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ORIGINAL ARTICLE

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Apoptotic and genotoxic effects of a methyl sulfonate ester that selectively generates N3-methyladenine and poly(ADP-ribose) polymerase inhibitors in normal peripheral blood lymphocytes

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Abstract Selective N3-adenine methylation represents a novel strategy for tumors with a phenotype of poor responsiveness to a number of anticancer agents currently used in the clinic. Resistance to N3-methyladenine-inducing agents, such as $MeOSO_2(CH_2)_2$-lexitropsin (Me-Lex), is due to high levels of N-methylpurine glycosylase (MPG). However, tumor cells with high MPG activity can be rendered susceptible to Me-Lex using poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors. *Purpose:* To evaluate the potential toxicity of Me-Lex, used as single agent or combined with PARP-1 inhibitors, in normal peripheral blood lymphocytes (PBL). *Methods:* PBL either resting or activated with phytohemagglutinin (PHA), obtained from healthy donors, were treated with graded concentrations of Me-Lex with or without PARP-1 inhibitor (3-aminobenzamide, AB, or NU1025, NU). MPG activity, apoptosis and sister chromatid exchanges (SCE) were evaluated. *Results:* (a) Me-Lex was cytotoxic mainly in PHA-activated PBL with low MPG activity; (b) combined treatment with Me-Lex and AB induced apoptotic effects as early as 24 h after drug exposure both in non-stimulated and PHA-activated PBL. When concentrations of PARP-1 inhibitors (25 μ*M* NU and 4 m*M* AB) that produced a twofold increase in Me-Lex cytotoxicity in tumor cells were compared, NU induced a less-pronounced increase in apoptosis in PBL treated with Me-Lex; (c) Me-Lex at concentrations that allowed cytogenetic analysis did not induce a significant number of SCE; (d) PARP-1 inhibitors provoked a dose-dependent increase in SCE, but 25 μ*M* NU was devoid of genotoxic effects and did not significantly increase SCE in PBL treated with Me-Lex. *Conclusions:* Me-Lex showed preferential cytotoxicity against mitogen-activated PBL. Our results also indicated that for each PARP-1 inhibitor it is necessary to define the concentration devoid of genotoxic effects in normal cells, but still capable of enhancing the efficacy of DNA-damaging agents in tumor cells.

Keywords Methylating agents · Poly(ADP-ribose) · Polymerase-1 inhibitors · Peripheral blood lymphocytes · N-methylpurine glycosylase

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Introduction

Selective N3-adenine (N3-A) methylation represents a novel chemotherapeutic strategy for tumors with a phenotype of drug resistance due to functional defects in the mismatch repair system and/or to high levels of O^6-alkylguanine-DNA alkyltransferase (OGAT) [28, 31]. These repair systems are the principal determinants influencing resistance of tumor cells to methylating agents that are cytotoxic through the formation of O^6-methylguanine adducts (e.g. temozolomide, TZM) [18, 23, 25]. Moreover, functional alterations in the mismatch repair system are also involved in resistance to a number of other antineoplastic agents with mechanisms of action distinct from DNA methylation [14].

A methylating agent that generates almost exclusively N3-A methyl adducts (N3-MeA) is $MeOSO_2(CH_2)_2$-lexitropsin (Me-Lex), a methyl sulfonate ester tethered to *N*-methylpyrrolecarboxamide dipeptide that targets A/T-rich sequences located in the DNA minor groove [12, 19, 35]. N3-MeA is an extremely toxic DNA lesion,

capable of inducing cell death and chromosome aberrations in human tumor cells or in normal murine embryonic stem cells with low levels of 3-methylpurine DNA glycosylase (MPG) [13, 28]. This enzyme catalyses the N-methylpurine excision that is the first step in the base excision repair (BER) process [34]. The BER system is a multiprotein complex in which a central role is played by poly(ADP-ribose) polymerase-1 (PARP-1). In fact, this enzyme acts as sensor of the nicks generated after the removal of the methylpurines, and coordinates the intervention of the different BER components involved in the DNA repair process [6, 7].

Tumor cells with high levels of MPG activity can be rendered susceptible to Me-Lex through the use of PARP-1 inhibitors [28]. In this case, the damage is due to the strand breaks generated by the interruption of the BER process after the removal of the methylated nucleotides by the concerted action of MPG and apurinic/apyrimidinic endonucleases.

The aim of the present study was to evaluate the potential cytotoxic and genotoxic effects of Me-Lex, used as single agent or combined with PARP-1 inhibitors, in normal peripheral blood lymphocytes (PBL). The results indicated that Me-Lex induced cytotoxic effects in mitogen-activated lymphocytes, especially in those characterized by low levels of MPG activity. Conversely, interruption of the N3-MeA repair process through the use of PARP-1 inhibitors provoked apoptosis in both resting and activated lymphocytes. Moreover, Me-Lex at concentrations that allowed chromosome analysis did not generate sister chromatid exchanges (SCE). PARP-1 inhibitors with different specificity and potency [1, 15, 16], used at concentrations (millimolar range for 3-aminobenzamide, AB; micromolar range for NU1025, NU) that equally enhanced tumor cell susceptibility to methylating agents, showed different abilities to induce SCE.

