Chronic lymphocytic leukemia (CLL) is a B-cell malignancy characterized by the progressive accumulation of clonally derived, CD5+, CD19+ B lymphocytes, most of which are G0/G1-phase, nonproliferating cells. CLL represents the quintessential example of a malignancy caused by failed programmed cell death rather than acute proliferation (1). Although most CLL cells are not active in DNA replication, they maintain DNA repair capacity, which may be associated with resistance to CLL therapy (2). Fludarabine, the most active single agent in the treatment of CLL, inhibits DNA replication and repair after incorporation into DNA (3, 4). The combination of fludarabine with DNA-damaging agents showed more than additive effects in apoptosis induction than the single agents alone in vitro (5, 6). This represents proof-of-concept that DNA damage activates DNA repair pathways within CLL cells, which increases the incorporation of the active metabolite of nucleoside analogues into DNA during DNA repair. This approach has contributed to the successful management of CLL (7–9). Combinations of fludarabine and cyclophosphamide were significantly better than fludarabine alone in terms of overall and complete response rates and progression-free survival, although overall survival did not differ between groups (10–12). As more effective treatments in CLL are still needed, we chose to determine if the combination of a novel nucleotide analogue, GS-9219, and DNA-damaging agents is synergistic in CLL cells in vitro.

GS-9219 is a cell-permeable double prodrug designed to limit plasma and systemic exposure to the highly cytotoxic nucleotide 9-(2-phosphonylmethoxyethyl)guanine (PMEG) and at the same time enrich lymphatic tissues with active metabolite, GS-9219 is a cell-permeable prodrug of the acyclic nucleotide analogue 9-(2-phosphonylmethoxyethyl)guanine (PMEG); the incorporation of the active metabolite PMEG diphosphate (PMEGpp) into DNA results in DNA chain termination due to the lack of a 3′-hydroxyl moiety. We hypothesized that the incorporation of PMEGpp into DNA during repair resynthesis would result in the inhibition of DNA repair and the accumulation of DNA breaks in chronic lymphocytic leukemia (CLL) cells that would activate signaling pathways to cell death.

Experimental Design: To test this hypothesis, CLL cells were irradiated with UV light to stimulate nucleotide excision repair pathways, enabling the incorporation of PMEGpp into DNA. The combination effects of GS-9219 and DNA-damaging agents and the signaling mechanisms activated in response to DNA repair inhibition by GS-9219, as well as changes in CLL cell viability, were investigated.

Results: PMEGpp was incorporated into DNA in CLL cells when nucleotide excision repair was activated by UV. Following PMEGpp incorporation, DNA repair was inhibited, which led to the accumulation of DNA strand breaks. The presence of DNA strand breaks activated the phosphatidylinositol 3-kinase protein kinase family members ataxia-telangiectasia mutated and DNA-dependent protein kinase. P53 was phosphorylated and stabilized in response to the inhibition of DNA repair. P53 targeted proteins, Puma and Bax, were up-regulated and activated. The combination of GS-9219 and DNA-damaging agents resulted in more cell death than the sum of the single agents alone.

Conclusion: GS-9219 inhibits DNA repair in CLL cells, an action that stimulates signaling pathways for apoptosis induction.

Abstract

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Translational Relevance

GS-9219 displayed significant in vivo efficacy in dogs with spontaneously occurring, advanced-stage non-Hodgkin’s lymphoma in preclinical studies, and it is currently in multicenter clinical trials for the treatment of hematologic malignancies. The primary mechanism of action of GS-9219 in replicating cells is incorporation of its active metabolite, 9-(2-phosphonylmethoxyethyl)guanine diphosphate (PMEGpp), into DNA and subsequent chain termination due to the lack of a 3′-hydroxyl moiety. Because most circulating chronic lymphocytic leukemia cells are quiescent, we hypothesized that there would be an increase in PMEGpp incorporation during repair resynthesis in response to DNA damage. Further, the accumulation of DNA breaks and the inhibition of DNA repair would subsequently activate signaling pathways to cell death. The understanding of cellular and molecular responses to DNA chain termination via the incorporation of PMEGpp into DNA may benefit the design of combination regimens, including GS-9219 and therapeutic agents that induce excision DNA repair, to achieve the greatest clinical outcome.

