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ORIGINAL ARTICLE

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Reversal of cytosine arabinoside (ara-C) resistance by the synergistic combination of 6-thioguanine plus ara-C plus PEG-asparaginase (TGAP) in human leukemia lines lacking or expressing p53 protein

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Abstract *Background*: Sequence-specific combinations of purine analogs, such as fludarabine or 6-mercaptopurine (6-MP), administered prior to cytosine arabinoside (ara-C) have been shown to abrogate ara-C resistance in human leukemia cells in vitro and in patients with relapsed acute myeloid or lymphoblastic leukemias. The two-drug combination of 6-MP plus ara-C results in greater cytotoxicity than that achieved with either ara-C or 6-MP alone. Further preclinical investigations have shown that the addition of PEG-asparaginase (PEG-ASNase) to the combination of 6-MP plus ara-C (6-MP + ara-C + PEG-ASNase) results in 15.6-fold synergism over that achieved with the two-drug regimen. This is due to increased DNA damage leading to apoptotic cell death. *Purpose:* Since the intravenous preparation of 6-MP is no longer available and since oral 6-thioguanine (6-TG) provides higher levels of intracellular thioguanine nucleotides than an isotoxic dose of oral 6-MP, we investigated the potential drug synergism of 6-TG plus ara-C plus PEG-ASNase (TGAP) in myeloid (HL60/S, HL60/SN3, U937) and lymphoblastic (CEM/0, CEM/ara-C/B, CEM/ara-C/I, MOLT-4) leukemia cell lines. The CEM clones, MOLT-4 and HL60/SN3 cell lines expressed functional or measurable p53 protein, while the other cell lines did not. *Methods*: The MTT and trypan blue dye exclusion assays were used to determine drug cytotoxicity. In addition, cellular apoptosis and cellular p53, p21/waf-1 and bcl-2 protein concentrations were determined by FACS analysis and ELISA assays. *Results*: Sequential exposure to 6-TG (24 h) plus ara-C (24 h) plus PEG-ASNase (24 h) produced 1.3- to 18.3-fold drug synergism over the two-drug combination of 6-TG plus ara-C. The molecular mechanism of synergism was due to the fact that the three-drug combination was capable of downregulating bcl-2 oncoprotein levels in these cell lines even when p53 was absent. *Conclusion*: These studies strongly demonstrate that the TGAP regimen is highly synergistic in p53-null and p53-expressing leukemia cell lines. We conclude that this combination regimen is collaterally sensitive with ara-C and further evaluation in an investigational phase I trial in relapsed leukemia patients is warranted.

Keywords 6-Thioguanine · Ara-C · Asparaginase · Ara-C resistance · Collateral sensitivity

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Introduction

Current chemotherapeutic treatments for relapsed acute lymphoblastic and acute nonlymphoblastic leukemia (ALL and ANLL) produce poor long-term results [28, 29]. The 5-year event-free survival is less than 10% with chemotherapy treatment alone for pediatric ALL patients who experience an early marrow relapse [11]. It is assumed that when relapse occurs, the leukemic blasts are clinically resistant to the prior chemotherapy [4, 5, 21, 28]. Thus, new chemotherapeutic regimens, consisting of drugs with different mechanisms of action to lessen the possibility of cross-resistance, are needed.

Cytosine arabinoside (ara-C) is a pyrimidine analog, which has long been used in the treatment of adult and pediatric patients with ALL and ANLL [4, 5, 21, 22, 36, 47]. To overcome the leukemic cell resistance to ara-C due to downregulation of deoxycytidine kinase (dCk) [5, 6, 22, 36, 47], sequence-specific combinations of purine analogs followed by ara-C, such as fludarabine plus ara-C or 6-mercaptopurine (6-MP) plus ara-C, have been shown to increase intracellular concentrations of ara-CTP in cells from patients with relapsed leukemia [7, 25, 48, 49]. The increased cytotoxicity found in vitro with 6-MP followed by ara-C has been attributed to the enhanced activation of dCk by the pretreatment of the leukemia cells with 6-MP, resulting in a fourfold increased intracellular concentration of ara-CTP leading to greater inhibition of DNA synthesis, and thus, a greater leukemic cell kill [48, 49]. The in vitro studies have demonstrated a dose-dependent effect of 6-MP in increasing the intracellular ara-CTP concentration [48]. A phase I trial of the combination regimen 6-MP plus ara-C (CCG-0933) in pediatric patients with relapsed leukemias has shown a response in 10 of 27 evaluable patients (5 ALL, 5 ANLL), 8 of whom had a complete response (CR) [49].

The protein inhibitors, L-asparaginase (ASNase) and PEG-ASNase, are important antileukemic agents used in both front-line therapy as well as in relapse protocols [3, 14, 15]. L-ASNases cause depletion of serum L-asparagine (Asn) by deaminating the amino acid to L-aspartic acid and ammonia. Since leukemic cells lack asparagine synthetase, depletion of serum Asn leads to considerable cell cytotoxicity [14, 15, 26]. The regimen comprising ara-C followed by native *E. coli* ASNase (Capizzi II regimen) has been shown to be synergistic and effective in comparison with high-dose ara-C (HDara-C) alone in patients with relapsed leukemia [14, 15]. In a separate study of 195 adult patients with refractory or relapsed leukemia, the CR rate from the HDara-C plus native ASNase regimen was 42% vs 12% from HDara-C alone. The overall survival of the patients treated with the combination was significantly greater than among those treated with HDara-C alone ($P = 0.046$) [16].

The inhibition of protein synthesis by ASNase decreases the cellular CTP and dCTP pools, enhancing the anabolism of ara-CTP and incorporation of ara-CTP into DNA [14]. In preclinical studies, the addition of PEG-ASNase to the combination of 6-MP and ara-C (6-MP + ara-C + PEG-ASNase) resulted in a 15.6-fold synergism over that achieved with the two-drug regimen. This is due to increased DNA damage leading to apoptotic cell death [39].

The antimetabolites 6-thioguanine (6-TG) and 6-MP have been used in the treatment of acute leukemias since the 1950s [12]. 6-TG and 6-MP are prodrugs that require conversion to the active triphosphate metabolite, thioguanylic acid for their cytotoxic effect [32, 42]. The tumor suppressor gene p53 initiates the cascade of signals toward apoptosis following DNA damage induced by ionizing radiation or cytotoxic agents [33]. Functional p53 can upregulate $p21^{WAF\text{-}1}$ which initiates the cascade through cyclins to a G_1 phase arrest. Antiapoptotic proteins, such as bcl-2, protect cells from the effect of chemotherapeutic agents, thereby inhibiting cell death [23, 45]. Preclinical studies have demonstrated that DNA damage caused by ara-C can trigger upregulation of p53 and $p21^{WAF\text{-}1}$ in human leukemic cell lines. In addition, combination regimens can downregulate bcl-2 protein levels in ara-C-resistant leukemic cell clones [40]. Since p53 has been shown to suppress the expression of bcl-2 and activate bax, another promoter of apoptosis in the bcl-2 family [27], the ratio of bcl-2 to p53 could be a better indicator of leukemic cell survival or apoptotic death following chemotherapy.

In this study, we investigated the potential synergism of the three-drug sequence-specific combination of 6-TG plus ara-C plus PEG-ASNase (TGAP) in myeloid (HL60, U937) and lymphoblastic (CEM, MOLT-4) leukemia cell lines. We evaluated the potential collateral sensitivity of the three-drug combination chemotherapy in the T-cell leukemia cell lines CEM/ara-C/B and CEM/ara-C/I, which are 850-fold and greater than 10^6-fold, respectively, more resistant to ara-C than the CEM/0 parent cell line. The results of the studies are presented here.

Materials and methods

Drugs

6-TG was purchased from Sigma Chemical Company (St. Louis, Mo.). Ara-C (cytarabine) was from Bedford Laboratories (Bedford, Ohio) and PEG-ASNase (Oncaspar) was from Enzon (Piscataway, N.J.). All other chemicals and reagents were of analytical grade.