Materials and methods

Cell culture

T-lymphoblastic leukemia Jurkat cells were cultured in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Gibco), 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories, McLean, Va.) at 37°C in a humidified atmosphere containing 5% CO_2.

PBL were obtained from healthy donors using a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). PBL (1×10^6/ml) were cultured in the medium described above, with or without 4 μg/ml phytohemagglutinin (PHA; Sigma, St Louis, Mo.).

Drugs

$MeOSO_2(CH_2)_2$-*N*-methylpyrrole dipeptide [$MeOSO_2(CH_2)_2$-lexitropsin, Me-Lex] was prepared as previously described [35]. TZM was kindly provided by Schering-Plough Research Institute (Kenilworth, N.J.). The PARP-1 inhibitor AB was purchased from Sigma, whereas the more specific and potent PARP-1 inhibitor NU (8-hydroxy-2-methylquinazolin-4[3H]-one) was obtained from Calbiochem (Darmstadt, Germany). Drug stock solutions were prepared by dissolving TZM and NU in dimethyl sulfoxide (DMSO) (100 m*M* and 25 m*M*, respectively), Me-Lex in 95% ethanol (10 m*M*) and AB in RPMI-1640 (16 m*M*). The final concentration of DMSO or ethanol in drug-treated cultures was always less than 0.5% (v/v) and did not contribute to toxicity (data not shown).

Drug treatment and cell growth evaluation

Inhibition of PARP-1 was obtained by treating the cells with 4 m*M* AB or 25 μ*M* NU, concentrations that have been reported to abrogate PARP-1 activity [1]. In selected experiments, graded concentrations of PARP-1 inhibitors were also tested (AB 0.5–4 m*M*, NU 12.5–100 μ*M*). Me-Lex (1.5–25 μ*M*) or TZM (62.5–250 μ*M*) were added to cell cultures immediately after AB or NU.

Jurkat cells were cultured in flasks (Falcon, Becton Dickinson Labware, Oxnard, Calif.) at a concentration of 3×10^5 cells/ml; resting or mitogen-activated PBL were cultured at a concentration of 1×10^6 cells/ml. Drug treatment of mitogen-activated PBL was performed 3 h after PHA exposure. Cell numbers were evaluated every 24 h by counting viable cells in quadruplicate. Cell viability was determined by trypan blue dye exclusion.

Assessment of apoptosis by flow cytometric analysis

Cells were harvested, washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at –20°C for 18 h. After centrifugation, cells were resuspended in 1 ml hypotonic solution containing 50 μg/ml propidium iodide (PI, Sigma), 0.1% sodium citrate, 0.1% Triton-X, and 10 μg/ml RNase (Boehringer Mannheim, Milan, Italy), and incubated at 37°C in the dark for 15 min. Data collection was gated utilizing forward light scatter and side light scatter to exclude cell debris and aggregates. PI fluorescence was measured on a linear scale using a FACSscan flow cytometer (Becton Dickinson, San Jose, Calif.). Apoptotic cells are represented by a broad hypodiploid peak, which is easily distinguishable from the narrow peak of cells with diploid DNA content in the red fluorescence channel.

Western blotting

Cell lysates and samples for electrophoresis were prepared as previously described [27]. Protein (80 μg per sample) was electrophoresed in 8% or 12% SDS-polyacrylamide mini-gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Equal protein loading was visualized by Ponceau S staining. Filters were blocked with blocking buffer (Boehringer Mannheim) and incubated with the monoclonal antibody directed against p53 (PAb1801) (Oncogene, Cambridge, Mass.) or with the monoclonal antibody against PARP-1 (C-2-10; Clontech, Palo Alto, Calif.), which allows detection of the native protein (116 kDa) and the apoptosis-related cleavage product (85 kDa). Immune complexes were visualized using a chemiluminescence kit (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions. Filters were exposed to X-OMAT AR autoradiographic films (Kodak, Rochester, N.Y.) for 10–45 s depending on the intensity of the signal.