GS-9219 inhibits the proliferation of stimulated T and B cells and various hematopoietic cell lines, with EC_{50} values ranging from 0.027 to 1.043 μmol/L. However, there is a >100-fold reduction in activity between quiescent and proliferating cells (EC_{50} values of 17.2 and 0.14 μmol/L, respectively), suggesting that GS-9219 had greater potency against highly proliferating cells (13). Because most CLL cells are quiescent, we postulated that PMEGpp might have limited opportunity to be incorporated into the DNA of indolent CLL cells. To increase the effectiveness of GS-9219 in CLL cells, we hypothesized that the incorporation of PMEGpp into DNA during DNA repair resynthesis would result in DNA chain termination in quiescent CLL cells. Further, the inhibition of DNA repair and the accumulation of DNA breaks would activate signaling pathways to cell death. To test this hypothesis, CLL cells were irradiated with UV to stimulate nucleotide excision repair pathways. This generated the opportunities for the incorporation of PMEGpp into the DNA of quiescent CLL cells as our model system. In this study, we have investigated the combination effects of GS-9219 and DNA-damaging agents in the inhibition of DNA repair and the signaling mechanisms activated in response to DNA repair inhibition by GS-9219 in CLL cells.

Materials and Methods

Isolation of CLL cells. All patients with CLL signed a written informed consent to participate in the laboratory protocol, which was approved by the M.D. Anderson Cancer Center Institutional Review Board. Whole blood was collected in heparinized tubes, diluted 1:3 with PBS, and layered onto Fico/Lite LymphoH (specific gravity, 1.077; Atlanta Biologicals). Cells were harvested from the interface, washed, counted, and sized using a Z2 Coulter particle counter and size analyzer (Coulter Corporation). Then, the cells were resuspended at 1 × 10^9/mL in RPMI 1640 without phenol red, supplemented with 10% autologous serum, and incubated at 37°C.

Chemicals and reagents. GS-9219, [3H]GS-9219 (25.7 Ci/mmol), and [14C]GS-9219 (57 mCi/mmol) were provided by Gilead Sciences, Inc. [3H]Thymidine (63.8 Ci/mmol) was from Moravek Biochemicals, Inc., and hydroxyurea was from Sigma-Aldrich. 4-Hydroperoxycyclophosphamide (4-HC) was provided by Dns. M. Colvin and S. Ludeman (Duke University, Durham, NC). KU-55933 was from KuDOS Pharmaceuticals. zVAD(Ome)-FMK was purchased from MP Biomedicals.

Analysis of metabolites by high-performance liquid chromatography. After incubation with [14C]GS-9219, cells were harvested, washed twice with cold PBS, and extracted with 60% methanol. The extract was evaporated, dissolved in water, and analyzed by high-performance liquid chromatography (HPLC) with a Partisil 10 SAX anion exchange column (Waters Corporation) and eluted at a flow rate of 1.3 mL/min with a 70-min linear gradient from 100% 5 mmol/L NH_4H_2PO_4 (pH 2.8) to 100% 0.75 mol/L NH_4H_2PO_4 (pH 3.6). The eluting metabolites were monitored for UV absorption at 262 nm. The mobile phase containing radioactive metabolites was mixed with scintillation fluid (Packard Instrument Co.), a ratio of 1:3 and quantitated by a flow-through scintillation counter (model A250, Packard Instrument Co.) connected in series to the HPLC system. The amount of metabolites was calculated from the specific activity of the radioactive compound, and the concentration of metabolites was calculated from a given number of cells of a determined mean volume (Coulter Corporation).

UV exposure. Cells were irradiated with UV-C light (254 nm) using a UVGL-25 lamp calibrated with a UVS-25 UV meter (UVP, Inc.).

Analysis of DNA repair resynthesis. [3H]Thymidine incorporation assays were done to determine DNA repair resynthesis as described previously (5). Each experiment was done in triplicate.

PMEG diphosphate (PMEGpp; ref. 13). PMEG is an acyclic nucleotide phosphonate analogue of 2′-deoxyguanosine-monophosphate that has shown significant anticancer activity in vitro and in animal model systems (14). The stable phosphate bond is resistant to the catabolic activities of phosphoesterase and nucleotidase, providing PMEG metabolic stability (15). However, the clinical development of PMEG has been limited by its poor membrane permeability and transporter-mediated toxicity due to the accumulation in target tissues (16, 17). The double-prodrug design of GS-9219 increased the accumulation of PMEGpp in lymphoid cells and tissues and decreased the systematic toxicity generated from PMEG (13).