Cell lines

The promyelocytic leukemia cell lines, HL60/S and HL60/SN3, were kindly provided by Dr. Peter Danenberg (USC Norris Cancer Center, Los Angeles, Calif.). HL60/S cells lack functional p53. The HL60/SN3 cells were stably transfected with a neomycin/wild-type functional p53 expression vector [9]. The myeloid leukemia cell line U937, and the T-cell lymphoblastic leukemia cell lines MOLT-4 and CCRF/CEM/0 were obtained from the American Type Culture Collection (Rockville, Md.). U937 cells lack p53 [20], while MOLT-4 and CEM/0 cells express functional p53 [2, 8]. Recently, using DNA arrays for p53, we have found that the U937 line has a deletion on exon 5 of the p53 gene and that the CEM/0 line has a base substitution at exon 6, position 248, from a cgg to cag, which leads to R248Q substitution, as has been reported recently [44, 50]. However, this missense mutation produces a p53 protein, which we have repeatedly shown binds as a functional tetramer protein to the consensus DNA sequence, hence, we consider this clone as a p53(+) cell line [8].

The ara-C-resistant cell lines, CEM/ara-C/B and CEM/ara-C/I, were developed in our laboratory by repeated treatments with ara-C and are 850-fold and greater than 10^6-fold, respectively, more resistant to ara-C than the parent CEM/0 cell line. Cell lines were cultured in RPMI-1640 (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% FBS (Gemini BioProducts), 1% HEPES buffer (Irvine Scientific) and 1% nonessential amino acids 100× (Irvine Scientific) at 37°C in an atmosphere containing 5% CO_2. The culture medium for MOLT-4 cells also contained 1% sodium pyruvate (Sigma Chemical Company).

Drug synergism studies

Myeloid and lymphoblastic leukemia cell lines were treated with single drugs alone or with the drug combinations at 37°C in an atmosphere containing 5% CO_2 as reported previously [48]. Cells were incubated with 6-TG alone for 48 h at drug concentrations ranging from 0.1 to 500 μ*M*. In experiments involving the two-drug combination, the cells were treated with various concentrations of 6-TG for 24 h followed by ara-C for 24 h at a dose ratio of 10:1 (6-TG:ara-C). In studies involving the three-drug combination, the cell lines were treated with a range of concentrations of 6-TG for 24 h followed by ara-C for 24 h followed by PEG-ASNase for 24 h at a dose ratio of 10:1:0.1 (6-TG:ara-C:PEG-ASNase). The drug concentrations for the combination treatments ranged from 0.1 to 50 μ*M* 6-TG, 0.01 to 5 μ*M* ara-C, and 0.001 to 0.5 IU/ml ASNase.

Cell viability

The viability of the leukemia cells following exposure to various drugs was determined by the MTT and trypan blue dye exclusion assays as reported previously [1, 8, 38, 46, 48]. The two cytotoxicity assays measure early, or mitochondrial membrane changes (MTT), vs late apoptotic loss of membrane integrity events. In addition, the trypan blue membrane integrity assay measures late apoptotic events and possible necrotic events induced by the DNA-damaging drugs 6-TG and ara-C [48]. The MTT assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase of viable cells to cleave the tetrazolium ring of MTT reducing the yellow salt to blue formazan crystals [8].

Determination of 50% growth-inhibition concentrations (IC_{50})

Leukemia cell survival was expressed as percent of control and plotted against drug concentration. The IC_{50} values for single-drug treatment, and the combination treatments were estimated from the cell viability data utilizing the median-effect principle and probit transformation. Thus, the dose to achieve the median effect values (Dm), equivalent to IC_{50} values, for each drug treatment was estimated [17, 38]. These principles allowed linearization of the nonlinear dose-response curves of the biology data for extrapolation of IC_{50} values.

Annexin V and propidium iodide flow cytometry apoptosis assay

Apoptosis following drug treatment was evaluated by flow cytometry with annexin V and propidium iodide (PI; R&D Systems, Minneapolis, Minn.). The current protocols in flow cytometry recommend the window settings for the classification of early vs late apoptotic cell subsets [52]. The window settings were based on the annexin V or PI stainings alone. In these studies, the percentiles obtained from FACS analyses of apoptotic cells correlated with the degree of cell viability loss as determined by the trypan blue dye exclusion test and caspase-3 activation (unpublished laboratory data). During early apoptosis, cells expose phosphatidyl serine onto the outer membrane and bind annexin V, while cells in late apoptosis/necrosis are permeable to PI, which then binds to the cellular DNA. Live cells are not stained with either annexin V or PI [52]. Cells were incubated with: (1) 10 μ*M* 6-TG for 24 h followed by 10 μ*M* ara-C for 24 h, (2) 10 μ*M* 6-TG for 24 h followed by 1 IU/ml PEG-ASNase, or (3) 10 μ*M* 6-TG for 24 h followed by 10 μ*M* ara-C for 24 h followed by 1 IU/ml PEG-ASNase for 4 h.

Quantitation of p53, $p21^{WAF-1}$, and bcl-2 proteins

The p53, $p21^{WAF-1}$ and bcl-2 proteins were quantitated in p53-expressing leukemia cell lines, HL60/SN3, MOLT-4, CEM/0 and CEM/ara-C/I, before and after drug exposure utilizing the antibody/antigen sandwich ELISA method, employing mouse monoclonal antibodies (Oncogene Research Products, Cambridge, Mass.). The cells were treated for a total of 24 h with 10 μ*M* 6-TG, 10 μ*M* ara-C or 1 IU/ml PEG-ASNase alone or in combination and the protein concentrations were determined in duplicate wells as reported previously [8]. The two- and three-drug sequential treatments consisted of 6-TG for 16 h followed by ara-C for 8 h (TGA), or 6-TG for 20 h followed by PEG-ASNase for 4 h (TGP), or 6-TG for 16 h followed by ara-C for 4 h followed by PEG-ASNase for 4 h (TGAP). The lower limit of the standard calibration lines from the ELISA assays were 0.01 ng/ml, 0.1 ng/ml, and 1 U/ml for the p53, p21, and bcl-2 proteins, respectively. Once the values of these proteins from the experimental treatments were calculated, they were expressed as fold increases over negative control, assayed at the same time, thus minimizing the possible reproducibility error. The lower limits and linearity characteristics of the standard calibration lines were superimposable on the ones obtained a few years earlier in the laboratory. Hence these assays were highly reproducible [8].

Results

Drug synergy studies

The myeloid (HL60/S, HL60/SN3, U937) and lymphoblastic (MOLT-4, CEM/ara-C/B, CEM/ara-C/I) leukemic cell lines were treated during exponential growth with a single drug, or with the two- and three-drug combinations of 6-TG, ara-C and PEG-ASNase at a dose ratio of 10:1:0.1. The MTT and trypan blue dye exclusion assays were used to determine cell viability after sequential incubation with these drugs. Cell survival was calculated as a percentage of untreated control cells (mean of triplicates; Tables 1 and 2). Higher cyto-

Table 1 IC_{50} values (μ*M*) of 6-TG, TGA and TGAP in myeloid and lymphoblastic leukemia cell lines as determined from the MTT cell viability assay following treatment with 6-TG alone (48 h), with the TGA regimen (6-TG for 24 h followed by ara-C for 24 h at a dose ratio of 10:1), or with the TGAP regimen (6-TG for 24 h followed by ara-C for 24 h followed by PEG-ASNase for 24 h at a dose ratio of 10:1:0.1). The IC_{50} values were calculated using the median effect principle and are reported as dose of 6-TG. The data show a greater cytotoxic effect of TGAP than of TGA overall

Cell line	p53 status	6-TG alone	TGA	TGAP	Fold sensitivity, TGAP vs TGA
HL60/S	–	148.2	14.4	8.2	1.8
HL60/SN3	+	90.6	23.6	3.7	6.3
U937	–	2.4×10^6	30.9	11.0	2.8
MOLT-4	+	7.3	9.9	1.7	5.7
CEM/0	+	54.7	20.1	1.1	18.4
CEM/ara-C/B	+	–	15.5	9.2	1.7
CEM/ara-C/I	+	56.1	12.8	9.6	1.3

Table 2 IC_{50} values (μM) of 6-TG, TGA and TGAP in myeloid and lymphoblastic leukemia cell lines as determined from the trypan blue dye exclusion cell viability assay following treatment with 6-TG alone (48 h), with the TGA regimen (6-TG for 24 h followed by ara-C for 24 h at a dose ratio of 10:1), or with the TGAP regimen (6-TG for 24 h followed by ara-C for 24 h followed by PEG-ASNase for 24 h at a dose ratio of 10:1:0.1). The IC_{50} values were calculated using the median effect principle and are reported as dose of 6-TG

