltransferase; MR, mismatch repair system; Me-Lex, MeOSO<sub>2</sub>(CH<sub>2</sub>)<sub>2</sub>-lexitropsin; MPG, *N*-methylpurine-DNA glycosylase; PARP-1, poly(ADP-ribose) polymerase-1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; AB, 3-aminobenzamide; NU1025, 8-hydroxy-2-methylquinazolin-4-[<sup>3</sup>H]one; PBS, phosphate-buffered saline; PI, propidium iodide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; ROS, reactive oxygen species; PFGE, pulsed field gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; IKK, I $\kappa$  kinase; TRAP, telomeric repeats amplification protocol; PCR, polymerase chain reaction; OD, optical density;  $\Delta\Psi_m$ , changes in mitochondrial membrane potential; AIF, apoptosis-inducing factor; IRC-191, 6-chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxy-acridine.

repaired by the *short patch* of base excision repair (BER) (Srivastava et al., 1998).

Despite being produced in low amounts ( $\sim 8\%$ ),  $O^6$ -MeG is generally considered the main cytotoxic and mutagenic lesion produced by the aforementioned compounds (Kyrtopoulos et al., 1997; Newlands et al., 1997). If not repaired by  $O^6$ -alkylguanine DNA alkyltransferase (AGT),  $O^6$ -MeG inappropriately pairs either with thymine or cytosine and triggers the intervention of the mismatch repair system (MR). However, MR fails to find a correct partner for the methylated base, generates nicks in the DNA, and activates the apoptotic machinery (D'Atri et al., 1998). In the presence of MR functional defects, tumors become tolerant to the damage provoked by  $O^6$ -MeG, and inhibition of AGT activity does not enhance cell killing induced by methylating agents (Liu et al., 1996; Levati et al., 1998). Moreover, in this case the increased level of unrepaired  $O^6$ -MeG and  $O^6$ -MeG:T mismatches deriving from AGT depletion can increase the mutagenic activity of these compounds and the chances of genetic damage with consequent secondary tumors.

Although the mechanisms involved in the cytotoxic and mutagenic activity of  $O^6$ -MeG have been extensively studied, little is known about the biochemical pathways leading to cell death induced by N3-MeA adduct. In fact, because of the wide spectrum of modified bases induced by methylating agents, it is difficult to correlate cell killing to any single methyl adduct. The only agent capable of generating almost exclusively N3-MeA ( $>99\%$ ) is  $\text{MeOSO}_2(\text{CH}_2)_2$ -lexitropsin (Me-Lex), a methyl sulfonate ester tethered to *N*-methylpyrrololeucine dipeptide that targets A/T-rich sequences located in the DNA minor groove (Zhang et al., 1993; Encell et al., 1996). Unrepaired N3-MeA per se seems to behave as a replication blocking lesion, presumably because it hampers the contact between DNA polymerase and adenine in the DNA template strand (Fronza and Gold, 2004). Me-Lex is highly toxic both in prokaryotic and eukaryotic cells, especially in cells with defective or low *N*-methylpurine-DNA glycosylase (MPG), which initiates the BER process cleaving the glycosyl bond of methylpurine (Tentori et al., 2000, 2001; Fronza and Gold, 2004). The resultant abasic site is further processed by the apurinic/apyrimidinic endonuclease, whereas removal of the deoxyribose phosphate and replacement of the methylated nucleotide takes place by means of the coordinate intervention of poly(ADP-ribose) polymerase-1 (PARP-1), DNA polymerase  $\beta$ , X ray repair cross-complementing 1, and ligase III (Srivastava et al., 1998).

Inhibitors of PARP-1 have been shown to enhance the antitumor activity of either the wide-spectrum methylating agent TMZ or the N3-MeA-selective Me-Lex, as a consequence of the disruption of BER process after the initial removal of the methylated base by MPG (Tentori et al., 2000, 2001, 2002, 2003). For Me-Lex, the enhancing effect of PARP-1 inhibition is especially evident in tumor cells poorly responsive to the agent because of high levels of MPG (Tentori et al., 2001).

The interest in methylating agents that selectively target N3-A is raised by the observation that they can be effective against MR-deficient tumors, which are instead resistant to TMZ and to other anticancer agents with different mechanisms of action (Tentori et al., 2000, 2001).

In this study, we demonstrate that necrosis provoked by Me-Lex in sensitive tumors is characterized by a burst of

poly(ADP-ribosylation) followed by a marked decrease of nuclear PARP-1 expression and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity. It is interesting that DNA damage provoked by N3-MeA induced up-regulation of nuclear telomerase activity.

## Materials and Methods

**Cell Lines and Culture Conditions.** The human B lymphoblastoid MT-1 and TK-6 cell lines were a generous gift of W. G. Thilly (Massachusetts Institute of Technology, Cambridge, MA). The MT-1 line was obtained from TK-6 by treatment with IRC-191 followed by selection for MNGG resistance. TK-6 cells are MR-proficient, whereas the MT-1 line is MR-deficient, harboring different missense mutations in both alleles of the hMSH6 locus (D'Atri et al., 1998).

Human promyelocytic leukemia cell line HL-60 and its methotrexate-resistant subline HL-60R were a generous gift of T. Shimada (Nippon Medical School, Tokyo, Japan). The dihydrofolate reductase/MSH3 locus of HL-60R is amplified and overproduces the hMSH3 protein. As a consequence, the cell line does not express the hMtsa heterodimer and is deficient in MR (Drummond et al., 1997).

Cell lines were cultured in RPMI-1640 medium (Invitrogen, Paisley, Scotland) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (Flow Laboratories, McLean, VA), at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere.

**Drugs.** Me-Lex was prepared as described previously (Zhang et al., 1993). The PARP-1 inhibitor 3-aminobenzamide (AB) was purchased from Sigma-Aldrich (St. Louis, MO), whereas the PARP-1 inhibitor 8-hydroxy-2-methylquinazolin-4[ $^3\text{H}$ ]one (NU1025) was obtained from Calbiochem (Darmstadt, Germany). Drug stock solutions were prepared by dissolving Me-Lex (10 mM) in 95% ethanol, AB (16 mM) in RPMI-1640 medium, and NU1025 (25 mM) in dimethyl sulfoxide. The final concentration of ethanol or dimethyl sulfoxide in drug-treated cultures was always less than 0.5% (v/v) and did not contribute to toxicity (data not shown).

For drug treatment, cells were cultured in flasks (Falcon; BD Labware, Oxnard, CA) at the concentration of  $3 \times 10^5$  cells/ml. Inhibition of PARP-1 was obtained by treating cells with 4 mM AB or 50  $\mu$ M NU1025, concentrations that have been described to inhibit PARP-1 activity (Tentori et al., 2001). Me-Lex was added to cell cultures, either alone or immediately after PARP-1 inhibitor, and used at concentrations ranging from 12.5 to 50  $\mu$ M. Cells were then incubated at 37°C.

**Analysis of Necrosis by Flow Cytometry.** Cells were exposed to Me-Lex alone or combined with AB. At different times after treatment, unfixed cells were washed, suspended in PBS, and labeled with 2  $\mu$ g/ml propidium iodide (PI). Necrotic cells that have lost plasma membrane integrity are positive for PI staining.

**5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine Iodide (JC-1) Analysis of Mitochondrial Membrane Potential and Determination of Reactive Oxygen Species (ROS) by Flow Cytometry.** Untreated or drug-treated cells were washed twice, suspended in PBS containing 10  $\mu$ g/ml 5 JC-1 monomer (Molecular Probes, Leiden, The Netherlands) and incubated at 37°C for 10 min. For ROS detection, untreated or drug-treated cells were incubated with 100  $\mu$ M dihydroethidium (Molecular Probes) at room temperature for 20 min.