The metabolic conversion of GS-9219 into PMEGpp is illustrated in Fig. 1A. The N^6-cyclopropyl modification on GS-9219 increases the lipophilicity and water solubility of PMEG (17, 18). The bisphosphonamidate modification neutralizes the negative phosphate charge of PMEG so that GS-9219 passes cell membranes more rapidly than PMEG (19). After entering cells, the phosphoramidate groups of GS-9219 are hydrolyzed by cathepsin A to generate the first metabolite, N^6-cyclopropyl-9-(2-phosphonylmethoxyethyl)diaminopurine (cpr-PMEDAP; ref. 13). The second metabolite step is the deamination of cpr-PMEDAP to PMEG by N^6-methyl-AMP aminohydrolase (13, 17). PMEG is subsequently phosphorylated by intracellular kinases, resulting in the formation of its active metabolite, PMEGpp (20), which competes with the natural substrate, dGTP, for incorporation by DNA polymerases (20, 21). The K<sub>i</sub> values for PMEGpp were 1/3 less than the K<sub>m</sub> values for dGTP (22). The lack of a 3′-hydroxyl moiety makes PMEGpp a de facto DNA chain terminator (23). The incorporation of PMEGpp into DNA during DNA replication causes DNA chain termination and stalled replication forks. Thus, the primary mechanism of action of GS-9219 in replicating cells is through DNA synthesis inhibition.

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Analysis of $[^3H]$PMEG incorporation into DNA. CLL cells were preincubated with 10 μmol/L $[^3H]$GS-9219 (1.29 Ci/mmol) for 4 h, irradiated with UV, and incubated for the indicated time period. Hydroxyurea (final concentration, 3 mmol/L) was added to each sample 30 min before irradiation. DNA was isolated by phenol-chloroform extraction and quantitated by NanoDrop (Thermo Scientific). Scintillation fluid was added to each sample, and radioactivity was quantitated.

Evaluation of DNA repair. DNA repair kinetics were determined as DNA single-strand breaks with the use of the single-cell gel electrophoresis (comet) assay in alkaline condition as described previously (24). Cellular responses to DNA damage were quantitated and expressed as tail moment. Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail (25, 26).

Quantitation of apoptosis. Apoptotic cell death was assessed by flow cytometry with the use of Annexin V-FITC (BD Biosciences) and propidium iodide (Sigma-Aldrich). During apoptosis induction, the pro-apoptotic protein Bax undergoes a conformational change that promotes oligomerization and causes the release of cytochrome c from mitochondria. Conformational changes of Bax were analyzed with a conformation-specific anti-Bax monoclonal antibody (BD Biosciences) by intracellular immunostaining followed by flow cytometric analysis.

Immunoblot analysis. Cells were harvested and lysed in ice-cold lysis buffer containing 0.1 mol/L sodium fluoride, 2 mmol/L sodium vanadate, 6.7 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L EDTA, 0.5% Triton X-100 and supplemented with the complete protease inhibitor mixture (Roche). Lysates were subjected to protein content determination using a detergent-compatible
protein assay kit (Bio-Rad). Equal amounts of total protein were loaded onto SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The blots were visualized and quantitated by Li-Cor Odyssey Imager (Li-Cor Biosciences). Sources of antibodies are as follows: monoclonal antibodies against total p53 (Calbiochem), phosphorylated Ser1981 of ataxia-telangiectasia mutated (ATM; Upstate), DNA-dependent protein kinase (DNA-PK; NeoMarkers), Bcl-2 (Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; Biomol International), and β-actin (Sigma-Aldrich); polyclonal antibodies against phosphorylated Ser15 of p53 (R&D Systems), Bax (BD Biosciences), Puma (ProSci, Inc.), ATM, and phosphorylated Ser2056 of DNA-PK (Abcam, Inc.).

RNA isolation and real-time reverse transcription-PCR. Total cellular RNA was isolated with the RNeasy kit from Qiagen. Total RNA (50 ng) was used for the one-step real-time PCR as described previously (27). The relative gene expression was analyzed by the comparative $C_{T}$ method using 18s rRNA as the endogenous control. The primers and probes for Bax, Bcl-2, and 18s rRNA and reaction mixtures were purchased from Applied Biosystems. The primers and probes for Puma were ordered from Sigma Genosys (28).