Cell line	p53 status	6-TG alone	TGA	TGAP	Fold sensitivity, TGAP vs TGA
HL60/S	–	102.5	6.7×10^{-4}	0.01[a]	0.06[a]
HL60/SN3	+	90.6	0.2	6.6×10^{-4}	235.8
U937	–	6.3	1.2	0.1	11.3
MOLT-4	+	0.06	0.1	0.0034	39.2
CEM/0	+	9.8	0.0014[a]	0.05[a]	0.03[a]
CEM/ara-C/B	+	–	0.2	0.02	15.1
CEM/ara-C/I	+	5.9	0.3	0.1	2.0

[a]Dose-response graphs of the drug combinations indicate more cytotoxicity with the TGAP regimen

toxicity was observed with the three-drug TGAP regimen and with the two-drug TGA regimen than with 6-TG alone in both myeloid and lymphoblastic leukemia cell lines. The MTT assay produced median drug concentrations (Dm) (equivalent to IC_{50} concentrations) in the U937 cell line of 2.4×10^6, 30.9 and 11 μM, for 6-TG alone, TGA, and TGAP, respectively. Since 6-TG was the highest drug concentration in the combination, the Dm was the estimated 6-TG concentration in the presence of the fixed combination ratio of 10:1:0.1.

Drug synergism was determined by a median effect principle computer program and the results are presented in Tables 1 and 2. The Dm (IC_{50}) concentrations in the MOLT-4 cell line were 7.3, 9.9 and 1.7 μM after the single-, two- and three-drug treatment, respectively. The cytotoxicity of the TGA regimen and the TGAP regimen were p53-independent. The Dm (IC_{50}) concentrations in the p53-null HL60/S cell line were 148.2, 14.4, and 8.2 μM, after the single-, two- and three-drug treatment, respectively. The Dm (IC_{50}) concentrations in the p53-expressing HL60/SN3 cell line were 90.6, 23.6 and 3.7 μM after the single-, two- and three-drug treatment, respectively. The three-drug TGAP regimen showed 1.3- to 18-fold greater drug synergism in comparison to the two-drug TGA regimen (Table 1).

The trypan blue dye exclusion assay also showed significant cytotoxicity of both the two- and three-drug regimens in myeloid and lymphoblastic leukemia cell lines (Table 2). The IC_{50} concentrations in the U937 cell line were 1.2 and 0.1 μM for the TGA and TGAP regimens, respectively. The IC_{50} concentrations in the MOLT-4 cell line were 0.1 and 3.4×10^{-3} μM, for the TGA and TGAP regimens, respectively. The TGAP regimen showed 11- to 39-fold greater drug synergism in these cell lines than the TGA regimen. The IC_{50} values showed that in the p53-null HL60/S cell line, there was a 17-fold greater sensitivity with the TGA regimen (IC_{50} 6.7×10^{-4} μM) than with the TGAP regimen (IC_{50} 0.01 μM), as determined by the median effect principle. However, the dose-response graphs of these two regimens plotted against the fraction affected suggested that the TGAP regimen was more cytotoxic. In the p53-expressing HL60/SN3 cell line, the TGAP regimen exhibited a 236-fold greater drug synergism than the TGA regimen with IC_{50} values of 0.2 μM and 6.6×10^{-4} μM, respectively (Table 2). The apparent lack of concordance in the IC_{50} values between the MTT and the trypan blue assay could be explained by the fact that the MTT assay measures early or mitochondrial membrane changes and the trypan blue assay measures late apoptotic (by loss of membrane integrity) events. In addition, the trypan blue assay also measures the possible necrotic events induced by the DNA-damaging drugs, 6-TG and ara-C.

Collateral sensitivity

There was a significant response to the three-drug TGAP regimen in the ara-C-resistant cell lines (Tables 1 and 2), indicating collateral sensitivity. The CEM/ara-C/B and CEM/ara-C/I are T-lymphoblastic leukemia cell lines, which apparently express functional p53 and are 850-fold and greater than 10^6-fold, respectively, more resistant to ara-C than the parent CEM/0 cell line. The CEM/ara-C/I cell line has a sensitivity to 6-TG similar to that of CEM/0, with IC_{50} values of 56.1 and 54.7 μM, respectively (Tables 1 and 2). In the CEM/0 cell line, treatment with the TGA and TGAP regimens resulted in IC_{50} values of 20.1 and 1.1 μM, respectively (Tables 1 and 2). In the CEM/ara-C/B cell line, the same treatments yielded IC_{50} values of 15.5 and 9.2 μM for TGA and TGAP, respectively. In the CEM/ara-C/I cell line, the IC_{50} values were 12.8 and 9.6 μM, respectively. There was a 1.3- to 18-fold synergism in these three cell lines with the TGAP regimen. The TGAP regimen induced a 0.5- to 1-$\log_{10}$ greater cell kill than the TGA regimen in the CEM/ara-C/I cell line as determined by the trypan-blue exclusion assay (Fig. 1).

Apoptosis studies following TGAP treatment

To evaluate apoptosis as the mechanism of cell death, flow cytometry with annexin-V and PI was performed on cells following drug treatment. Apoptosis was observed after a 4-h incubation with ASNase, after 2 h with ara-C and after 24 h with 6-TG in the HL60 cell lines (data not shown). The maximum degree of apoptosis was observed

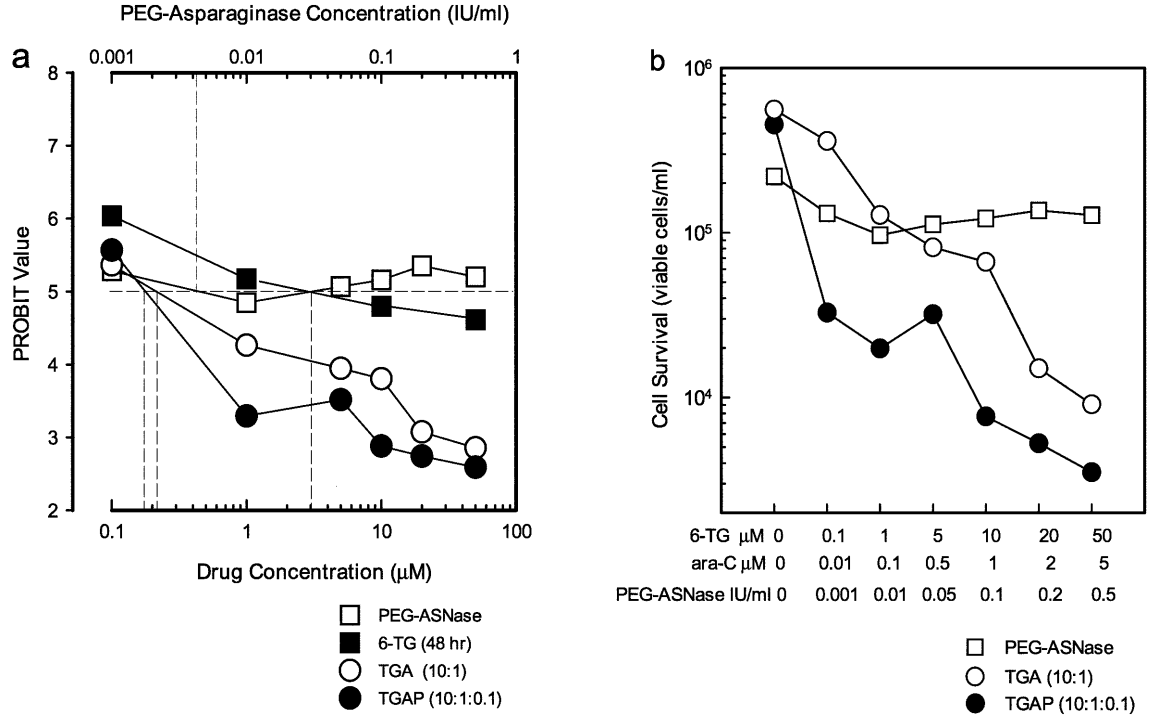

Fig. 1a, b The CEM/ara-C/I T-cell ALL cell line was treated with PEG-ASNase alone for 24 h (❑), 6-TG alone for 48 h (■), 6-TG for 24 h followed by ara-C for 24 h at a dose ratio of 10:1 (○) or 6-TG for 24 h followed by ara-C for 24 h followed by PEG-ASNase for 24 h at a dose ratio of 10:1:0.1 (●). Cell viability was determined by the trypan blue exclusion assay. **a** Cell viability expressed as percent of control and transformed statistically by Probit analysis; **b** cell survival versus drug concentration. IC_{50} values of the two- and three-drug combinations are one-tenth the IC_{50} value of 6-TG used alone. With the three-drug combination of 6-TG + ara-C + PEG-ASNase, there is a 0.5- to 1-$\log_{10}$ greater cell kill than with the two-drug combination of 6-TG + ara-C

after 24 h exposure to ASNase or ara-C (data not shown). The duration of drug exposure for the combination regimens was based on these results.