Data collection was gated using forward light scatter and side light scatter to exclude cell debris and aggregates. Cell fluorescence was recorded using a FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488-nm argon laser.

**Pulsed Field Gel Electrophoresis (PFGE).** PFGE of high molecular weight DNA fragments and preparation of plugs were performed as described previously (D'Atri et al., 1998). Electrophoresis was carried out using a switch back power supply (Hofer, San Francisco, CA). The gel (1.5% agarose) was run at 200 V at 4°C in  $0.5 \times$  Tris borate-EDTA containing ethidium bromide (0.5  $\mu$ g/ml),

with the ramping rate changing from  $T_1 = 0.5$  s and  $T_2 = 10$  s for 18 h, with a forward-to-backward ratio of 3. DNA was visualized under UV light and photographed using a Kodak digital camera setup.

**Analysis of Poly(ADP-Ribose)-Modified Proteins.** After treatments, cells were washed three times with 5 ml of ice-cold PBS + 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride and suspended at the concentration of  $10^7$ /ml in a denaturing buffer using a procedure described previously (Shah et al., 1995). In brief, cells were disrupted by sonication on ice (sonication buffer: 62.5 mM Tris-HCl, pH 6.8, 4 M urea, 10% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, and 0.003% bromophenol blue) twice for 30 s (60 W) and heated for 15 min at 65°C. Samples were electrophoresed in 7.5% SDS-PAGE and transferred onto a nitrocellulose filter. The membrane was saturated overnight and then incubated for 3 h with a rabbit polyclonal antibody against poly(ADP-ribose) polymers (BIOMOL Research Laboratories, Plymouth Meeting, PA). After several washings with PBS containing 0.2% Tween 20, the membrane was incubated for 1 h in the presence of donkey anti-rabbit antibody conjugated to horseradish peroxidase. Visualization of immunoreactive bands was performed by an enhanced chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ).

**Electrophoretic Mobility Shift Assay (EMSA).** Cells were suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM  $MgCl_2$ , 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), incubated on ice for 15 min, and then homogenized by 15 passages through a 25-gauge needle. Nuclei were collected by centrifugation at 1000g for 5 min at 4°C and suspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M  $NaCl$ , 1.5 mM  $MgCl_2$ , 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml pepstatin, 0.5 mM spermidine, and 5  $\mu$ g/ml aprotinin). After 15 min of incubation on ice, the nuclear extracts were collected by centrifugation at 10,000g for 2 min. Nuclear extracts (5  $\mu$ g of protein) were then incubated with  $^{32}P$  end-labeled double-stranded NF- $\kappa$ B consensus or mutated oligonucleotides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Complexes were analyzed by nondenaturing 4% PAGE, and specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65 (Rel A or NF- $\kappa$ B3) or p50 (Rel B or NF- $\kappa$ B1), for NF- $\kappa$ B. Supershifted antibodies were obtained from Santa Cruz Biotechnology, Inc. The gels were subsequently dried and autoradiographed with intensifying screens.

**Western Blot Analysis.** Nuclear, cytosolic, and mitochondrial fractions were prepared using fractionation kits from Medical and Biological Laboratories (Watertown, MA). Equal amount of proteins per sample were electrophoresed in 8% or 12% SDS-polyacrylamide gels. Afterward, proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences Inc.), and protein loading was visualized by Ponceau S staining. Filters were blocked with blocking buffer (Amersham Biosciences Inc.) and incubated with monoclonal antibodies directed against AIF, p65, p50, and p53 (Santa Cruz Biotechnology, Inc.), PARP-1, manganese superoxide dismutase, and cytochrome *c* (BD Biosciences), and actin and lamin (Sigma-Aldrich). Immune-complexes were visualized using a chemiluminescence kit (Amersham Biosciences Inc.), according to the manufacturer's instructions. Filters were exposed to Hyperfilm autoradiographic films (Amersham Biosciences Inc.) for 1 to 15 s, depending on the intensity of the signal.

**MPG Activity.** MPG activity was assayed as described previously (Tentori et al., 2001). Tumor cells ( $10^7$ ) were sonicated at 4°C in 0.5 ml of buffer I (50 mM Tris-HCl, 3 mM dithiothreitol, and 2 mM EDTA, pH 8.3), with freshly added 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride. Various amounts of cell extracts were incubated with 15  $\mu$ g (15,000 cpm) of freshly dissolved calf thymus DNA methylated by *N*-[ $^3H$ ]methyl-*N*-nitrosourea (19 Ci/mmol; Amersham Biosciences Inc.), in a total volume of 100  $\mu$ l of buffer II (20 mM Tris-HCl, 1 mM dithiothreitol, 60 mM NaCl, and 1 mM EDTA, pH 8). After 1 h at 37°C, the reaction was stopped on ice

by the addition of 30  $\mu$ l of 2 M NaCl containing 0.5 mg/ml calf thymus DNA and 1 mg/ml bovine serum albumin. DNA was ethanol-precipitated and samples were centrifuged at 10,000g for 15 min. Three hundred microliters of the supernatants were transferred to a scintillation tube and counted. MPG activity was determined for protein and time-limiting conditions and expressed as femtomoles of methylpurines released per milligram of proteins per hour.

**Kinase Assay.** Cell lysates were prepared using a high salt buffer containing 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% Nonidet P-40, 10% glycerol, 20  $\mu$ M dithiothreitol, and protease inhibitors. Lysates were incubated with anti-I $\kappa$ B kinase (IKK) $\alpha$  antibodies (BD Biosciences Pharmingen, San Diego, CA) in the presence of 15  $\mu$ l of protein A-Sepharose at 4°C for 12 h. After extensive washing, endogenous IKK activity was determined using GST-I $\kappa$ B $\alpha$  (1-54) as substrate (Amici et al., 2001). Western blot analysis was performed as kinase loading control.

**Telomerase Assay.** The telomeric repeats amplification protocol (TRAP) assay, based on PCR amplification of telomerase extension products, was performed as described previously (Tentori et al., 2001). Total cell extracts were prepared by lysing the cells in ice-cold extraction buffer [0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 1 mM  $MgCl_2$ , 1 mM EGTA, 0.25 mM sodium deoxycholate, 150 mM NaCl, 10% glycerol, 5 mM  $\beta$ -mercaptoethanol, and 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride]. After centrifugation at 20,000g, supernatants were collected, protein concentration was determined, and aliquots of supernatants were used for the TRAP assay. In selected experiments, the pellet obtained after high-speed centrifugation was further solubilized and assayed for telomerase activity.

For the analysis of telomerase activity in the nuclear and cytosolic compartments, cell extracts were prepared using a commercially available fractionation kit from Medical and Biological Laboratories.