Measurement of MPG activity

MPG activity was assayed as previously described [30, 32]. PBL (2×10^7) were sonicated at 4°C in 0.5 ml buffer I (50 m*M* Tris-HCl, 3 m*M* dithiothreitol, and 2 m*M* EDTA, pH 8.3) with freshly added 1 m*M* 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride. After removal of cell debris by centrifugation at 15,000 rpm at 4°C for 10 min, the supernatants were immediately tested for MPG activity. Various amounts of cell extract were incubated with 10 μg (10,000 cpm) of freshly dissolved calf thymus DNA methylated by

N-[^{3}H]-methyl-N-nitrosourea (19 Ci/mmol, Amersham) in a total volume of 100 µl buffer II (20 m*M* Tris-HCl, 1 m*M* dithiothreitol, 60 m*M* NaCl, and 1 m*M* EDTA, pH 8). After 1 h at 37°C, the reaction was stopped on ice by the addition of 30 µl 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 10,000 *g* for 15 min. The supernatants in 300-µl aliquots were transferred to a scintillation tube and counted. The MPG activity was determined for protein and time-limiting conditions and expressed as femtomoles of methyl purines released per 10^6 cells.

OGAT assay

OGAT activity was determined as previously described [33]. Cells were washed twice with PBS and stored as pellets (1×10^6 cells) at –80°C until used. Cells were sonicated in 0.5 ml buffer I and centrifuged as described above for the MPG assay. Supernatants were used for the assay and OGAT activity was determined by measuring the transfer of [^{3}H]-methyl groups from calf thymus DNA methylated by N-[^{3}H]-methyl-N-nitrosourea to the OGAT protein. The OGAT activity was expressed as femtomoles of methyl groups per 10^6 cells.

Analysis of chromosome aberrations and SCE

Mitogen-activated PBL, untreated or drug-treated, were incubated 1 h after drug exposure with 5-bromo-2′-deoxyuridine (BrdU, Sigma) at a final concentration of 0.5 µ*M* for 72 h. Colchicine (0.8 µg/ml, Sigma) was added to the cultures 2 h before harvesting. Metaphase preparations were obtained according to standard techniques, treating cells first with a hypotonic solution (KCl 0.075 *M*) and then with a fixative mixture of methanol/acetic acid (3/1). Air-dried slides were stained with 5% Giemsa in phosphate buffer for breakage evaluation.

For SCE evaluation, slides were also stained according to the method of Perry and Wolff [26]. SCE were generally scored on 30 metaphases. Statistical analysis was performed using the Mann-Whitney test.

Results

PARP-1 inhibitors increase apoptosis in resting or PHA-activated human PBL treated with Me-Lex

Non-stimulated or PHA-activated PBL were treated with graded concentrations of Me-Lex (1.5–25 µ*M*) as a single agent or combined with the PARP-1 inhibitor AB. The latter agent was used at a concentration (4 m*M*) previously reported to potentiate cytotoxicity of Me-Lex and of other antineoplastic agents [15, 27, 28]. After 24 h of culture, cells were harvested and tested for apoptosis induction. The results (Fig. 1) represent the mean of nine independent experiments performed with PBL derived from nine different healthy donors. The data indicate that at 24 h Me-Lex induced limited apoptotic effects, mainly in PHA-activated PBL, at 12.5 and 25 µ*M*. In contrast, addition of AB markedly increased apoptosis either in non-stimulated or PHA activated PBL. Apoptosis triggered by the drug combination was accompanied by PARP-1 cleavage and p53 induction (Fig. 2).

For comparison, the same samples were treated with TZM, that generates low levels of N3-MeA adducts (9% of total methyl adducts). The results of the flow cytometric analysis indicated that treatment with 250 µ*M* TZM as a single agent induced apoptotic effects only in PHA-activated PBL at 72 h after drug exposure (Fig. 3). In the case of resting PBL, TZM did not induce apoptosis at any drug concentration tested (data not shown). This is consistent with the view that apoptosis induced by O^6-methyl guanine requires DNA post-replicative intervention of mismatch repair [8]. Furthermore, cell extracts obtained from the PBL analyzed showed comparable OGAT levels (activity range 31–37 fmol/10^6 cells), thus explaining the similar susceptibility to TZM detected in these samples.

In PHA-activated cells, AB induced only a modest increase in apoptosis in cells exposed to 250 µ*M* TZM at 24 h (Fig. 3). Combined treatment of resting PBL with TZM and AB did not significantly increase apoptosis (data not shown).

Inhibition of PARP-1 activity was also accomplished using NU, which is regarded as a more specific and potent PARP-1 inhibitor than AB. Our previous studies had shown that a comparable potentiation of TZM cytotoxicity can be achieved with 25 µ*M* NU and 4 m*M* AB [27, 30]. In order to define whether this also might occur for Me-Lex cytotoxicity in tumor cells, MPG-proficient leukemia Jurkat cells were treated with a fixed concentration of Me-Lex (12.5 *M*), as a single agent or combined with graded concentrations of AB or NU. The results of cell growth analysis are expressed in terms of the ratio between the percentage growth inhibition detected in cells treated with the drug combination and that observed in cells exposed to Me-Lex only. The data indicate that 25 µ*M* corresponded to the minimal NU concentration that is devoid of growth-inhibitory effects (data not shown) and capable of increasing by twofold