Following sequential drug exposure, the myeloid cell lines exhibited an increase in the frequency of apoptotic cells in comparison with untreated cells. In the FACS analyses the guidelines from the current flow cytometry protocols in setting the windows for early or late apoptotic cell compartments were followed. The control analyses clearly showed that more than 95% of the untreated cells were clustered in the lower left quadrant, indicating that the control cells were negative for both annexin V and PI (viable cells). These data were verified by the trypan blue dye exclusion data. The HL60 cell lines had a higher frequency of apoptotic cells than the U937 cell line. After sequential treatment with TGA, 18% of HL60/SN3 cells were in early apoptosis and 64% in late apoptosis/necrosis (Table 3). In the HL60/SN3 cell line the TGAP regimen resulted in 20% of the cells being in early apoptosis and 69% in late apoptosis/necrosis. The TGAP regimen resulted in a greater number of total apoptotic events, 81% versus 89%, in this cell line (Fig. 2). In the HL60/S cell line, there was greater cytotoxicity with the TGA regimen, with 31% of the cells in early apoptosis and 40% in late apoptosis/necrosis, whereas the TGAP regimen resulted in 21% of cells in early apoptosis and 31% in late apoptosis/necrosis. The myeloid cell line, U937, showed less apoptosis than other cell lines with the combination drug treatments with total apoptosis/necrosis of 29% and 22% for the TGA and TGAP regimens, respectively.

The TGA and TGAP regimens were effective in inducing apoptosis in the ara-C-sensitive and ara-C-resistant CEM and MOLT-4 lymphoblastic leukemia cells (Table 3, Fig. 2). In the ara-C-sensitive CEM/0 parent cell line, the sequential treatment with TGA resulted in 15% of cells in early apoptosis and 25% in late apoptosis/necrosis. Following treatment with the TGP regimen, 15% of the cells were in early apoptosis and 23% were in late apoptosis/necrosis. Following treatment with the TGAP regimen, 11% of the cells were in early apoptosis and 24% in late apoptosis/necrosis. Total apoptotic events were the same following treatment with the TGA, TGP and TGAP regimens (39%, 39% and 35%, respectively). In the ara-C-resistant CEM/ara-C/I cell line, following treatment with the TGA regimen, 19% of the cells were in early apoptosis and 35% in late apoptosis/necrosis. Following treatment with the TGP regimen, 24% of the cells were in early apoptosis and 35% in late apoptosis/necrosis, whereas the TGAP regimen resulted in 19% of cells in early apoptosis and 38% in late apoptosis/necrosis (total apoptosis 53% vs 65% vs 58%). MOLT-4 showed greater apoptosis than the CEM lines with 89%, 74% and 69% of the cells in total apoptosis/necrosis following treatment with the TGA, TGP and TGAP regimens, respectively.

Table 3 Apoptosis following treatment with drug combinations. FACS analysis with annexin-V and PI of lymphoblastic and myeloid leukemia cell lines following sequential treatment with 10 μ*M* 6-TG, 10 μ*M* ara-C, and 1 IU/ml PEG-ASNase. The results are expressed as percent of events counted. The TGA combination regimen consisted of 6-TG (24 h) followed by ara-C (24 h), the TGP regimen consisted of 6-TG (48 h) followed by PEG-ASNase (4 h), and the TGAP regimen consisted of 6-TG (24 h) followed by ara-C (24 h) followed by PEG-ASNase (4 h) (one event = one cell)

Cell line	Drug treatment	Treatment duration (h)	Unstained cells (% of total events)	Early apoptosis (% of total events)[a]	Early + late apoptosis (% of total events)[b]
HI60/SN3	TGA	48	18	18	81
	TGAP	52	10	20	89
HL60/S	TGA	48	28	31	70
	TGAP	52	37	21	51
U937	TGA	48	63	3	29
	TGP	52	82	3	17
	TGAP	52	72	4	22
MOLT-4	TGA	48	10	19	89
	TGP	52	26	13	74
	TGAP	52	30	23	69
CEM/0	TGA	48	60	15	39
	TGP	52	60	15	39
	TGAP	52	64	11	35
CEM/ara-C/l	TGA	48	46	19	53
	TGP	52	35	24	65
	TGAP	52	41	19	58

[a]Annexin V-positive and PI-negative quadrant
[b]Annexin V-positive and PI-positive quadrant

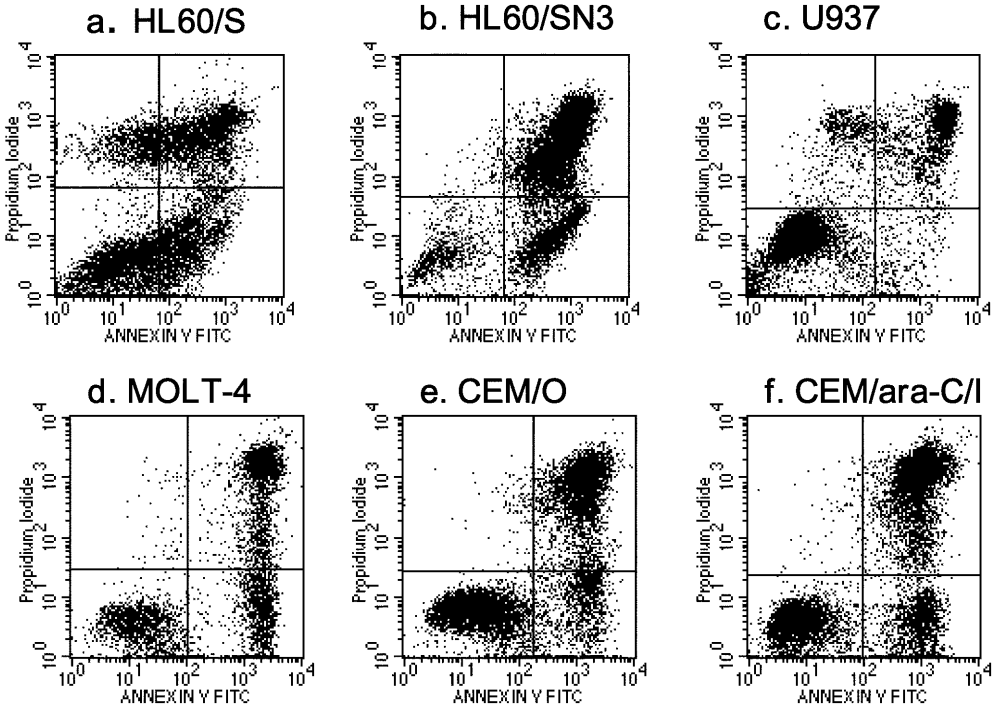

Fig. 2a–f Determination of apoptosis by flow cytometry with annexin V and PI in various leukemia cell lines (**a** HL60/S, **b** HL60/SN3, **c** U937, **d** MOLT-4, **e** CEM/0, **f** CEM/ara-C/I) following treatment with TGAP. Cells were treated with 10 μ*M* 6-TG (24 h) followed by 10 μ*M* ara-C (24 h) followed by 1 IU/ml PEG-ASNase (4 h)

Expression of p53 and bcl-2 oncoproteins

To characterize the mechanisms of apoptosis following treatment with combination drug regimens, we compared the levels of the proapoptotic protein p53 in leukemia cells with and without drug exposure.