The telomerase reaction was carried out in 40  $\mu$ l of the reaction mixture consisting of 20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM  $MgCl_2$ , 1 mM EGTA, 0.05% Tween 20, 0.1  $\mu$ g of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 0.1  $\mu$ M T4 gene 32 protein, and 50  $\mu$ M of each deoxynucleotide triphosphate. Samples were incubated at room temperature for 15 min to allow telomerase to extend TS primer. The reaction was stopped in ice, and 2 units of *Taq*DNA polymerase, 0.16  $\mu$ l of [ $\alpha$ - $^{32}P$ ]dCTP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), and 0.1  $\mu$ g of CX oligonucleotide (5'-CCCTTACCCTTACCCTTACCCTAA-3') were added to each single PCR tube. Amplification of the telomeric products was performed by PCR (94°C, 30 s; 50°C, 30 s; and 72°C, 1 min; 31 cycles). After TRAP assay, 40  $\mu$ l of the PCR reaction was separated on a 10% nondenaturing polyacrylamide gel. Thereafter, gels were fixed and exposed to X-ray films (Eastman Kodak, Rochester, NY) at -80°C. The signal of the telomeric ladder was quantified by bidimensional densitometry using a Kodak apparatus (Imaging densitometer, Image Analysis Software, Rochester, NY). Optical density/mm<sup>2</sup> (OD) was corrected for the background (i.e., lane relative to lysis buffer).

Assay of alkaline phosphatase activity as an internal control for the quality of the cell extract was performed using a commercially available kit (Sigma-Aldrich).

## Results

**N3-A Methylation Induces PARP-1 Activation, Early DNA Fragmentation, and Loss of Mitochondrial Membrane Potential.** We previously demonstrated that Me-Lex is capable of inducing cell killing in MR-deficient leukemia cells (Tentori et al., 2000 and 2001). Because PARP-1 activation has been suggested to contribute to cell death induced by a number of genotoxic agents through depletion of NAD<sup>+</sup> and ATP (Ha and Snyder, 1999), it was investigated whether treatment with Me-Lex activates PARP-1. To this end, MR-deficient leukemia MT-1 cells were exposed to 25  $\mu$ M Me-



Lex, a concentration capable of killing 60 to 70% of the cells at 24 h (Tentori et al., 2001), and analyzed for poly(ADP-ribose) (PAR) formation at 15 and 30 min after drug exposure by Western blot analysis with an antibody anti-PAR. The results indicate that Me-Lex induced a robust burst of PAR formation shortly after treatment, which was abrogated by pretreatment with the potent and specific PARP-1 inhibitor NU1025 (Fig. 1A). PARP-1 activation was transient because PAR formation was no longer detectable 60 min after drug exposure (data not shown). Treatment with 25  $\mu$ M Me-Lex induced PARP-1 activation also in MR-proficient TK-6 leukemia cell line (Fig. 1A).

Analysis of high molecular weight DNA fragmentation at 3 h after treatment revealed the presence of a predominant 50-kb fragment in the sample treated with 25  $\mu$ M Me-Lex. Addition of the PARP-1 inhibitor AB inhibited the 50-kb fragment formation at this early time point (Fig. 1B).

Analysis of cell growth by colony-forming ability assay shows that Me-Lex markedly inhibited cell growth both in MT-1 and TK-6. Sensitivity to Me-Lex was also assessed in MR-proficient HL-60 leukemia cell line and its MR-deficient subline HL-60R. The results indicate that Me-Lex induced comparable growth inhibition in both lines, suggesting that a functional MR is not required for N3-MeA antitumor activity (Table 1).

We next investigated whether PARP-1 activation induced by Me-Lex might cause mitochondrial membrane depolarization. Changes in mitochondrial membrane potential ( $\Delta\Psi_m$ ) were monitored using the fluorescent probe JC-1. This fluorescent mitochondria-specific dye possesses a dual emission wavelength: as  $\Delta\Psi_m$  drops, green JC-1 monomer fluorescence increases, whereas red J-aggregate fluorescence decreases. The results indicate that Me-Lex (25  $\mu$ M) induced a reduction of  $\Delta\Psi_m$  in about 40% of MT-1 cell population, as early as 3 h after treatment. The percentage of cells with normal mitochondrial potential (top left quadrant) dramatically decreased from 90 to 8% within a time frame of 6 h (Fig. 2). Pretreatment with the PARP-1 inhibitor AB (4 mM) protected cells from mitochondrial potential depolarization (Fig. 2).

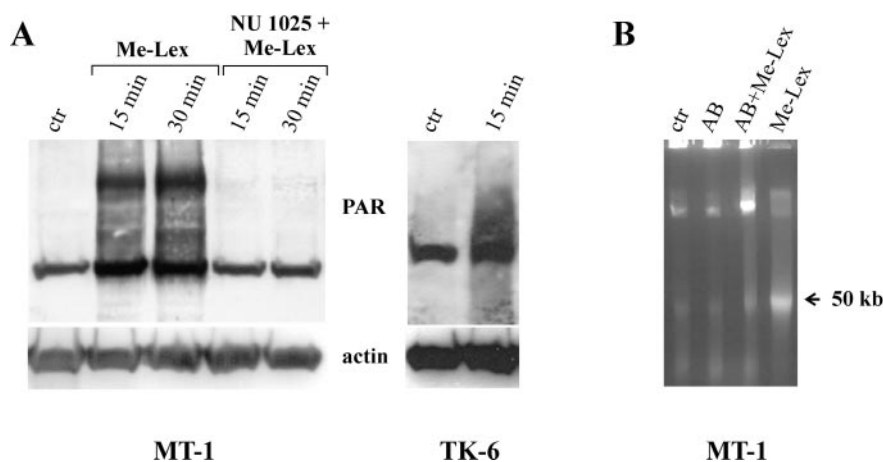
Because disruption of mitochondrial membrane potential is usually accompanied by increased ROS production, untreated or Me-Lex-treated cells were analyzed for ROS generation using a previously described technique based on the superoxide-induced conversion of the oxidant sensitive dye

dihydroethidium to ethidium, which intercalates within DNA (Virag et al., 1998). Flow cytometry analysis revealed that 25  $\mu$ M Me-Lex induced an increase of ROS production in  $63 \pm 8\%$  of cells 3 h after drug exposure. This effect was blocked by pretreatment with AB.

**Toxicity Induced by N3-MeA Is Accompanied by Down-Regulation of PARP-1 Expression and NF- $\kappa$ B Activity.** Analysis of nuclear proteins by Western blot analysis in MT-1 cells revealed a profound down-regulation of PARP-1 expression as early as 3 h after treatment with 25  $\mu$ M Me-Lex (Fig. 3). Similar results were obtained in TK-6 leukemia cells treated with the same Me-Lex concentration, which induced necrosis in  $46 \pm 5\%$  of the cell population 10 h after drug exposure (Fig. 3). At the same time points, no down-regulation of PARP-1 expression in the mitochondrial fraction was observed, whereas in the same samples AIF and cytochrome *c* expression slightly decreased at 10 h. No nuclear translocation of AIF or cytosolic mobilization of cytochrome *c* were detected (data not shown).

Because PARP-1 has been shown to regulate the function of NF- $\kappa$ B (Chang and Alvarez-Gonzalez, 2001; Hassa et al., 2001, 2003), a transcription factor modulated by a number of anticancer agents (Das and White, 1997), we have investigated whether the marked reduction of PARP-1 protein induced by Me-Lex was accompanied by a parallel decline of NF- $\kappa$ B activity. Analysis of the transcription factor binding activity by EMSA in MT-1 and TK-6 leukemia cells showed a constitutive activation of NF- $\kappa$ B in both cell lines (Fig. 4). In MT-1 cells, Me-Lex induced a time- and dose-dependent decrease of NF- $\kappa$ B activity. NF- $\kappa$ B reduction was evident starting from 6 h after treatment with 25  $\mu$ M Me-Lex (Fig. 4A), and with 12.5  $\mu$ M Me-Lex 10 h after drug exposure (Fig. 4B). Preincubation of nuclear extracts with antibody to either the p50 or the p65 subunits of NF- $\kappa$ B revealed that both antibodies shifted the band to a higher molecular mass and that treatment with Me-Lex induced a decrease of both components (Fig. 4A). A similar NF- $\kappa$ B down-regulation was observed in TK-6 leukemia cells (Fig. 4B).