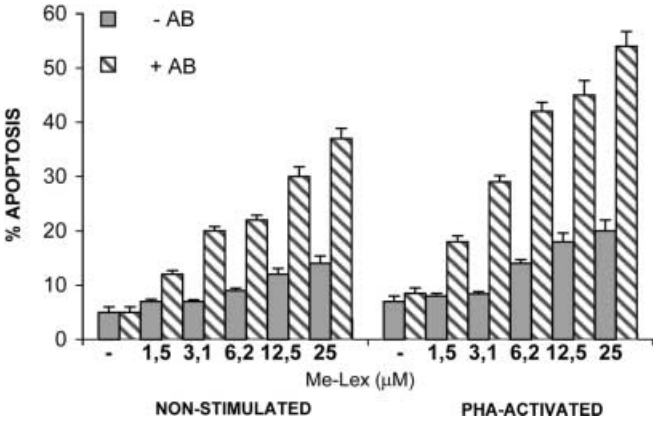

Fig. 1 Me-Lex plus PARP-1 inhibitor induces early apoptosis in resting and mitogen-activated PBL. Non-stimulated or PHA-activated PBL from nine healthy donors were treated with graded concentrations of Me-Lex as a single agent (*solid columns*) or combined with 4 m*M* AB (*hatched columns*). Data are percentages of apoptotic cells evaluated by flow cytometry at 24 h after treatment, and represent the mean of nine independent experiments +SE (*bars*) calculated following angular transformation of the percentage values. Differences between the percentage of apoptotic cells induced by Me-Lex plus AB and those induced by Me-Lex only were all statistically significant ($P<0.01$, Student's *t*-test)

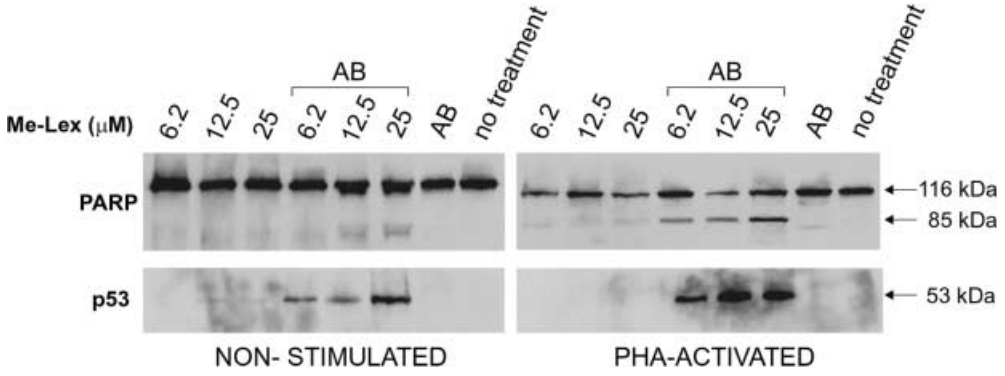

Fig. 2 Me-Lex plus AB induces PARP-1 proteolytic cleavage and p53 induction. *Upper panel* Immunoblotting of PARP-1 protein. Arrows point to the 116-kDa native protein and to the 85-kDa apoptotic fragment. *Lower panel* Immunoblotting of p53 protein. The expression of PARP-1 and p53 were evaluated 24 h after treatment. Equal loading of proteins was checked by red S Ponceau staining (data not shown). The data are representative of one of two different experiments with comparable results

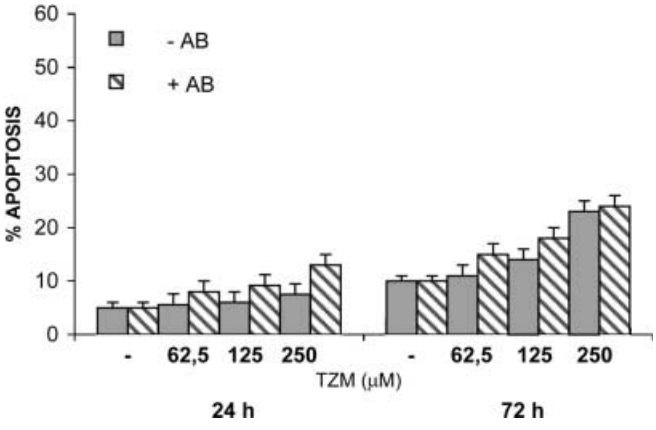

Fig. 3 Analysis of apoptosis in PBL treated with TZM with or without PARP-1 inhibitor. PHA-activated PBL from six healthy donors were treated with graded concentrations of TZM as a single agent (*solid columns*) or combined with 4 m*M* AB (*hatched columns*). Data are percentages of apoptotic cells evaluated by flow cytometry at 24 and 72 h after treatment, and represent the mean of six independent experiments +SE (*bars*), calculated following angular transformation of the percentage values

the cytotoxicity of Me-Lex. This concentration of NU was comparable to 4 m*M* AB (Fig. 4). Moreover, no further increase in Me-Lex cytotoxicity was observed when NU concentrations higher than 25 μ*M* were tested.