Results

Intracellular metabolism of GS-9219 in CLL cells. The intracellular metabolism of GS-9219 and the concentration of each metabolite were first studied in CLL cells to determine the optimal concentration of GS-9219 for the investigations of DNA repair inhibition. Twenty-one CLL cell samples were incubated with 10 μmol/L [14C]GS-9219 for 24 hours and subjected to HPLC analysis to determine the concentration of each metabolite. The major metabolites of GS-9219 in CLL cells were cpr-PMEDAP and PMEGp, suggesting that the rate-limiting intracellular steps of PMEGpp generation were the deamination of cpr-PMEDAP to generate PMEG and the phosphorylation of PMEGp (Fig. 1B). Thus, the intracellular metabolism of GS-9219 was similar in B-CLL cells and activated T cells (13). Although there was heterogeneity of PMEGpp accumulation among samples, the median PMEGpp concentration was ∼6 μmol/L, which is similar to dGTP levels in CLL cells (29). Because the incorporation efficiencies ($V_{max}/K_m$) of PMEGp by many DNA polymerases are only 2-fold less than those of dGTP (23), we postulated that when CLL cells are treated with micromolar range of extracellular GS-9219, there would be a favorable ratio of PMEGp to compete with dGTP for the incorporation into DNA during DNA repair resynthesis in CLL. The plasma pharmacokinetics of GS-9219 and metabolites were studied after a 30-min i.v. infusion to healthy beagle dogs (13). The plasma concentration achieved in these dogs was reported to be ∼5 μmol/L following i.v. infusion of the 3 mg/kg dose of GS-9219. Based on the pharmacokinetic study in the dogs and the intracellular metabolism study in primary CLL cells (Fig. 1B), 1 and 10 μmol/L GS-9219 were chosen for the following investigations.

Inhibition of DNA repair by GS-9219 in UV-irradiated CLL cells. The ability of CLL cells to undergo DNA repair after UV irradiation has been documented (3, 30). We examined
the activation of the nucleotide excision repair pathways in CLL cells exposed to a range of UV doses (0-32 J/m²) by [³H]thymidine incorporation (Fig. 2A). Most CLL cells are quiescent, but they are metabolically active. The incorporation of [³H]thymidine was not completely inhibited by hydroxyurea even when there was no UV irradiation, likely indicating the low level of maintenance DNA repair activity in response to metabolic stress under in vitro culture conditions (31). CLL cells displayed a UV dose-dependent increase in [³H]thymidine incorporation during DNA repair resynthesis, reaching saturation levels at ~4 J/m². A UV dose of 2 J/m² was chosen for the subsequent studies because it stimulated DNA repair but caused less cell death than 4 J/m² UV in CLL cells (data not shown). DNA repair resynthesis was inhibited in a concentration-dependent manner when CLL cells were pretreated with 1 and 10 μmol/L GS-9219 followed by UV irradiation (Fig. 2B). The low-level maintenance DNA repair might result in the incorporation of PMEGpp into DNA without UV irradiation (~20 fmol PMEG/μg DNA). The amount of PMEG incorporated into DNA increased after UV irradiation in a time-dependent manner. There was ~68 fmol PMEG/μg DNA (3 pmol PMEG/10⁷ cells) in CLL cells treated with the combination of GS-9219 and UV for 24 hours (Fig. 2C). To further examine the effects of GS-9219 in the inhibition of DNA repair in CLL cells, the DNA repair process was followed using the single-cell gel electrophoresis (comet) assay (Fig. 2D). GS-9219 itself did not produce obvious DNA damage in quiescent CLL cells, presumably because PMEGpp had limited opportunities to be incorporated into DNA. Most DNA damage caused by 2 J/m² UV was rapidly repaired, as the mean tail moments were close to the basal value 4 hours after UV irradiation, indicating the completion of DNA repair. However, with the presence of PMEGpp in GS-9219-pretreated CLL cells, DNA breaks generated by the removal of UV-induced photoproducts could not be repaired by DNA repair resynthesis, as the mean tail moments were greater than those treated with UV alone and did not return to the basal level 4 hours after UV irradiation. These results suggest that the incorporation of PMEGpp into DNA inhibited DNA repair in quiescent CLL cells.