When the same cell lines were treated with the wide-spectrum methylating agent TMZ (6.2–250  $\mu$ M), a reduction of NF- $\kappa$ B activity was observed only in TK-6 cells 48 h after exposure to 12.5 or 25  $\mu$ M TMZ (Fig. 4C). In fact, TK-6 cells are sensitive to TMZ, being characterized by low AGT activity and functional MR. The late appearance of NF- $\kappa$ B down-regulation reflects the timing of TMZ cytotoxicity, which



**Fig. 1.** Me-Lex induces PARP-1 activation and early DNA fragmentation. A, PARP-1 inhibitor abrogates PAR formation induced by Me-Lex. MT-1 or TK-6 cells were incubated for 15 min with the PARP-1 inhibitor NU1025 (50  $\mu$ M) before treatment with 25  $\mu$ M Me-Lex and then analyzed by immunoblot for PAR formation and actin expression at the indicated times. B, analysis of DNA fragmentation in drug-treated MT-1 cells. Cells were cultured for 3 h in medium only or in the presence of 25  $\mu$ M Me-Lex  $\pm$  4 mM AB. Then, high molecular weight DNA was extracted and analyzed by PFGE. The size of 50-kilobase DNA fragments, estimated using molecular weight markers, is indicated. The figure is a photograph of an agarose gel stained with ethidium bromide. Representative of two independent experiments.

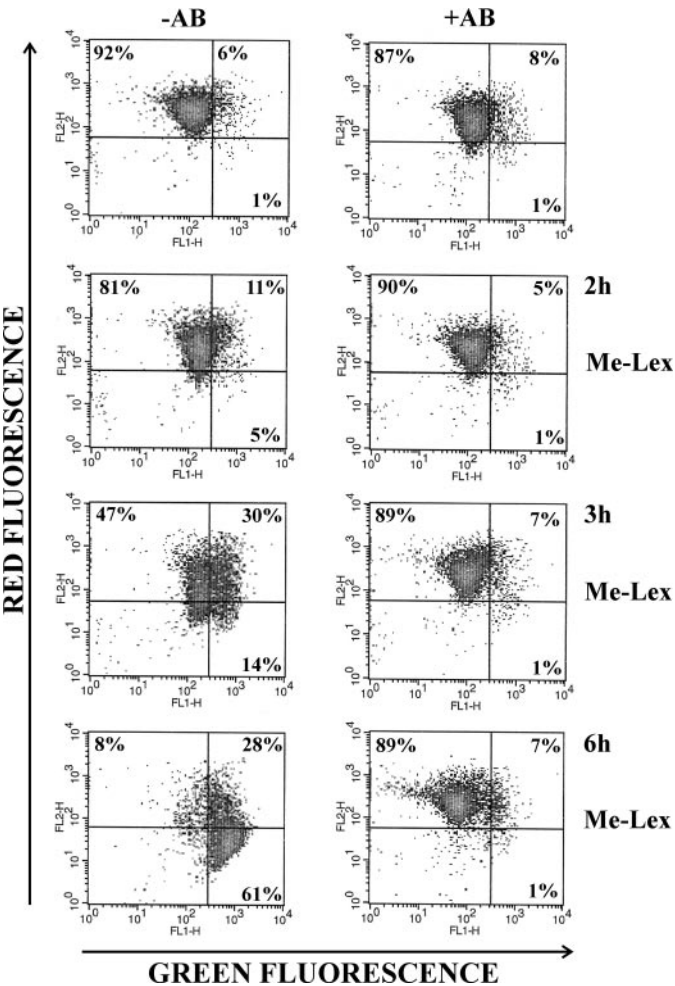
derives from the processing of O<sup>6</sup>-MeG and occurs during the second cycle of DNA replication after adduct generation. In MT-1 cells, no modulation of NF-κB was detected even at 10-fold higher concentration (Fig. 4C). These results are consistent with the intrinsic resistance of MT-1 cells to O<sup>6</sup>-MeG damage because of MR deficiency (D'Atri et al., 1998).

TABLE 1

Chemosensitivity to Me-Lex of MR-proficient and MR-deficient leukemia cell lines determined by colony forming ability

Cells were incubated with graded concentrations of Me-Lex for 4 h and then plated at 1 cell/well using a 96-well plate for each drug concentration tested. After 10 days, plates were analyzed for colony forming ability (Tentori et al., 2003). Cell line chemosensitivity to Me-Lex was evaluated in terms of IC<sub>50</sub>, i.e., the concentration of the drug expressed in micromolar, capable of inhibiting colony-forming ability by 50%. The IC<sub>50</sub> was calculated on the regression line in which the number of colonies was plotted versus the drug concentrations. Each value represents the mean of three independent experiments ± S.E.

Cell Line	MR Status	Me-Lex IC <sub>50</sub> μM
TK-6	+	2 ± 0.3
MT-1	—	2.5 ± 0.2
HL-60	+	5.4 ± 0.2
HL-60 R	—	5.0 ± 0.4

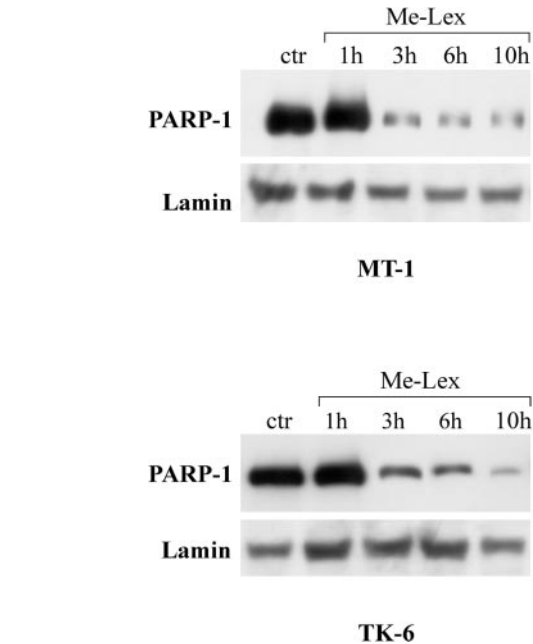


**Fig. 2.** Determination of Me-Lex-induced mitochondrial depolarization by flow cytometry. MT-1 cells were treated with 25 μM Me-Lex (left) or pretreated with 4 mM AB before exposure to 25 μM Me-Lex (right). Cells were then analyzed at the indicated time points for changes in JC-1 dye fluorescence (FL-1, green fluorescence; FL-2, red fluorescence). Percentage numbers in the top left quadrant and right quadrants indicate proportion of cells with normal and depolarized mitochondria, respectively. Representative of three independent experiments.

**PARP-1 Inhibition Counteracts Down-Regulation of NF-κB and Necrosis Induced by Me-Lex.** Pretreatment of MT-1 leukemia cells with two different PARP-1 inhibitors (AB or NU1025) prevented the marked reduction of NF-κB activity induced by Me-Lex 10 h after treatment (Fig. 4B). PARP-1 inhibitor counteracted reduction of NF-κB activity induced by Me-Lex also in TK-6 cells (Fig. 4B).