We treated the p53-expressing HL60/SN3, MOLT-4, CEM/0 and CEM/ara-C/I cell lines with 10 μ*M* 6-TG (24 h, 52 h), 10 μ*M* ara-C (8 h, 28 h) or 1 IU/ml PEG-ASNase (4 h, 24 h) alone or in combination. The two- and three-sequential drug combinations were the TGA, TGP, and TGAP regimens as defined above. The reduced duration of exposure was necessary to have sufficient numbers of cells after treatment(s) for nuclear extraction. P53 and bcl-2 protein concentrations were determined as described in Materials and methods. The negative control values for p53 averaged (mean ± SD) 0.13 ± 0.007 ng/10^6 cells (%CV 5.6%), 0.26 ± 0.026 ng/10^6 cells (%CV 10.1%), 1.95 ± 0.025 ng/10^6 cells (%CV 2.46%) and 1.18 ± 0.29 ng/10^6 cells (%CV 25.2%) for

the HL-60/SN-3 MOLT-4, CEM/0 and CEM/ara-C/I cell lines, respectively. The negative control of bcl-2 oncoprotein averaged (mean ± SD) 182 ± 22.67, 725.5 ± 642.1, 108.5 ± 5.7, and 132.6 ± 6 U/10^6 cells for the HL-60/SN3, MOLT-4, CEM/0, and CEM/ara-C/I cell lines, respectively. P21 was expressed in low quantities in all cell lines that expressed p53.

Following treatment with single drugs, the upregulation of p53 in four human leukemic lines was dependent upon the duration of drug exposure with an overall increase in p53 protein seen after 52 h of 6-TG, 28 h of ara-C, and 4 h of PEG-ASNase exposure in comparison with control untreated cells (Fig. 3). A 0.7- to 1.4-fold change over the untreated control values in the p53 protein concentration was observed following a 52-h exposure to 6-TG, with p53 increased in the HL60/SN3, MOLT-4 and CEM/0 cell lines. P53 was decreased in the CEM/ara-C/I cell line after 6-TG treatment (Fig. 3a). P53 was uniformly increased in all the cell lines by 1.92- to 2.67-fold following a 28-h exposure to ara-C (Fig. 3b). Upregulation of p53 by PEG-ASNase was seen after a 4-h exposure in the MOLT-4 and CEM/ara-C/I cell lines, but was not seen until after a 24-h exposure in the HL60/SN3 cell line. In the CEM/0 cell line, p53 levels decreased after treatment with PEG-ASNase (Fig. 3c). Prolonged exposure to PEG-ASNase resulted in a decrease in the p53 protein level in MOLT-4 and CEM cell lines, probably due to inhibition of protein synthesis. Bcl-2 was downregulated following prolonged exposure to 6-TG and PEG-ASNase in MOLT-4 and CEM cell lines and appeared to be independent of the p53 status.

The p53 protein levels in the HL60/SN3 cell line were increased by all the combination regimens (Fig. 4). The largest increases in p53 protein levels were observed after treatment with the TGA regimen and the TGAP regimen as compared to untreated control cells. The p53 protein levels were increased after treatment with the TGP regimen only in the MOLT-4 and CEM/0 cell lines and increased with TGA only in the CEM/ara-C/I cell line. Changes in the bcl-2 protein levels were variable in the CEM/0 and CEM/ara-C/I lines (Fig. 4). The bcl-2 to p53 ratio in the leukemic cells decreased by 30% to 61% in the HL60/SN3 and MOLT-4 cells overall. The bcl-2 to p53 ratio was lowest after treatment with the TGA regimen (58% and 61% for HL60/SN3 and MOLT-4, respectively), and after treatment with the TGAP regimen (44% and 49%, respectively; Table 4). The bcl-2 to p53 ratio decreased slightly after treatment with the TGP regimen only in the CEM/0 cell line and with the TGA regimen only in the CEM/ara-C/I cell line.

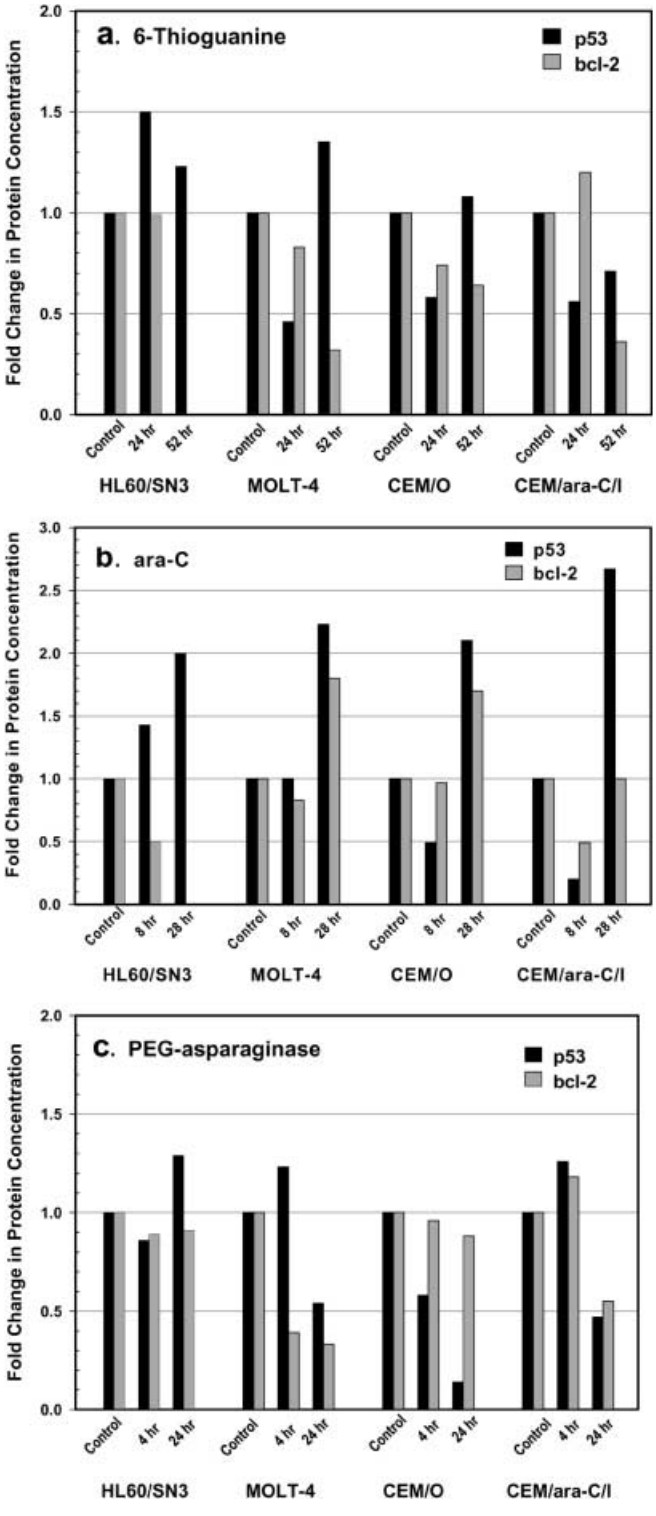

Fig. 3a–c Expression of p53 and bcl-2 proteins following single drug treatments. The p53 protein levels in leukemia cells with or without drug exposure were compared. The p53-expressing HL60/SN3, MOLT-4, CEM/0 and CEM/ara-C/I cell lines were treated with (**a**) 10 μ*M* 6-TG, (**b**) 10 μ*M* ara-C or (**c**) 1 IU/ml PEG-ASNase. The upregulation of p53 was dependent upon the duration of drug exposure with overall increases in p53 protein seen after 52 h of 6-TG, 28 h of ara-C, and 4 h of PEG-ASNase in comparison with untreated control cells. Bcl-2 was downregulated following prolonged exposure to 6-TG and PEG-ASNase in MOLT-4 and CEM cell lines and appeared to be independent of p53

Discussion

Combination regimens with nucleoside analogs and other classes of antineoplastic drugs have been the cornerstone of antileukemic therapies [5, 7, 15, 19, 25, 49]. The sequence-specific combination of 6-MP followed by