Apoptosis induction in response to inhibition of DNA repair. The consequences of inhibition of DNA repair were first evaluated by flow cytometric analysis with the use of Annexin V-FITC staining to quantitate apoptosis induction. When CLL cells were incubated with GS-9219 alone for 24 hours, <5% of cells showed Annexin V positivity. Results of the comet assay and Annexin V-FITC staining suggested that GS-9219 by itself did not cause DNA damage. The combination of 10 μmol/L GS-9219 with 2 J/m² UV induced more than additive in apoptosis induction than the sum of single agents alone (Fig. 3A). Besides UV, the combination effects of GS-9219 with the cyclophosphamide prodrug, 4-HC, were also investigated. The combination of 10 μmol/L GS-9219 with 4-HC also showed more Annexin V positivity than the sum of single agents alone (Fig. 3B). Taken together, these results support a mechanism by which DNA-damaging agents stimulated DNA repair within CLL cells, and during DNA repair resynthesis, PMEGpp was incorporated into DNA to inhibit repair, causing initiation of apoptosis.

Sensing mechanisms of DNA damage followed by PMEGpp incorporation. Sensors of DNA damage, including ataxia-telangiectasia and Rad3-related (ATR), ATM, and DNA-PK, can detect various insults to the genome and initiate signal transduction cascades to DNA repair, cell cycle, or apoptotic machineries (32). It has been reported that ATR activity responds mainly to replicative stress and is down-regulated in noncycling CLL cells (33). To investigate the DNA damage–sensing pathways stimulated on inhibition of DNA repair in CLL cells treated with the combination of GS-9219 and UV, the changes of ATM and DNA-PK were first evaluated by immunoblot analysis with specific antibodies against total and phosphorylated ATM and DNA-PK catalytic subunit (DNA-PKcs). The levels of the phosphorylated ATM increased slightly in CLL cells treated with GS-9219 or UV alone for 4 hours compared with the untreated control cells (Fig. 4A, lanes 1-3). After long-term culture in vitro, the low-level maintenance DNA repair activity could lead to the incorporation of PMEGpp into DNA and activate ATM (Fig. 4A, lane 6). In GS-9219-pretreated CLL cells, the phosphorylation of ATM was detected 4 hours after UV irradiation and remained for at least 24 hours (Fig. 4A, lanes 4 and 7). The protein levels of phosphorylated ATM were greater in cells treated with the combination of GS-9219 and UV than in those treated with UV or GS-9219 alone. Unlike the early activation of ATM, DNA-PK was activated only in 24-hour samples treated with the combination of GS-9219 and UV. The changes of ATM and DNA-PKcs from 4 samples

Fig. 3. Apoptosis induction in response to the inhibition of DNA repair. A, CLL cells were preincubated with or without GS-9219 for 4 h, irradiated with or without 2 J/m² UV. Apoptosis was assessed 20 h after UV irradiation by flow cytometry with the use of Annexin V-FITC. Black box, 2 J/m² UV; white box, GS-9219; gray box, GS-9219 and UV combination treatment. ***, P < 0.0001, between combination treatment of 10 μmol/L GS-9219 with UV and the sum of 10 μmol/L GS-9219 and UV alone (n = 20); bars, SE. B, CLL cells were preincubated with or without 10 μmol/L GS-9219 for 4 h; 60 μmol/L 4-HC was added for 30 min; and the cells were washed into fresh medium. Apoptosis was assessed 20 h after adding 4-HC. Black box, 4-HC 60 μmol/L; white box, GS-9219; gray box, GS-9219 and 4-HC combination treatment. **, P < 0.01, between combination treatment of 10 μmol/L GS-9219 with 60 μmol/L 4-HC and the sum of 10 μmol/L GS-9219 and 60 μmol/L 4-HC alone (n = 10); bars, SE.
were quantitated and expressed as fold increases over untreated control samples (Supplementary Fig. S1A). The activation of ATM and DNA-PK in response to DNA chain termination followed by PMEGpp incorporation was further investigated including more time points to elucidate the temporal order of the activation of these two protein kinases in samples from five patients (Fig. 4B; Supplementary Fig. S1B). In GS-9219–pretreated CLL cells, the phosphorylation of ATM was detected 1 hour after UV irradiation, gradually increased over time, and was maintained at least 20 hours after UV irradiation. ATM is a key upstream regulator of p53, and the phosphorylation of p53 at the Ser15 site is a marker of ATM activity in response of DNA damage (34). The phosphorylation and accumulation of p53 was concomitant with that of ATM (Fig. 4B; Supplementary Fig. S1B). The activation of DNA-PK was not detected in the early time points and was not as pronounced as the activation of ATM under the same conditions (Fig. 4B; Supplementary Fig. S1B).