Under normal conditions, NF-κB is present in the cytoplasm most commonly as a p65/p50 heterodimer that is retained in an inactive state by its association with the inhibitory IκBα protein (Karin et al., 2004). Upon NF-κB activation, IκBα undergoes phosphorylation, ubiquitination, and degradation, thus allowing nuclear translocation of p50/p65 complex. The phosphorylation of IκBα is catalyzed by an IKK complex. We next investigated whether Me-Lex induced down-regulation of constitutive NF-κB activation was caused by inhibition of IKK activity. The results demonstrate a constitutive IKK activity in control MT-1 cells, whereas immunoprecipitated IKK from Me-Lex treated cells showed abrogation of kinase activity that was reversed by pretreatment with the PARP-1 inhibitor AB. Immunoblotting analysis of cell extracts from untreated and drug treated cells showed not significant changes in the protein levels of the IKK subunit (Fig. 4D).

Me-Lex induced necrosis in MT-1 cells, which are endowed with low levels of MPG activity. Pretreatment with PARP-1 inhibitor protected cells from this type of cell death (Tentori et al., 2001). Similar results were obtained in TK-6 cells, which are characterized by comparable levels of MPG activity (MT-1, 153 ± 31 and TK-6, 217 ± 23 fmol/mg). In fact, 25 μM Me-Lex induced necrosis in TK-6 cells as evidenced by PI uptake 10 h after treatment. Necrosis did not seem to be secondary to apoptosis, because at earlier times (1, 3, and 6 h)



**Fig. 3.** Time-course analysis of PARP-1 expression in MT-1 or TK-6 cells after treatment with Me-Lex. Nuclear extracts (10 μg) from untreated MT-1 cells or TK-6 cells exposed to 25 μM Me-Lex were resolved on 8% SDS-PAGE gels, electrotransferred on polyvinylidene difluoride membranes, and probed for PARP-1. The same blots were stripped and re-probed with lamin as nuclear marker protein. Representative of two independent experiments.

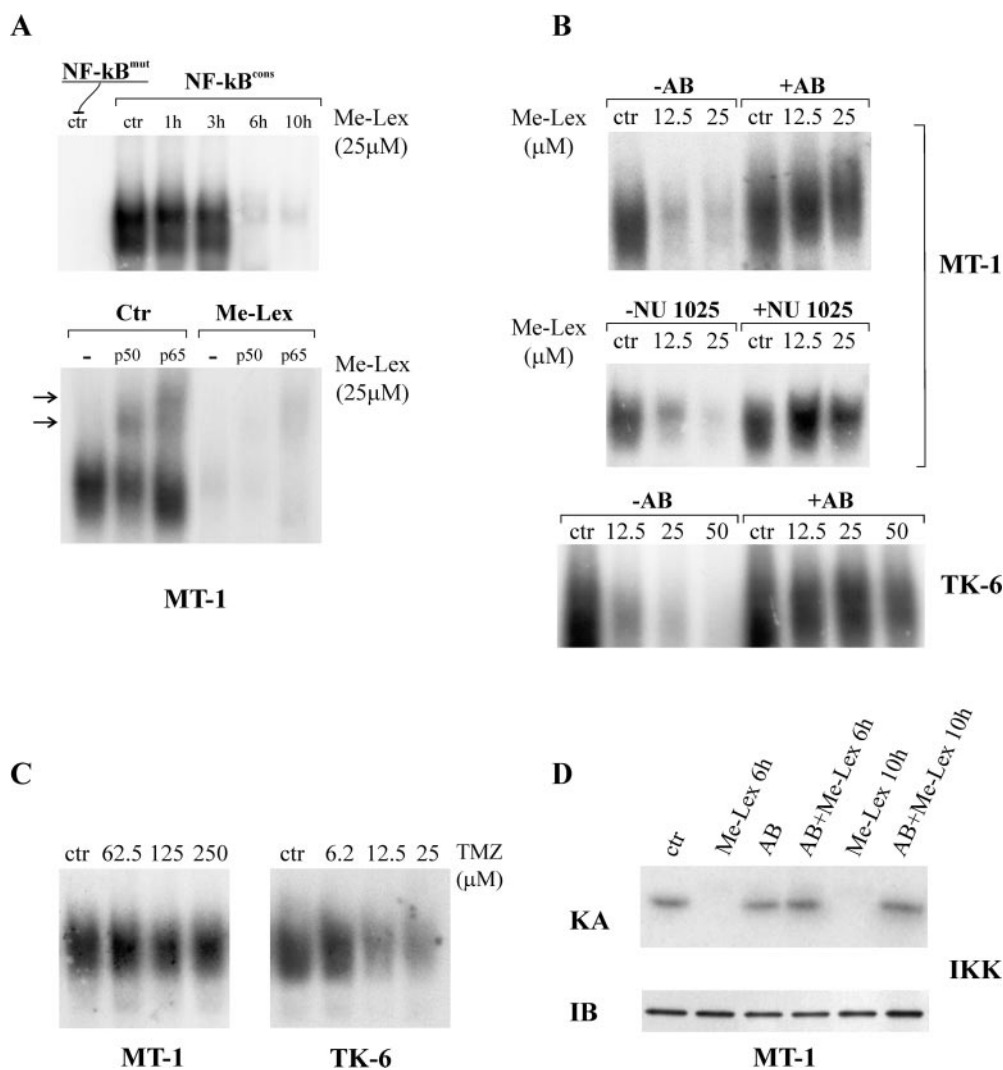
a negligible percentage of cells (>7%) stained positively with annexin V. The percentage of necrotic cells induced by Me-Lex in TK-6 cells dramatically decreased when cells were pretreated with AB (Fig. 5A). However, protection from cell death afforded by the PARP-1 inhibitor was only transient, because cells treated with the drug combination eventually died by apoptosis because of interruption of methylpurine repair caused by PARP-1 inhibition (Tentori et al., 2001). Cleavage of PARP-1 was indeed observed in cells treated with Me-Lex + PARP-1 inhibitor (Fig. 5B). Moreover, treatment with Me-Lex + AB provoked a marked induction of p53 starting from 1 h after treatment (Fig. 5B).

**Me-Lex Differently Modulates Telomerase Activity in the Nuclear and Cytoplasmic Compartment.** Because previous studies have shown that telomerase, a ribonuclear protein involved in telomere elongation and cell survival, can be modulated by DNA damage, we have investigated whether treatment with Me-Lex might affect telomerase function. Kinetics analysis of telomerase activity, using total cell extracts (corresponding to equal number of cells) prepared from MT-1 cells untreated or exposed to Me-Lex (25  $\mu$ M), revealed a consistent decrease of telomeric ladder (41% reduction with respect to untreated control) as early as 3 h after treatment. This reduction reached about 90% at 10 h

(Fig. 6A). At this time, in untreated MT-1 cells telomerase activity was detectable in as low as 25 ng of total cell extract, whereas in cells treated with 25  $\mu$ M Me-Lex for 10 h telomerase was not observed even at a 10-fold higher protein concentration. However, it should be noted that in drug-treated cells, telomerase activity could be detected starting from a protein concentration of 500 ng and higher (data not shown). A dose-dependent down-regulation of telomerase activity also was observed in total cell extracts from TK-6 cells 10 h after drug exposure (Fig. 6A). At 24 h after treatment with Me-Lex, telomerase activity was no longer detected even at high protein concentration (Tentori et al., 2001). High concentrations of TMZ instead did not induce a substantial reduction of telomerase in MT-1 cells 10 h after treatment (data not shown).

All samples showed comparable levels of alkaline phosphatase activity (data not shown). This enzyme possesses stability similar to that of telomerase, thus serving as an internal control for the quality of the extracts.