On the basis of these results, PHA-activated human PBL were treated with graded concentrations of Me-Lex as a single agent or combined with 4 m*M* AB or 25 μ*M* NU. The results of the flow cytometric analysis performed at 24 h after treatment demonstrated that in Me-Lex-treated PBL, NU induced a less-pronounced increase in apoptosis than AB (Fig. 5).

Cytotoxicity of Me-Lex used as single agent is more evident in PHA-stimulated PBL with low MPG activity

PBL obtained from healthy donors were tested for MPG activity, and among the samples analyzed, three PBL samples with low MPG activity (2.5–13 fmol/10^6 cells) and three with high MPG activity (50–67 fmol/10^6 cells) were selected and exposed to Me-Lex. PBL were analyzed for apoptosis induction at 24 and 48 h after treatment. In PHA-activated PBL Me-Lex concentrations ranging between 1.5 and 6.2 μ*M* induced limited apoptotic effects at 24 h after treatment even in PBL with low MPG activity (data not shown). After 48 h of culture, the methylating agent was more cytotoxic in cells with low MPG activity than in those characterized by high enzymatic activity (Fig. 6). Differences in PBL sensitivity to Me-Lex tended to diminish at higher drug concentrations (12.5–25 μ*M*) (data not shown).

Moreover, only a marginal percentage of apoptotic cells were detected in resting PBL, regardless MPG level. In particular, the mean percentage of apoptotic cells induced by 6.2 μ*M* Me-Lex in PBL with low MPG activity was 6% ($n=3$, range 3–10%) and in PBL with high enzymatic activity was 5% ($n=3$, range 3–8%).

Influence of PARP-1 inhibitors on SCE induction after treatment with methylating agents

Since previous studies had demonstrated that AB is capable of inducing SCE formation in a dose-dependent fashion [4], we investigated whether the more potent inhibitor NU might have a similar effect. PBL were activated for 3 h with PHA and then exposed to graded concentrations of AB (1–4 m*M*) or NU (25–100 μ*M*). The results, illustrated in Fig. 7, indicate that NU induced a dose-dependent increase in SCE formation. The data also confirmed the ability of AB to induce SCE formation. In addition, PBL treated with 4 m*M* AB showed a comparable level of SCE with respect to PBL treated with 100 μ*M* NU.

In order to explore whether 25 μ*M* NU might increase SCE induction in PBL, cells were treated with

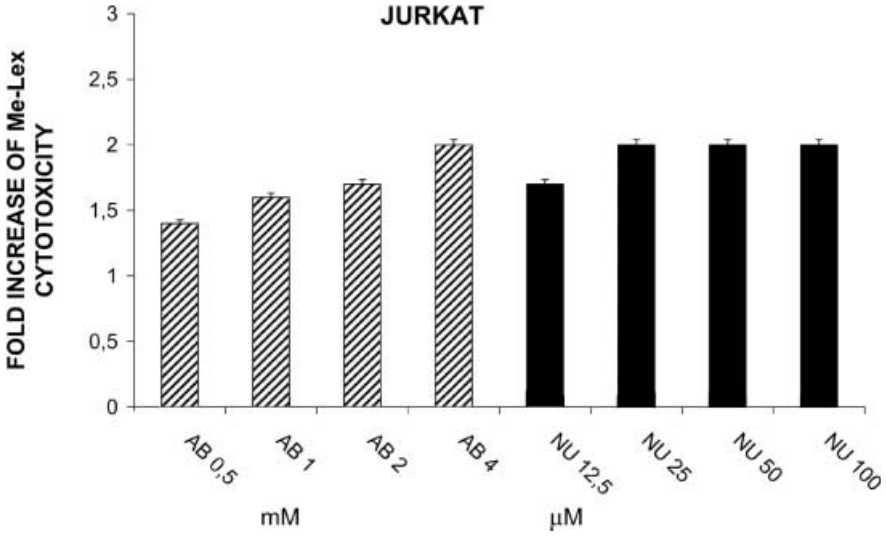

Fig. 4 Comparative analysis of the enhancing effect mediated by AB or NU on Me-Lex cytotoxicity in Jurkat cells. Leukemia Jurkat cells were exposed to graded concentrations of AB or NU and to a fixed concentration of Me-Lex (12.5 μ*M*). After 48 h, cell growth was evaluated in terms of number of viable cells. Histograms represent the ratio between the percentage of growth inhibition detected in cells treated with the drug combination and that observed in cells exposed to Me-Lex only. Data are the means + SE (*bars*) from three repeated experiments

graded concentrations of the methylating agents and analyzed for SCE formation at 72 h of culture. The results were compared with those obtained with 4 m*M* AB, a concentration that induced an increase in Me-Lex cytotoxicity comparable to that induced by 25 μ*M* NU in Jurkat cells.