KU-55933, the specific ATM inhibitor (35), was used to confirm the contributions of ATM to the change of p53 phosphorylation status in CLL cells treated with the combination of GS-9219 and UV. KU-55933 treatment decreased the level of the phosphorylated p53, which was induced in response to the combination of GS-9219 and UV in a concentration-dependent manner (Fig. 4C). However, the phosphorylation of p53 was not inhibited completely that there was no apparent reduction of cleaved PARP in CLL cells treated with the combination of GS-9219, UV, and KU-55933 compared with the positive control (Supplementary Fig. S2A, lanes 4 to 7 and 12), and high concentration KU-55933 alone induced >10% Annexin V positivity, which was approximately half of that induced by the combination treatment of GS-9219 and UV (Supplementary Fig. S2B). CLL samples containing ATM gene deletion were used to further confirm the contributions of ATM to the change of p53 phosphorylation status. The phosphorylation of p53 was less in CLL samples containing ATM gene deletion than CLL samples with wild-type ATM after the combination treatment of GS-9219 and UV (Fig. 4D). Taken together, ATM was activated to initiate signal transductions to the downstream target p53, which was subsequently phosphorylated and accumulated in CLL cells treated with the combination of GS-9219 and UV.

**Molecular mechanisms of apoptosis induction in CLL cells treated with GS-9219 and UV.** To investigate possible p53-mediated mechanisms of apoptosis induction on inhibition of DNA repair, the change to p53 and its downstream apoptotic proteins were evaluated by immunoblot analysis. There was a minor increase in total p53 protein when cells were irradiated with 2 J/m² UV alone (Fig. 5A, lanes 2 and 5). There was more accumulation of phosphorylated and total p53 in the 24-hour samples than in the 4-hour samples in GS-9219–treated CLL cells (Fig. 5A, lanes 3 and 6). When CLL cells were preincubated with GS-9219 for 4 hours followed by UV irradiation, the increases in total p53 and phosphorylated p53 protein levels were all greater in cells treated with the combination of GS-9219 and UV than in those treated with UV or GS-9219 alone (Fig. 5A, lanes 4 and 7). PARP cleavage was only seen in the samples treated with the combination of GS-9219 and UV for 24 hours (Fig. 5A, lane 7). The immunoblots of four CLL samples were quantitated and expressed as fold increase over untreated control samples (Fig. 5B). There was ~10-fold more total p53 in cells after the combination treatment of GS-9219 and UV for
24 hours compared with the untreated control cells. The phosphorylation and accumulation of p53 are necessary for its transcription activity. It has been shown that treatment of CLL cells with fludarabine induced the expression of the proapoptotic genes, Bax and Puma, in a p53-dependent manner (36). Puma transcripts increased ~5-fold after 24 hours of the combination treatment (Fig. 5C), as well as the protein level (Fig. 5B). Although Bax mRNA and protein did not change as much as Puma, Bax underwent a conformational change from its inactive form to the active conformation in response to the combination treatment (Fig. 5D). Others have suggested that p53 is a negative regulator of Bcl-2 expression in hematopoietic cells (37). The transcripts and protein level of Bcl-2 did not vary significantly in our model system, and this may be due to different mechanism of regulation.

Discussion

Eukaryotic cells have developed elaborate DNA damage sensing and signaling pathways to maintain viability under a variety of natural and environmental stress. These pathways affect processes such as cell cycle progression, DNA repair, and apoptosis. The responses of CLL cells to the inhibition of a DNA repair process documented in the present studies are summarized in Fig. 6. Treatment of CLL cells with low doses of UV initiated nucleotide excision repair, which facilitated incorporation of PMEGpp into the repair patches. The incorporated analogue caused DNA chain termination and DNA strand breaks, which were sensed by ATM on inhibition of DNA repair. The activated ATM initiated signaling to the downstream target, p53, which was subsequently phosphorylated and accumulated to exert its apoptotic functions. Thus, p53-targeted apoptotic genes, Puma and Bax, were up-regulated and activated when DNA repair was inhibited, likely contributing to cell death. Thus, GS-9219 inhibited DNA repair in primary CLL cells and induced signal transduction mechanisms for apoptosis activation, which was to a greater extent than the sum of cell death caused by each agent alone.