We then analyzed whether DNA damage induced by Me-Lex might differently affect subcellular localization of telomerase activity. The results of TRAP assay in MT-1 cells indicate that the level of telomerase activity in cytoplasmic extracts of untreated cells was 2-fold higher than that



**Fig. 4.** Treatment with Me-Lex induces reduction of NF- $\kappa$ B and IKK kinase activity. A, MT-1 cells were treated with 25  $\mu$ M Me-Lex. Nuclear extracts (5  $\mu$ g) were incubated with  $^{32}$ P end-labeled double-stranded NF- $\kappa$ B consensus or mutated oligonucleotides and subjected to EMSA at the indicated times. Antibody mediated supershift analysis was carried out by including antibodies (1  $\mu$ g) against p50 or p65 subunits of NF- $\kappa$ B in the binding reaction of nuclear extracts from untreated MT-1 cells or cells treated with 25  $\mu$ M Me-Lex for 10 h. Arrows indicate supershifted bands. B, MT-1 or TK-6 cells were treated with graded concentration of Me-Lex, as single agent or in combination with 4 mM AB or 25  $\mu$ M NU1025. Nuclear extracts were prepared 10 h after treatment and analyzed by EMSA. C, influence of treatment with TMZ on DNA binding activity of NF- $\kappa$ B in susceptible TK-6 or resistant MT-1 cells. Nuclear extracts (5  $\mu$ g) from TK-6 or MT-1 cells, untreated or treated with the indicated concentrations of TMZ, were incubated with  $^{32}$ P end-labeled double-stranded NF- $\kappa$ B consensus oligonucleotides and subjected to EMSA 48 h after drug exposure. D, total cell extracts were prepared from MT-1 cells untreated or treated with 4 mM AB or with 25  $\mu$ M Me-Lex  $\pm$  4 mM AB for the indicated times. The IKK protein was immunoprecipitated and the kinase assay (KA) was performed. Immunoprecipitated IKK protein was visualized by immunoblot (IB) in all samples.



present in nuclear extracts (Fig. 6A). Me-Lex induced a dose-dependent reduction of telomeric ladder only in the cytosolic compartment 10 h after drug exposure. It is surprising that the drug induced a 2- to 4-fold increase of telomerase activity in the nuclear fraction at 12.5 and 25  $\mu$ M, respectively (Fig. 6A).

Kinetics analysis of telomerase activity revealed that the increase of telomerase activity induced by Me-Lex in the nuclear extracts was detected starting from 6 h after treatment (Fig. 6B). A complete abrogation of telomerase was

observed at later times (i.e., 18 h; data not shown). PARP-1 inhibition counteracted the reduction of telomerase in total cell extracts or in the cytosolic compartment, slightly affecting the increase in the nuclear fraction induced by Me-Lex (Fig. 6B).

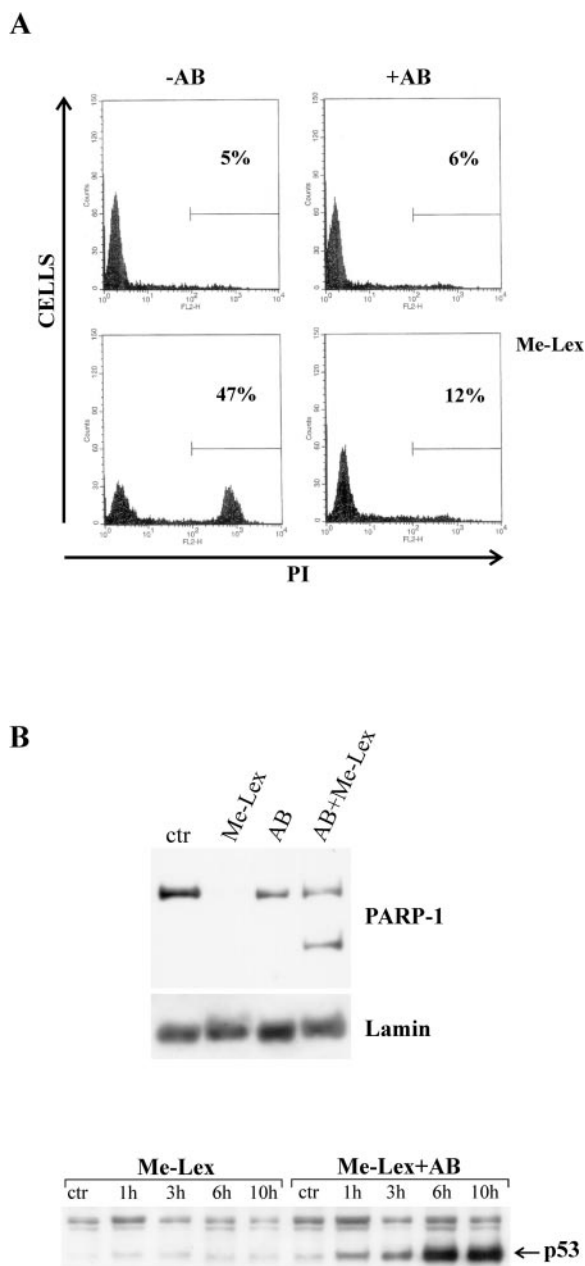
Detection of a profound telomerase reduction in total cell extracts seemed to be in contrast with the increase of activity detected in the nuclei of Me-Lex-treated cells. Therefore, we have investigated whether the decrease of telomerase activity observed in total cell extracts was caused by the loss of nuclear components, which would have masked up-regulation of nuclear telomerase. To this end, we assayed telomerase activity in the pellet obtained after high-speed centrifugation of total cell lysate. After removing supernatant, the residual pellet was solubilized with the nuclear extraction buffer present in the commercial fractionation kit (Medical and Biological Laboratories). The results of TRAP assay showed a 2-fold increase of the telomeric ladder in the pellet of drug-treated cells with respect to that of untreated control, both in MT-1 and TK-6 cells (data not shown). Therefore, the pellet probably contained nuclear-associated proteins, including telomerase. These results suggest that telomerase reduction observed in total cell extracts substantially reflected decrease of telomerase in the cytosolic compartment.

## Discussion

Herein, we demonstrate for the first time that Me-Lex, a compound capable of generating >99% N3-MeA adducts, induced a prompt activation of PARP-1, loss of mitochondrial membrane potential, and a decrease in nuclear PARP-1 expression and NF- $\kappa$ B activity. Moreover, appearance of DNA fragmentation was followed by up-regulation of telomerase in the nuclear compartment with a concomitant decrease of activity in the cytosol.

In addition to being involved in DNA repair as a component of BER, PARP-1 also acts as mediator of necrotic cell death (Ha and Snyder, 1999). In fact, extensive activation of PARP-1 and consequent consumption of NAD<sup>+</sup> during the synthesis of poly(ADP-ribose) lead to ATP depletion in the effort to resynthesize the nicotinamide derived dinucleotide. In the leukemia cell line TK-6, and in its subline MT-1, Me-Lex provoked a strong activation of PARP-1. These cell lines are both characterized by low MPG activity and similar susceptibility to necrosis induced by Me-Lex. Sensitivity to Me-Lex is not influenced by the functional status of MR because the methylating agent induced comparable growth inhibition in MR-deficient MT-1 or MR-proficient TK-6 and in other leukemia cell lines (i.e., HL60 and its HL-60 R subline) that differ in MR status.