In regard to Me-Lex, the concentrations tested (1.5–3.1 μ*M*) were lower than those used for apoptosis induction because exposure of PHA-activated PBL to 6.2–25 μ*M* Me-Lex markedly reduced the mitotic index and did not allow chromosomal analysis. The results, illustrated in Fig. 8, show that: (a) 1.5–3.1 μ*M* Me-Lex did not generate SCE; (b) TZM induced a significant increase in SCE with respect to untreated control only at 250 μ*M*, a concentration that is far above that achievable in patients [10]; (c) AB induced SCE and in association with 250 μ*M* TZM produced additive effects; (d) treatment with 25 μ*M* NU, a concentration devoid of genotoxic effects, did not significantly enhance SCE formation when combined with the methylating agents.

In regard to clastogenicity, although a certain degree of variability between donors was observed, chromosome damage in the groups treated with 3.1 μ*M* Me-Lex was limited (breaks/cell 0–0.14) and was not significantly enhanced by PARP-1 inhibition.

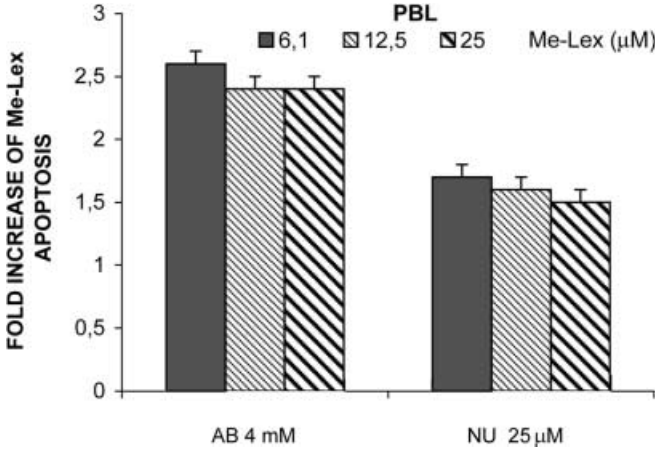

Fig. 5 Comparative analysis of the enhancing effect of AB and NU on Me-Lex cytotoxicity in PBL. PHA-activated PBL were exposed to graded concentrations of Me-Lex combined with 4 m*M* AB or 25 μ*M* NU. After 24 h of culture, cells were harvested and apoptosis was evaluated by flow cytometry. Histograms represent the ratio between the percentage of apoptotic cells detected in PBL treated with the drug combination and that observed in samples exposed to Me-Lex only. Data are the means + SE (*bars*) of three repeated experiments

Discussion

This study was aimed at investigating the potential toxicity of Me-Lex, which selectively methylates DNA at N3-A, as a single agent or combined with PARP-1 inhibitors, in resting or mitogen-activated normal lymphocytes. The interest in this compound, and in pharmacological modalities capable of potentiating its antitumor activity, is justified by the observation that Me-Lex is cytotoxic in tumor cells that are resistant to traditional methylating drugs, that generate a wide spectrum of DNA lesions, and to other DNA-targeting chemotherapeutic agents with different mechanisms of action. However, no data are presently available on the toxicity of this agent in normal human PBL. Moreover, while the genotoxic properties of benzamide-based PARP-1 inhibitors are well-known, the genotoxic potential of more potent and specific inhibitors of PARP-1 have not yet been investigated.

The results reported here indicate that Me-Lex was cytotoxic mainly for PHA-activated PBL. In this regard, it should be noted that N3-MeA behaves as a replication-blocking lesion capable of blocking the required contact between DNA polymerases and the purinic nucleotides [13]. Moreover, it has also been reported that N3-MeA is a strong inhibitor of S phase progression [13]. Therefore, our findings on the preferential

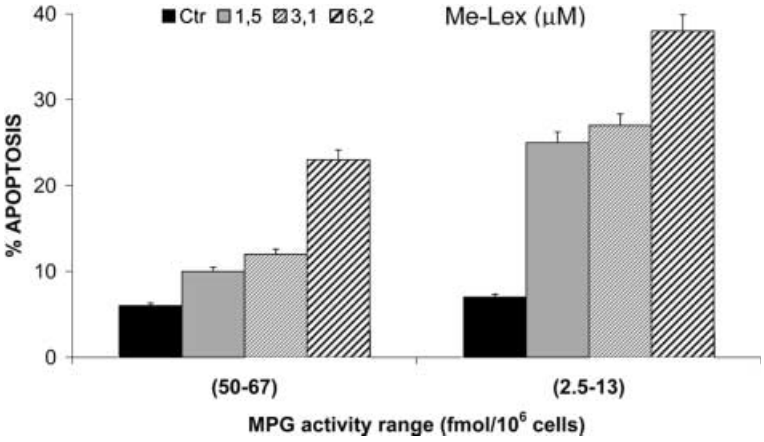

Fig. 6 Analysis of apoptosis in PHA-activated PBL characterized by different levels of MPG activity. Three PBL with low (2.5–13 fmol/10^6 cells) and three with high (50–67 fmol/10^6 cells) MPG activity were selected. After PHA activation for 3 h, PBL were exposed to graded concentrations of Me-Lex and cultured for 48 h. Data are percentages of apoptotic cells evaluated by flow cytometry, and are the means of three independent experiments + SE (*bars*)

toxicity of Me-Lex in mitogen-activated PBL are consistent with the hypothesis that N3-MeA requires DNA synthesis to induce cytotoxic effects. In fact, resting PBL were not as sensitive to Me-Lex as PHA-activated PBL.