GS-9219 has substantial antiproliferative activity against activated lymphocytes and tumor cell lines of hematologic malignancies (13). Tumor cell proliferation was significantly reduced as measured by positron emission tomography/computed tomography scans in dogs with advanced-stage, spontaneously occurring non-Hodgkin's lymphoma. Further, clinical efficacy studies showed that GS-9219 has significant single-agent activity in these dogs. However, GS-9219 had only weak cytotoxicity against quiescent lymphocytes (13) and CLL cells (Fig. 3) when used as a single agent. Although
Fig. 6. A model for the signaling pathways of cell death in CLL cells treated with GS-9219 and DNA-damaging agents.

the maintenance DNA repair activity led to a limited amount of PMEG incorporation. DNA-damaging agents such as UV that initiated DNA repair increased significantly the incorporation of the analogue into repair patches and resulted in more than additive cell death. UV is a well-characterized inducer of nucleotide excision repair; it has been shown that 2.73 × 10⁻² lesions are generated per kilobase pair of DNA per joule per square meter (38). These occurred as ∼75% cyclobutane pyrimidine dimers and 25% 6-4 photoproducts. Using this as a reference, we calculated that the UV dose of 2 J/m² would generate ∼1.7 × 10⁵ such DNA lesions per CLL cell. When CLL cells were preincubated with 10 μmol/L GS-9219 for 4 hours followed by 2 J/m² UV radiation, there were ∼1.8 × 10⁵ PMEG molecules incorporated per CLL cell after 24 hours of combination treatment (Fig. 2C), indicating that a large proportion of the repair patches had been terminated by the analogue.

4-HC is the prodrug of the active form of the clinically used drug cyclophosphamide that causes multiple types of DNA adducts, including ∼67% phosphorriester monoadducts, 26% N⁷ guanine monoadducts, and 7% N⁷ guanine-N⁷ guanine diadducts (39). These DNA adducts are repaired by several repair processes. The use of the combination of fludarabine with cyclophosphamide in the clinic was supported by data (38). These DNA adducts are repaired by several repair processes; including

- 25% cytosine dimers
- 26% 6-4 photoproducts
- 7% phosphotriester monoadducts
- 7% guanine dimer
- 2.73 × 10⁵ PMEG molecules per cell

linked to early CLL progression, resistance to chemioimmuno-therapy, and poor prognosis (40). The ATM protein is a central component of the signal transduction pathway that is activated in response to DNA double-strand breaks or changes in the chromatin structure (41, 42). ATM protein also contributes to the induction of p53-dependent apoptosis (43). ATM deletion or mutation was found in 10% to 20% of untreated CLL patients. A recent study further divided CLL tumors with a chromosome 11q deletion into two subgroups based on the status of the remaining ATM allele (44). Their data showed that ionizing radiation and cytotoxic agents including fludarabine, chlorambucil, and cyclophosphamide could induce ATM activation and, subsequently, the phosphorylation of p53 in CLL samples with at least one wild-type ATM allele. However, the activation of ATM and the phosphorylation of p53 were severely compromised in CLL samples with no wild-type ATM allele. Patients with complete loss of ATM activity due to biallelic ATM defects, had less response to cytotoxic chemotherapy in vitro and a poorer clinical outcome. Although the remaining ATM allele status is unknown in the two CLL samples with the 11q deletion, there was less phosphorylation of p53 in response to the combination treatment of GS-9219 and UV compared with the CLL samples with wild-type ATM (Fig. 4D). The phosphorylation of p53 in response to the combination treatment of GS-9219 and UV was reduced by KU-55933, consistent with the conclusion that ATM was a major upstream kinase for p53 phosphorylation in the response to inhibition of DNA repair. More investigations are needed to understand the contributions of other kinases to the phosphorylation of p53 when DNA repair is inhibited on PMEGpp incorporation because KU-55933 did not inhibit the phosphorylation of p53 completely, and there was no apparent reduction of cleaved PARP in CLL cells treated with the combination of GS-9219, UV, and KU-55933 compared with the positive control (Supplementary Fig. S2A). Although pharmacologic inhibitors are powerful tools to study the relationships among upstream kinases and p53, 10 μmol/L KU-55933 alone induced >10% Annexin V positivity, which was approximately half of that induced by the combination treatment of GS-9219 and UV (Supplementary Fig. S2B). CLL samples containing an ATM gene deletion may be another way to further study if lack of ATM function would lead to resistance to cell killing by the combination of GS-9219 and DNA-damaging agents.