Cell death provoked by Me-Lex was preceded by loss of mitochondrial membrane potential and ROS generation. Pre-treatment with PARP-1 inhibitors blocked ADP-ribose polymer formation, preserved mitochondrial membrane integrity, and prevented necrosis. These results strongly suggest that necrosis was the result of PARP-1 overactivation. Our findings are consistent with those reported with high concentrations of the wide-spectrum methylating agents MNNG or MNU in different model systems (Mizumoto and Farber, 1995; Ha and Snyder, 1999; Yu et al., 2002). Thus, it is conceivable to hypothesize that N3-MeA is the DNA lesion



**Fig. 5.** Treatment with PARP-1 inhibitor + Me-Lex protects leukemia cells from necrosis, induces PARP-1 cleavage and p53 stabilization. **A**, TK-6 cells were exposed to 25  $\mu$ M Me-Lex  $\pm$  4 mM AB and analyzed by flow cytometry 10 h after treatment. Unfixed cells were washed, suspended in PBS and labeled with 2  $\mu$ g/ml PI. Percentages of PI-positive necrotic cells are indicated. Representative of three independent experiments. **B**, MT-1 cells were treated with 25  $\mu$ M Me-Lex  $\pm$  4 mM AB and analyzed for PARP-1 cleavage 10 h after treatment or for p53 induction at the indicated times by Western blot analysis. Representative of two independent experiments. Similar results were obtained in TK-6 cells (data not shown).

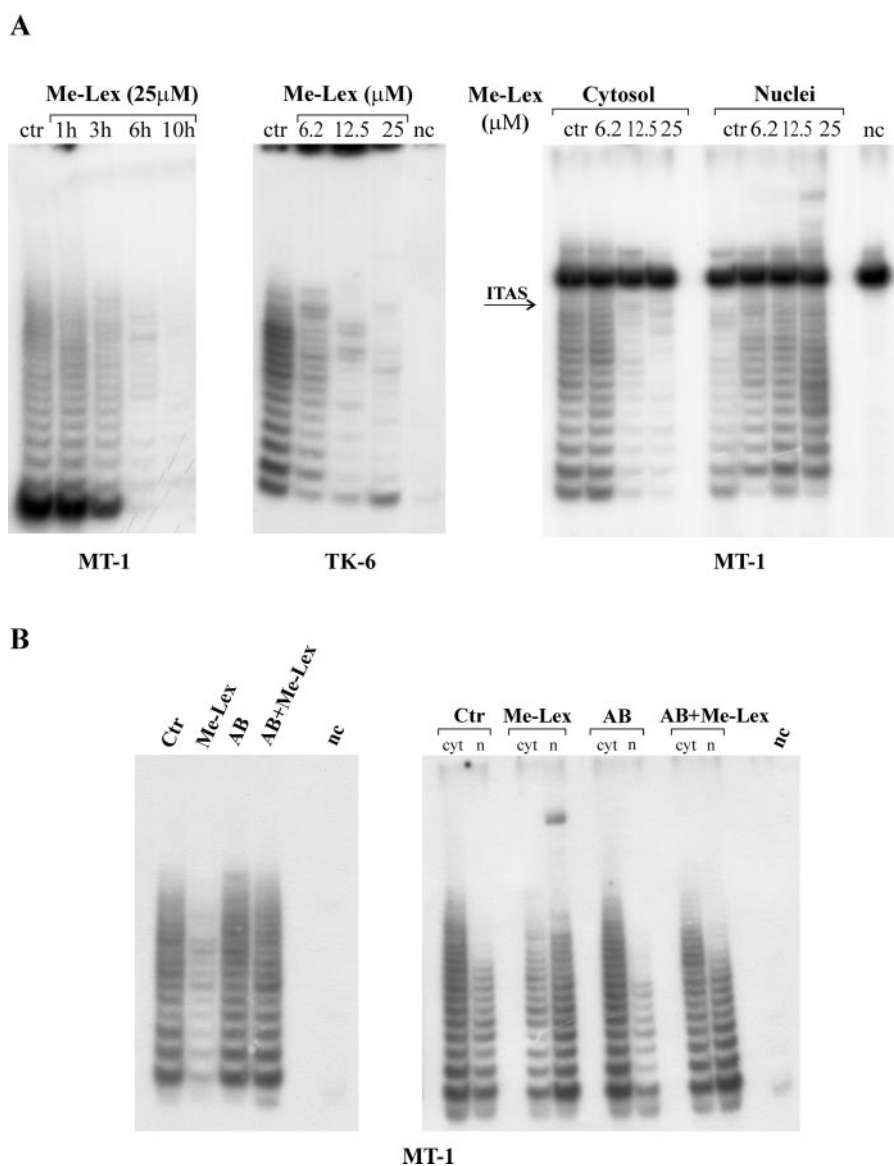
responsible of PARP-1 activation and cytotoxicity also in the case of high doses of MNNG and MNU.

It has been recently demonstrated that mitochondrial membrane depolarization associated with cell death signaling induced by PARP-1 activation leads to AIF nuclear translocation, which in turn triggers chromatin condensation independently of caspases (Yu et al., 2002; Alano et al., 2004). In cell death induced by Me-Lex, high molecular weight DNA fragmentation was evident 3 h after treatment in the absence of AIF nuclear translocation. These data indicate that cytotoxicity mediated by Me-Lex is unlikely to be the result of AIF-dependent apoptosis, whereas it resembles necrosis. In fact, high molecular weight fragmentation has been associated with either apoptotic or necrotic cell death (Kataoka et al., 1995; Slagsvold et al., 2003).

Activation of PARP-1 by Me-Lex occurring 15 to 30 min after drug exposure, was followed a few hours later by a marked decrease in nuclear PARP-1 expression and NF- $\kappa$ B activity. PARP-1 has been shown to act as NF- $\kappa$ B coactivator through a direct protein-protein interaction with both p50 and p65 subunits (Hassa et al., 2001, 2003). Indeed, PARP-

1-deficient cells are defective in NF- $\kappa$ B-dependent transcription activation in response to inflammatory stimuli (Oliver et al., 1999). Because of the functional link existing between PARP-1 and NF- $\kappa$ B it is conceivable that the decrease of PARP-1 protein in the nucleus, which occurs in Me-Lex-treated cells undergoing necrosis, might induce an impairment of the DNA binding activity of NF- $\kappa$ B complexes. However, it cannot be excluded that Me-Lex might directly affect NF- $\kappa$ B ability to interact with DNA.

Modulation of NF- $\kappa$ B has been implicated in the cellular response to genotoxic damage. Inhibition of this transcription factor would promote cell death, whereas NF- $\kappa$ B activation after treatment with anticancer drugs is generally regarded as a hallmark of tumor resistance (for review, see Karin et al., 2004). In fact, the leukemia lines used in this study express high basal levels of NF- $\kappa$ B activity in accordance with the observation that this transcription factor is constitutively activated in leukemic progenitors (Zaninoni et al., 2003; Birkenkamp et al., 2004). In both TK-6 and MT-1 cells, Me-Lex induced an early and dose-dependent decrease of NF- $\kappa$ B DNA binding activity that paralleled the cytotoxic