Among the mitogen-activated PBL analyzed, Me-Lex (1.5–6.2 μ*M*) was especially cytotoxic in those cells characterized by low MPG activity. The MPG enzyme would be expected to play a more important role in resistance to the N3-A-selective methylating agent Me-Lex than to wide-spectrum methylating compounds such as TZM. In fact, samples characterized by different susceptibilities to Me-Lex, presumably due to different levels of MPG activity, showed comparable sensitivity to TZM. This was likely to have been due to the low levels of N3-MeA adducts generated by TZM and to the prevalent role played by O^6-methylguanine in the cytotoxicity induced by this agent [23]. Indeed, these PBL were endowed with similar levels of OGAT enzyme activity.

Apoptosis induced by Me-Lex plus PARP-1 inhibitor was observed as early as 24 h after drug treatment in both resting and in PHA-activated PBL, and was accompanied by PARP-1 cleavage and p53 induction. In PHA-activated cells, the enhancing effect mediated by the PARP-1 inhibitor tended to diminish at 48 h of culture due to the late appearance of Me-Lex-induced cell death, especially in PBL with low MPG activity. Conversely, in resting PBL, in which Me-Lex cytotoxicity was less pronounced, inhibition of PARP-1 resulted in a prolonged increase in the apoptotic effects of Me-Lex. We have previously demonstrated that cytotoxicity derived from the interruption of the repair of N-methylpurines does not require DNA synthesis. In particular, tumor cells arrested in G_1 phase as a consequence of adenovirus-mediated p53 overexpression are susceptible to combined treatment with PARP-1 inhibitor and TZM [29]. These observations, together with the data obtained in resting PBL using Me-Lex plus PARP-1 inhibitor, underline the potential role of the association of methylating agents and PARP-1 inhibitors in the chemotherapeutic treatment of tumors with a low fraction of actively proliferating cells. In contrast to the combination of Me-Lex plus PARP-1 inhibitor, the association of TZM with PARP-1 inhibitors showed limited toxicity toward resting PBL.

In regard to the pharmacological modality to achieve abrogation of PARP-1 activity, a number of studies have indicated that the benzamide derivatives lack specificity and are likely to possess additional mechanisms of action unrelated to PARP-1 inhibition [5, 17, 20, 21, 22]. Moreover, the potency of benzamides is limited since millimolar concentrations are generally required to achieve potentiation of cytotoxicity of methylating agents. In addition, AB at these high concentrations induced a dose-dependent induction of SCE formation in normal cells [4, 11, 24]. Due to the availability of more potent and specific PARP-1 inhibitors, we sought to determine whether these new compounds also induce SCE formation, at least at the concentrations required to increase the cytotoxic activity of methylating agents in tumor cells. The results of this study showed that NU induced SCE formation at concentrations higher than 25 μ*M* (Fig. 7).

When the minimal concentrations of AB (i.e. 4 m*M*) and NU (i.e. 25 μ*M*) required for producing a twofold increase in Me-Lex cytotoxicity in tumor cells were compared, NU did not substantially increase SCE in PBL treated with either Me-Lex or TZM. On the other hand, all groups exposed to treatments including AB showed SCE levels higher than those observed in samples treated with Me-Lex or Me-Lex plus NU. Moreover, when these concentrations of AB and NU were compared for their ability to increase apoptosis in PBL treated with Me-Lex, 4 m*M* AB was found to enhance the cytotoxic effects of Me-Lex to a higher extent than 25 μ*M* NU. The finding that NU in combination with Me-Lex induces less apoptosis than AB might have been due to the fact that the NU concentration used was 160-fold lower than that of AB, while this compound is only 40-fold more potent as a PARP-1 inhibitor than AB [1]. Similarly, the high concentration of AB required to abrogate PARP-1 activity might also account for the increased SCE formation. Interestingly, NU at a concentration of 100 μ*M*, that is 40-fold lower than that of AB, induced the same level of SCE as AB. Me-Lex at concentrations that allowed cell division and cytogenetic