DNA-PK is one of the central enzymes involved in double-strand break repair through its role in nonhomologous end joining; it also seems to play a critical role in triggering apoptosis in response to excessive or unreparable DNA damage (45). The autophosphorylation activity of DNA-PK at several serine/threonine sites is stimulated by double-strand break ends. In contrast to the phosphorylation of ATM and p53, which occurred at 1 hour, the autophosphorylation of DNA-PK was observed at later time points and was concomitant with PARP cleavage in CLL cells treated with the combination. This suggested that DNA-PK activation was triggered by the fragmented chromosomal DNA generated by apoptosis. Whether there is functional redundancy between ATM and DNA-PK in response to the combination treatment of GS-9219 and DNA-damaging agents remains to be elucidated.

CLL represents an example of a malignancy caused by failed programmed cell death due to the overexpression of antiapoptotic proteins such as Bcl-2 and Mcl-1 (46). The balance between the
proapoptotic and antiapoptotic proteins controls cell viability. It has been known that the expression of several of the proapoptotic Bcl-2 family members such as Bax and the BH3-only proteins Puma, Noxa, and Bid depends on transcriptional transactivation by p53 in different tissues and cells (47–49). Our data showed that p53 was phosphorylated and accumulated, accompanying the increases of the mRNA and protein of Puma and Bax after the inhibition of DNA repair by GS-9219. Deletion of loci involving the TP53 was found in 3% to 27% CLL patients and was associated with poor progression-free survival and responses to fludarabine and cyclophosphamide treatment (11). Whether the status of 17p can be an independent prognostic marker for GS-9219 treatment remains to be answered. Puma has been described to function either by directly activating the proapoptotic proteins Bax and Bak or by neutralizing antiapoptotic members of the Bcl-2 family in different studies (50, 51). Whether Puma plays a direct role in Bax activation or functions as a sensitizer to release Bax after the combination treatment of GS-9219 and UV remains to be elucidated. It has been suggested that p53 mediated the apoptotic pathways in quiescent lymphocytes after the inhibition of DNA repair by fludarabine through transactivation of Fas receptor and ligand (5). It would be worth investigating if the combination treat-

ment of GS-9219 and DNA-damaging agents can trigger signaling to activate extrinsic apoptotic pathways in CLL cells. GS-9219 displayed significant in vivo efficacy in dogs with spontaneously occurring, advanced-stage non-Hodgkin's lymphoma in preclinical studies, and it is currently in clinical trials in hematologic malignancies at multiple centers. The understanding of cellular and molecular responses to DNA chain termination via the incorporation of the active metabolite of GS-9219, PMEGpp, into DNA may benefit the design of combination regimens including GS-9219 and other DNA-damaging agents to achieve the greatest clinical outcome.

Disclosure of Potential Conflicts of Interest

A.S. Ray, D.B. Tumas, and H. Reiser are employed by and have an ownership interest in Gilead Sciences, Inc. W. Plunkett received a research grant from Gilead Science, Inc.

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References


11. Catovsky D, Richards S, Matutes E, et al. Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled tri-
14. Rose WC, Crosswell AR, Bronson JJ, Martin JC. In vivo antitumor activity of 9-[2-(phosphonyl-

methoxy)ethyl]guanine and related phospho-
15. Holy A. Antiviral acyclic nucleoside phospho-
17. Naenssens L, Hatse S, Segers C, et al. 9-[2-

Phosphonylmethoxyethyl)]-β-cyclopophyll-2,6-
diaminopurine: a novel produg of 9-[2-phos-

phonylmethoxyethyl]guanine with improved antitumor efficacy and selectivity in chorio-
18. Compton ML, Toole JJ, Paborsky LR. 9-(2-

Phosphonylmethoxyethyl)-β-cyclopophyll-2,6-
diaminopurine (cpr-PMEDAP) as a prodrug of 9-


ic phosphorylation of 9-(2-phosphonylmethoxy-

it inhibitory potencies of acyclic phosphono-

methoxyalkyl nucleotide analogs toward DNA poly-

22. Kramata P, Downey KM, Paborsky LR. Incor-

poration and excision of 9-(2-phosphonylmethoxy-

23. Kramata P, Downey KM. 9-(2-Phosphonyl-

24. Yamachi T, Nowak BJ, Keating MJ, Plunkett W. DNA repair initiated in chronic lymphocytic leukaemia lymphocytes by 4-hydroperoxycyclo-

25. Olive PL, Banath JP, Durand RE. Detection of etoposide resistance by measuring DNA dam-
29. Balakrishnan K, Nimmanapalli R, Ravandi F, Keating MJ, Gandhi V. Forodesine, an inhibitor of purine nucleoside phosphorylase, induces ap-

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