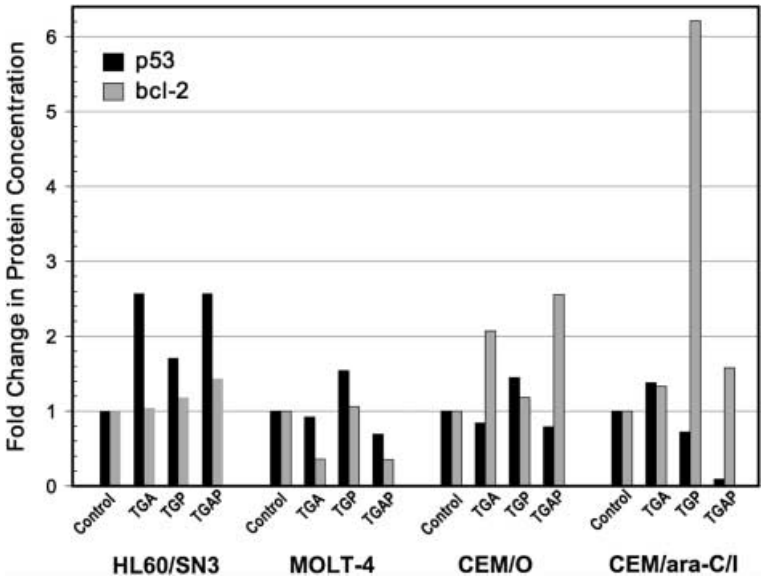

Fig. 4 Expression of p53 and bcl-2 proteins following treatment with the TGA regimen (10 μ*M* 6-TG for 16 h + 10 μ*M* ara-C for 8 h), the TGP regimen (10 μ*M* 6-TG for 20 h + 1 IU/ml PEG-ASNase for 4 h), or the TGAP regimen (10 μ*M* 6-TG for 16 h + 10 μ*M* ara-C for 4 h + 1 IU/ml PEG-ASNase for 4 h). Total treatment duration was 24 h, with controls (untreated cells) obtained after 24 h of growth. P53 and bcl-2 protein concentrations were determined utilizing commercially available antibodies in an antibody-antigen sandwich ELISA as described in Materials and methods

Table 4 P53 and bcl-2 protein concentrations in p53-expressing cell lines (HL60/SN3, MOLT-4, CEM/0 and CEM/ara-C/l) after treatment with the TGA, TGP or TGAP combination regimens. The TGA regimen consisted of 6-TG (16 h) followed by ara-C (8 h), the TGP regimen of 6-TG (20 h) followed by PEG-ASNase (4 h), and the TGAP regimen of 6-TG (16 h) followed by ara-C (4 h) followed by PEG-ASNase (4 h). The p53 and bcl-2 proteins were quantitated by ELISA. One bcl-2 unit is the amount of bcl-2 protein found in 5.6×10^4 HL60 cells (Bcl-2 ELISA protocol)

Cell line	Drug treatment	Treatment duration (h)	P53 protein (ng/10^6 cells)	Bcl-2 protein (U/10^6 cells)	Bcl-2/p53 ratio
HL60/SN3	Vehicle control	24	0.14	1152.0	8228.5
	TGA	24	0.36	1197.6	3327
	TGP	24	0.24	1353.9	5641
	TGAP	24	0.36	1642.4	4562
MOLT-4	Vehicle control	24	0.26	725.5	2790.4
	TGA	24	0.24	262.5	1094
	TGP	24	0.40	766.6	1916.5
	TGAP	24	0.18	255.6	1420
CEM/0	Vehicle control	24	1.95	108.5	56
	TGA	24	1.63	224.7	138
	TGP	24	2.83	127.6	45
	TGAP	24	1.55	277.8	179.2
CEM/ara-C/l	Vehicle control	24	1.18	132.6	112.3
	TGA	24	1.63	176.6	108.3
	TGP	24	0.85	823.6	969
	TGAP	24	0.11	209.7	1906.4

ara-C has synergistic antileukemic effects in the CEM human T-cell ALL cell lines, sensitive and resistant to ara-C [39, 49]. These studies demonstrated collateral sensitivity by increasing the ara-CTP concentration and led to the development of a phase I clinical trial with intravenous 6-MP followed by a continuous infusion of ara-C in pediatric patients with relapsed ALL and ANLL and non-Hodgkin's lymphoma with bone marrow involvement [48, 49]. The success of 6-MP plus ara-C in the in vitro studies has been translated into the clinical setting, with a 37% overall response rate and 80% of responding patients showing a CR [49]. The molecular mechanism by which 6-MP could contribute to cytotoxicity has been suggested to be via its DNA hypomethylation ability as was reported previously [19, 30]. Therefore, 6-MP anabolites have the potential to augment ara-C activation to ara-CTP [48]. The Capizzi II regimen utilizes the synergism between ara-C, an S-phase DNA-damaging agent, and *E. coli* ASNase, a protein inhibitor which may inhibit proteins responsible for the repair of DNA damaged by ara-CTP [14, 15, 16].

Since the intravenous preparation of 6-MP is no longer available and since oral 6-TG provides higher levels of intracellular TG nucleotides than an isotoxic dose of oral 6-MP [31], we investigated the potential synergism of the TGAP regimen in both myeloid (HL60, U937) and lymphoblastic (CEM, MOLT-4) leukemia cell lines. In addition, we evaluated the potential collateral sensitivity of the TGAP regimen in T-cell leukemia cell lines with different degrees of resistance to ara-C, the CEM/ara-C/B and CEM/ara-C/I cell lines. These cell lines are 850-fold and greater than 10^6-fold,

respectively, more resistant to ara-C than the CEM/0 parent cell line. They were developed in our laboratory with repeated doses of ara-C, and are theoretical models representing the relapsed leukemia patient population [35].

There was significant activity of the TGAP regimen in ara-C-resistant lymphoblastic leukemia cell lines, demonstrating collateral sensitivity with ara-C. In particular, in the CEM/ara-C/I cell line, which is over 10^6-fold resistant to ara-C, there was over 1-$\log_{10}$ cell kill with a submicromolar dose of ara-C in combination with a submicromolar dose of 6-TG followed by PEG-ASNase (Fig. 1b). This three-drug combination at doses of 10 μM 6-TG plus 1 μM ara-C plus 0.1 IU/ml PEG-ASNase resulted in greater than 80% cell kill with respect to untreated control cells. Even at relatively low concentrations of the three drugs, significant cytotoxicity was achieved. These concentrations have been achieved in patients. Since all three drugs have different mechanisms of action, there was less likelihood that cross-resistance could have developed [1, 4, 22].

Leukemia cells at diagnosis appear to express functional p53 with a low rate (2–10%) of mutations [53, 54]. Leukemia cells from relapsed patients, however, have a much higher rate of mutations (up to 50%) which may explain in part the poor outcome of relapsed patients to subsequent therapies with p53-dependent mechanisms [24, 34, 44, 53, 54]. Thus, mutations in p53 leading to inactivation of the protein may be associated with resistance to chemotherapy and decreased survival of patients with hematological malignancies [23, 54].

Bcl-2, an antiapoptotic protein, is expressed in most leukemias, lymphoblastic and myeloid [10, 18]. High expression of the protein may protect cells from drug-induced apoptosis and confer resistance to chemotherapy [13]. However, bcl-2 levels alone do not predict clinical response in ALL and ANLL [41, 51]. Thus, the ratio of bcl-2 to p53 may be a more important molecular parameter in predicting leukemic cell survival or apoptotic death where a high or increasing bcl-2 to p53 ratio would favor cell survival and a low or decreasing ratio would favor apoptosis.

There was little difference in the cytotoxicity of 10 μM 6-TG between p53-null and p53-expressing HL60 cells after exposure for 24 h (data not shown). However, after exposure for 48 h, a difference could be seen, with more cytotoxicity in the p53-expressing HL60/SN3 cells. The upregulation of p53 on exposure to 6-TG occurred after 24 h in the HL60/SN3 cell line and occurred after more than 48 h in leukemic cell lines constitutively expressing p53, i.e. MOLT-4 and CEM (Fig. 3). This is in agreement with the observation of Morgan et al. that DNA fragmentation occurs after 24 h exposure to 6-TG in HL60 cells but not in CEM cells [37].