**Fig. 6.** Differential modulation of telomerase activity induced by Me-Lex in nuclear and cytoplasmic compartments. **A**, analysis of telomerase activity in total cell extracts prepared from MT-1 (left) or TK-6 cells (middle), untreated or exposed to Me-Lex (MT-1, 1–10 h; TK-6, 10 h). Telomerase activity was tested by TRAP assay using total cell extracts corresponding to equal number of cells ( $2 \times 10^3$ ). As negative control (nc), cell extract was replaced by an equal volume of lysis buffer. ODs were MT-1 untreated control, 4250; 1 h Me-Lex, 4200; 3 h Me-Lex, 2898; 6 h Me-Lex, 1232; 10 h Me-Lex, 383; TK-6 untreated control, 5854; 6.2  $\mu$ M Me-Lex, 3498; 12.5  $\mu$ M Me-Lex, 1358; and 25  $\mu$ M Me-Lex, 1071. Cytosolic and nuclear extracts (right) corresponding to equal number of MT-1 cells ( $2 \times 10^3$ ) were analyzed for telomerase activity by TRAP assay 10 h after treatment with the indicated concentrations of Me-Lex. Arrow points to an internal telomerase assay standard (ITAS). Representative of two independent experiments. ODs for cytoplasmic extracts were untreated control, 7693; 6.2  $\mu$ M Me-Lex, 7578; 12.5  $\mu$ M Me-Lex, 2990; and 25  $\mu$ M Me-Lex, 2048. ODs for nuclear extracts were untreated control, 3620; 6.2  $\mu$ M Me-Lex, 5437; 12.5  $\mu$ M Me-Lex, 6132; and 25  $\mu$ M Me-Lex, 7701. **B**, analysis of telomerase activity in total cell extracts (left) or in cytosolic and nuclear fractions (right) of MT-1 cells, untreated or exposed to 25  $\mu$ M Me-Lex  $\pm$  AB 6 h after treatment. ODs for total extracts were untreated, 5082; Me-Lex, 1973; AB, 5308; and AB + Me-Lex, 5290. ODs for cytoplasmic extracts were untreated, 5683; Me-Lex, 2682; AB, 5670; and AB + Me-Lex, 4699. ODs for nuclear extracts were untreated, 2200; Me-Lex, 4617; AB, 1671; and AB + Me-Lex, 3881.



effect. Me-Lex also inhibited the activity of IKK complex, the kinase that phosphorylates I $\kappa$ B $\alpha$  thereby allowing the release and consequent nuclear translocation of NF- $\kappa$ B (Karin et al., 2004). This might hamper further recruitment of NF- $\kappa$ B protein to the nucleus and contribute, together with NF- $\kappa$ B down-regulation, to silence gene transcription in cells undergoing necrosis (Fig. 7). In contrast, the wide-spectrum methylating agent TMZ, which requires an intact MR function for O<sup>6</sup>-MeG mediated toxicity, provoked a delayed down-regulation of NF- $\kappa$ B activity only in TK-6 cells in concomitance with the appearance of cytotoxicity.

It has been previously reported that the enzymatic activity of PARP-1 is dispensable for its function as NF- $\kappa$ B coactivator (Hassa et al., 2001). Inhibition of PARP-1 did not affect basal NF- $\kappa$ B activity of leukemia cells but prevented the reduction of NF- $\kappa$ B activity induced by Me-Lex. This was probably the consequence of protection from necrosis afforded by PARP-1 inhibitor. It should be noted that combined treatment with Me-Lex + AB was accompanied by p53 induction and PARP-1 cleavage. Despite the fact that leukemia cells treated with Me-Lex + PARP-1 inhibitor were committed to die via apoptosis, this cell death modality was not accompanied by down-regulation of NF- $\kappa$ B, at least within the time frame investigated in this study. Apoptosis induced by the drug combination is probably the result of strand breaks deriving from the interruption of BER process provoked by PARP-1 inhibition (Tentori et al., 2001).

In the present study, we show that N3-MeA is capable of signaling a prompt increase, even though transient, of telomerase activity in the nucleus. This is probably the consequence of nuclear translocation of the enzyme from the cytoplasm because the reduction of activity detected in this compartment paralleled up-regulation of telomerase in the nucleus. Telomerase is a ribonuclear protein devoted to the maintenance of telomeres, the specialized structures located at the ends of chromosomes. Although elongation of telomeres requires nuclear localization of telomerase, functional telomerase has been found both in the nucleus and cytoplasm of cancer cells (Akiyama et al., 2003; Kyo et al., 2003). It is interesting that in untreated MT-1 and TK-6 cell lines, expression of telomerase was higher in the cytosol with respect to the nuclear compartment.

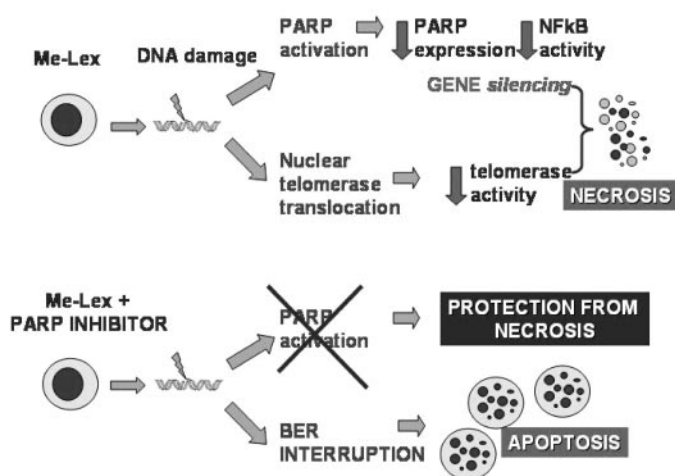


Fig. 7. Schematic drawing of PARP-1 involvement in cell death pathways induced by Me-Lex as single agent or combined with PARP-1 inhibitor.

Beside being involved in cellular immortalization, telomerase seems to play a role also in cellular response to DNA damage and repair (Shin et al., 2004). In fact, up-regulation of total cellular telomerase followed by a decline of activity at late stages of cell death was previously reported in tumor cells treated with the topoisomerase II inhibitor etoposide (Sato et al., 2000; Moriarty et al., 2002; Klapper et al., 2003). Telomerase up-regulation also was detected in cells exposed to radiation, indicating an involvement in chromosome healing (Neuhof et al., 2001). The molecular mechanisms inducing telomerase nuclear translocation in Me-Lex-treated cells have yet to be identified. However, because Me-Lex is capable of inducing chromosome damage in MT-1 cells (Tentori et al., 2000), it can be hypothesized that broken chromosomes might mimic dysfunctional telomeres and recruit telomerase activity in the nucleus in an attempt to repair the damage.

Inhibition of PARP-1 prevented reduction of telomerase in total cellular extract and cytosolic compartment, as a consequence of protection from necrosis. However, treatment with AB slightly affected nuclear up-regulation of telomerase induced by Me-Lex. These results suggest that the DNA modifications, which follow treatment with Me-Lex, might trigger the increase of telomerase in the nucleus independently on PARP-1 activity.

It has been recently demonstrated that NF- $\kappa$ B, beside acting as transcription regulator of human telomerase reverse transcriptase (Yin et al., 2000), is also capable of directly interacting with human telomerase reverse transcriptase through its p65 subunit, allowing nuclear translocation of telomerase upon a proliferative stimulus (Akiyama et al., 2003). The results presented here indicate that up-regulation of nuclear telomerase induced by Me-Lex takes place concomitantly with the drug-induced decrease of NF- $\kappa$ B, excluding an involvement of NF- $\kappa$ B in nuclear translocation of telomerase. Therefore, other still unknown mechanisms, independent on the transcription factor and triggered by DNA damage, are probably responsible for telomerase shuttling to the nucleus.

In conclusion, the present study provides new insight in tumor cell response to N3-MeA, which might represent a novel target for anticancer drugs to be exploited for the treatment of tumors unresponsive to classical wide-spectrum methylating agents. Moreover, these results underline the central and paradoxical role of PARP-1 in cell death induced by N3-MeA. In fact, although inhibition of PARP-1 protects from necrosis, it also triggers apoptosis as a result of inhibition of methylpurine repair mediated by BER (Fig. 7).

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