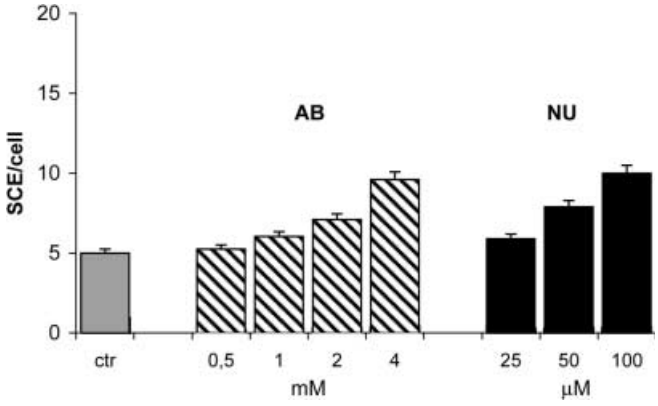

Fig. 7 PARP-1 inhibitors induce a dose-dependent increase in SCE formation. PHA-activated PBL were treated with graded concentrations of AB (*hatched columns*) or NU (*black columns*) and analyzed for SCE after 72 h of culture. The *gray column* indicates the number of SCE per cell present in untreated controls. *Bars* represent the SE calculated from two independent experiments. AB (4 m*M*) and NU (50–100 μ*M*) induced a significantly higher number of SCE per cell with respect to control cultures ($P < 0.01$)

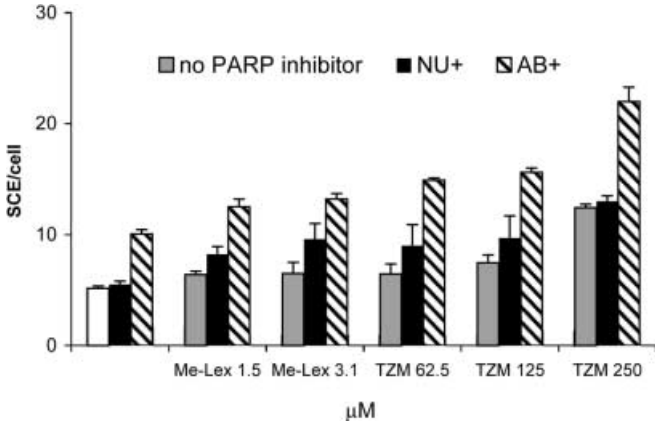

Fig. 8 Analysis of SCE in PBL treated with Me-Lex or TZM with or without PARP-1 inhibitors. PHA-activated PBL were exposed to graded concentrations of Me-Lex (*gray columns*) combined with 4 m*M* AB (*hatched columns*) or 25 μ*M* NU (*black columns*). After 72 h of culture, cells were harvested and analyzed for SCE formation. The *empty column* represents untreated control. Histograms represent the mean number of SCE per cell observed in three independent experiments + SE (*bars*). The numbers of SCE observed in the samples treated with AB or AB plus Me-Lex were always significantly higher than the numbers detected in samples treated with NU, NU plus Me-Lex or Me-Lex only ($P < 0.05$). The numbers of SCE observed in the samples treated with AB plus TZM were always significantly higher than the numbers detected in samples treated with NU plus TZM or TZM only ($P < 0.05$). Differences between groups treated with NU or NU plus Me-Lex and groups treated with Me-Lex only were not statistically significant. Differences between groups treated with NU plus TZM and groups treated with TZM only were not statistically significant. TZM induced a statistically significant increase in SCE numbers with respect to control only at a concentration of 250 μ*M* ($P < 0.01$)

analysis did not induce a significant number of SCE or chromosome breaks. Despite a lack of clastogenicity at these lower Me-Lex concentrations, cytotoxic activity remains in cells with low MPG activity, especially when combined with PARP-1 inhibition [28].

In conclusion, the present report provides a preclinical in vitro evaluation of the toxic potential of Me-Lex as a prototypical selective N3-A-methylating compound that can be effectively used either as a single agent or, in the case of MPG-proficient tumors, combined with PARP-1 inhibitors. Moreover, Me-Lex showed preferential toxicity in mitogen-activated PBL, whereas the drug combination was also cytotoxic for resting lymphocytes. Since the majority of circulating PBL are in the G_0 phase of the cell cycle, it can be assumed that a combined treatment might possess an elevated toxic potential for normal cells. However, this pharmacological strategy might be specifically considered for localized/regional treatment of tumors, including those with a drug-resistance phenotype and low proliferation rate.

The increasing interest in PARP-1 inhibitors is justified by their ability to increase the antitumor effects not only of methylating drugs, but also of γ-irradiation and, as recently reported, of topoisomerase I inhibitors [2, 3, 9]. Our results indicated that the use of PARP-1 inhibitors could produce SCE in normal lymphocytes, potentially increasing the genotoxic risk when used in combination protocols with methylating agents. Therefore, in the process of selecting the most powerful and selective inhibitors, it is necessary to define for each compound the concentration that is devoid of genotoxic properties in normal cells but still capable of enhancing the efficacy of DNA-damaging agents in tumor cells.

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