Downregulation of bcl-2 was seen in the MOLT-4 and CEM/0 cell lines beginning 24 h after exposure to 10 μM 6-TG, but was more pronounced after 52 h of exposure (Fig. 3). Downregulation of bcl-2 on exposure to 1 IU/ml PEG-ASNase was observed after 4 h in the HL60/SN3, MOLT-4 and CEM/0 cell lines and the protein concentration was not significantly changed at 24 h. The effect of 10 μM ara-C on bcl-2 protein levels was variable. A decrease in the protein levels was seen after 24 h exposure in CEM/0 cell line, but not in the MOLT-4 or HL60/SN3 cell lines, the p53-expressing cell lines. This was likely due to the high cytotoxicity of ara-C at a dose of 10 μM in these cell lines (Tables 1 and 2).

The collateral sensitivity seen with the two- and three-drug combination regimens in the ara-C-resistant cell lines CEM/ara-C/B and CEM/ara-C/I suggests that the addition of 6-TG could reverse ara-C resistance. In addition to other modes of development of resistance to ara-C, resistance could also arise from the drug's ability to induce DNA hypermethylation and inactivate dCk, the enzyme catalyzing the rate-limiting step in the conversion of ara-C to its active metabolite [4, 5, 19, 21, 43, 47]. It has been shown that demethylating agents such as 5-aza-cytidine (5-aza-C), can reactivate dCk expression and reverse ara-C resistance [5]. Both 6-MP and 6-TG also have the ability to resensitize ara-C by hypomethylating DNA and allowing increased intracellular conversion of ara-C to ara-CTP, the active metabolite, resulting in an increased cytotoxic effect [5, 30, 48]. The addition of PEG-ASNase to 6-TG and ara-C allowed an independent mechanism of cell killing to enhance cytotoxicity and possibly lessen the development of further drug resistance in these cell clones. This is a separate mechanism for drug synergism than the one determined for the fludarabine plus ara-C-containing regimens [6, 19, 25]. Thiopurines do not appear to inhibit ribonucleotide reductase and they do not deplete dCTP as does fludarabine [25, 40]. However, thiopurines decrease DNA methylation [30, 43].

DNA hypermethylation occurs in the promoter region of many genes including dCk, which then become "silenced" [5, 43]. Agents such as 5-aza-C and thiopurines cause DNA hypomethylation thus, allowing re-expression of dCk in ara-C-resistant leukemic cells [5, 48]. Re-expression of cytosolic dCk increases the cellular ara-CTP concentration by approximately fivefold after treatment with 6-MP followed by ara-C [48]. The differences in the mechanisms of drug synergism between fludarabine plus ara-C and 6-MP plus ara-C has been demonstrated clinically in relapsed pediatric patients with leukemias: CR was reinduced in patients in relapse from fludarabine plus ara-C regimens with 6-MP plus ara-C [49]. Future investigations will examine DNA hypomethylation of ara-C by 6-TG in the TGAP regimen.

Overall, our present findings suggest that TGAP is a better combination regimen in inducing drug synergism in ara-C-resistant cell lines than found in previous studies on the combination of 6-MP plus ara-C or 6-MP plus ara-C plus PEG-ASNase [39, 48]. The flow cytometry analyses demonstrated that following TGAP treatment, cells of all cell lines sensitive and refractory to ara-C underwent apoptotic cell death. Therefore, these studies strongly indicate that the three-drug combination of TGAP has a significant drug synergistic anti-

leukemic effect in both p53-null and p53-expressing cell lines and reverses ara-C resistance. These results support the clinical use of this combination in relapsed leukemia, of which approximately 50% of cases have functional p53. A pilot phase I clinical trial is currently in progress at our institution using of oral 6-TG followed by ara-C continuous infusion followed by intramuscular injection of ASNase in pediatric patients with relapsed or refractory leukemias.

References

1. Adamson PC, Poplack DG, Balis FM (1994) The cytotoxicity of thioguanine vs. mercaptopurine in acute lymphoblastic leukemia. Leuk Res 18:805
2. Aldridge DR, Radford IR (1998) Explaining differences in sensitivity to killing by ionizing radiation between human lymphoid cell lines. Cancer Res 58:2817
3. Asselin Bl, Whitin JC, Coppola DJ, Rupp IP., Sallan SE, Cohen HJ (1993) Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol 11:1780
4. Avramis VI, Biener R, Krailo M, Finkelstein J, Ettinger L, Willoughby M, Siegel SE, Holcenberg JS (1987) Biochemical pharmacology of high dose 1-β-D-arabinofuranosylcytosine in childhood acute leukemia. Cancer Res 34:1775
5. Avramis VI, Mecum RA, Nyce J, Steele DA, Holcenberg JS (1989) Pharmacodynamic and DNA methylation studies of high-dose 1-β-D- arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. Cancer Chemother Pharmacol 24:203
6. Avramis VI, Weinberg KI, Sato JK, Lenarsky C, Willoughby ML, Coates TD, Ozkaynak MF, Parkman R (1989) Pharmacology studies of 1-β-D-arabinofuranosylcytosine in pediatric patients with leukemia and lymphoma after a biochemically optimal regimen of loading bolus plus continuous infusion of the drug. Cancer Res 49:241
7. Avramis VI, Wiersma S, Krailo MD, Ramilo-Torno LV, Sharpe A, Liu-Mares W, Kwock R, Reaman GH, Sato JK (1998) Pharmacokinetic and Pharmacodynamic studies of fludarabine and cytosine arabinoside administered as loading boluses followed by continuous infusion after a phase I/II trial in pediatric patients with relapsed leukemias. Clin Cancer Res 4:45
8. Avramis VI, Nandy P, Kwock R, Solorzano MM, Mukherjee SK, Danenberg P, Cohen LJ (1998) Increased p21/WAF-1 and p3 protein levels following sequential three drug combination regimen of fludarabine, cytarabine and docetaxel induces apoptosis in human leukemia cells. Anticancer Res 18:2327
9. Banerjee D, Lenz H-J, Schnieders B, Manno DJ, Ju JF, Spears CP, Hochhauser D, Danenberg K, Danenberg P, Bertino JR (1995) Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. Cell Growth Differ 6:1405
10. Banker DE, Groudine M, Norwood T, Appelbaum FR (1997) Measurement of spontaneous and therapeutic agent-induced apoptosis with bcl-2 protein expression in acute myeloid leukemia. Blood 89:243
11. Barrett AJ, Horowitz MM, Pollock BH, Zhang MJ, Bortin MM, Buchanan GR, Camitta BM, Ochs J, Graham-Pole J, Rowlings PA, Rimm AA, Klein JP, Shuster JJ, Sobocinski KA, Gale RP (1994) Bone marrow transplants from HLA-identical siblings as compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission. N Engl J Med 331:1253
12. Burchenal JH, Murphy ML, Ellison RR, et al (1953) Clinical evaluation of a new antimetabolite, 6-mercaptopurine, in the treatment of acute leukemia and allied diseases. Blood 8:965
13. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Guyotat D (1993) High expression of bcl-2 protein in acute myeloid leukemia is associated with poor response to chemotherapy. Blood 81:3091
14. Capizzi RL, White C (1988) The pharmacological basis for the efficacy of high-dose Ara-C and sequential asparaginase in adult acute myelogenous leukemia. Yale J Biol Med 61:11
15. Capizzi RL, Poole BL, Cooper MR, Richards F 2nd, Stuart JJ, Jackson DV Jr, White DR, Spurr CL, Hopkins JO, Muss HB, Rudnik SA, Wells R, Gabriel D, Ross D (1984) Treatment of poor risk acute leukemia with sequential high dose ara-C and asparaginase. Blood 63:694
16. Capizzi RL, Davis R, Powell B, Cuttner J, Ellison RR, Cooper MR, Dillman R, Major WB, Dupre E, McIntyre OR (1988) Synergy between high-dose cytarabine and asparaginase in the treatment of adults with refractory and relapsed acute myelogenous leukemia – a Cancer and Leukemia Group B Study. J Clin Oncol 6:499
17. Chou T-C (1991) The median-effect principle and the combination index for quantitation of synergism and antagonism. In: Chou TC, Rideout DC (eds) Synergism and antagonism in chemotherapy. Academic Press, San Diego New York Boston London Sydney Tokyo Toronto, p 61
18. Couston-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, Raimondi SC, Behm FG, Arico M, Campana D (1996) Clinical relevance of bcl-2 overexpression in childhood acute lymphoblastic leukemia. Blood 87:1140
19. Dinndorf PA, Avramis VI, Wiersma S, Krailo MD, Liu-Mares W, Seibel NL, Sato JK, Mosher RB, Kelleher JF, Reaman GH (1997) Phase I/II study of idarubicin given with continuous infusion fludarabine followed by continuous infusion cytarabine in children with acute leukemia: a report from the Children's Cancer Group. J Clin Oncol 15:2780
20. Dou QP, Lui VW (1995) Failure to dephosphorylate retinoblastoma protein in drug-resistant cells. Cancer Res 55:5222
21. Ellison RR for the Acute Leukemia Cooperative Group B (1968) Arabinosylcytosine: a useful agent in the treatment on acute leukemia in adults. Blood 32:507
22. Estey EH, Keating MJ, McCredie KB, Freireich EJ, Plunkett W (1990) Cellular ara-CTP pharmacokinetics, response, and karyotype in newly diagnosed acute myelogenous leukemia. Leukemia 4:95
23. Findley HW, Gu L, Yeager AM, Zhou M (1997) Expression and regulation of bcl-2, bcl-xl, and bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. Blood 89:2986
24. Fu CH, Nandy P, Siegel SE, Avramis VI (1998) Increase in the bcl-2 to p53 ratio in leukemic cells from patients (pts) is associated with relapsed leukemia. Abstracts of the Eighth International Congress on Anti-Cancer Treatment (abstract no. O 179). 3–6 February 1998, Paris, p 118
25. Gandhi V, Estey E, Keating MJ, Plunkett W (1993) Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia during therapy. J Clin Oncol 11:116
26. Ho D, Whitecar J, Luce J, Frei E (1970) L-Asparaginase requirement and the effect of L-asparaginase on the normal and leukemic human bone marrow. Cancer Res 30:466
27. Jones NA, Turner J, McIlwrath AJ, Brown R, Dive C (1998) Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak upregulation and the functional status of p53. Mol Pharmacol 53:819
28. Kaufman D, Chabner BA (1996) Clinical strategies for cancer treatment: the role of drugs. In: Chabner BA, Longo DL (eds) Cancer chemotherapy and biotherapy: principles and practice, 2nd edn. Lippincott-Raven, Philadelphia New York, p 1
29. Keating MJ, Estey E, Kantarjian H (1993) Acute leukemia. In: DeVita VT, Hellman S, Rosenberg SA (eds) Cancer principles and practice of oncology. 4th edn. J.B. Lippincott Co., Philadelphia, p 1938
30. Lambooy L, Leegwater PAJ, Van Den Heuvel LP, Boekkerink JP, De Abreu RA (1998) Inhibition of DNA methylation in

