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

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/1, 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

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]. In the 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 asparaginase 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 antimitabolites 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, thio- guanylic 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 p21WAF-1 which initiates the cascade through cyclins to a G1 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 p21WAF-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/1, which are 850-fold and greater than 106-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/1, were developed in our laboratory by repeated treatments with ara-C and are 850-fold and greater than 106-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% CO2. The culture medium for MOLT-4 cells also contained 1% sodium pyruvate (Sigma Chemical Company).