malignant MOLT F4 lymphoblasts by 6-mercaptopurine. Clin Chem 44:556
31. Lennard L, Davies HA, Lilleyman JS (1993) Is 6-thioguanine more appropriate than 6-mercaptopurine for children with acute lymphoblastic leukemia? Br J Cancer 68:186
32. Liliemark J, Pettersson B, Engberg B, Lafolie P, Masquelier M, Peterson C (1990) On the paradoxically concentration-dependent metabolism of 6-mercaptopurine in WEHI-3b murine leukemia cells. Cancer Res 50:108
33. Lowe SW, Ruley HE, Jacks T, Housman DE (1993) P53-dependent apoptosis modulates the cytotoxicity of anticancer drugs. Cell 74:957
34. Marks DI, Kurz BW, Link MP, Ng E, Shuster JJ, Lauer SJ, Carroll D, Brodsky I, Haines DS (1997) Altered expression of p53 and mdm-2 proteins at diagnosis is associated with early treatment failure in childhood acute lymphoblastic leukemia. J Clin Oncol 15:1158
35. Martin-Aragon S, Mukherjee SK, Taylor BJ, Ivy SP, Fu CH, Ardi VC, Avramis VI (2000) Cytosine arabinoside (ara-C) resistance confers cross-resistance or collateral sensitivity to other classes of anti-leukemic drugs. Anticancer Res 20:139
36. Momparler RL (1974) A model for the chemotherapy of acute leukemias with 1-β-D-arabinofuranosylcytosine. Cancer Res 34:1775
37. Morgan CJ, Chawdry RN, Smith AR, Siravo-Sagraves G, Trewyn RW (1994) 6-Thioguanine-induced growth arrest in 6-mercaptopurine-resistant human leukemia cells. Cancer Res 54:5387
38. Nandy P, Lien EJ, Avramis VI (1994) Antileukemic activity studies and cellular pharmacology of the analogues of 2-hydroxy-1H-isoindole-1,3-dione (HSID) alone and in combination with cytosine arabinoside (ara-C) against human leukemia cells CEM/0. Acta Oncol 33:953
39. Nandy P, Periclou AP, Avramis VI (1998) The synergism of 6-mercaptopurine plus cytosine arabinoside followed by PEG-asparaginase in human leukemia cell lines (CCRF/CEM/0 and CCRF/CEM/ara-C/7A) is due to increased cellular apoptosis. Anticancer Res 18:727
40. Nandy P, Lien EJ, Avramis VI (1999) Inhibition of ribonucleotide reductase by a new class of isoindole derivatives: drug synergism with cytarabine (ara-C) and induction of cellular apoptosis. Anticancer Res 19:1625
41. Naumovski L, Martinovsky G, Wong C, Chang M, Ravendranath Y, Weinstein H, Dahl G (1998) Bcl-2 expression does not correlate with patient outcome in pediatric acute myelogenous leukemia. Leuk Res 22:81
42. Nelson JA, Carpenter JW, Rose LM, Adamson DJ (1975) Mechanism of action of 6-thioguanine, 6-mercaptopurine and 8-azaguanine. Cancer Res 35:2872
43. Nyce J (1989) Drug-induced DNA hypermethylation and drug resistance in human tumors. Cancer Res 49:5829
44. O'Connor PM, Jackman J, Insoo B, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S, Fornance AK Jr, Kohn KW (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency in 123 anticancer agents. Cancer Res 57:4285
45. Olvati ZN, Millman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerize in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609
46. Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MWJ, Heyenbrok MW, Haehlen K, Veermen AJP (1990) In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 76:2327
47. Plunkett W, Iacoboni S, Estey E, Danhauser L, Liliemark JO, Keating M (1985) Pharmacologically directed Ara-C therapy for refractory leukemia. Semin Oncol 12:20
48. Ramilo-Torno LV, Avramis VI (1995) Intracellular pharmacodynamic studies of the synergistic combination of 6-mercaptopurine and cytosine arabinoside in human leukemia cell lines. Cancer Chemother Pharmacol 35:191
49. Sato JK, Ramilo-Torno L, Krailo M, Carcich S, Mosher R, Seibel N, Steinherz P, Reaman G, Avramis VI (1999) Phase I clinical and pharmacodynamic study of continuous infusion (CI) mercaptopurine (MP) followed by continuous infusion cytosine arabinoside (ara-C) in relapsed leukemia. Proc Am Soc Clin Oncol 18:199a (abstract 767)
50. Sigal A, Rotter V (2000) Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res 60:6788
51. Tsurusawa M, Saeki K, Katano N, Fujimoto T (1998) Bcl-2 expression and prognosis in childhood acute leukemia. Children's Cancer and Leukemia Study Group. Pediatr Hematol Oncol 15:143
52. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J Immunol Methods 184:39
53. Wada M, Bartram CR, Nakamura H, Hachiya M, Chen DL, Borenstin J, Miller CW, Ludwig L, Hansen-Hagge TE, Ludwig WD, Reiter A, Mizoguchi H, Koeffler HP (1993) Analysis of p53 mutations in a large series of lymphoid hematologic malignancies of childhood. Blood 82:3163
54. Wattel E, Preuhomme C, Hecquet B, Vanrumbeke M, Quesnel B, Dervite I, Morel P, Fenaux P (1994) P53 mutations are associated with resistance to chemotherapy and short survival in hematological malignancies. Blood 84:3148