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3-amino thioacridone inhibits DNA synthesis and induce DNA damage in T-cell acute lymphoblastic leukemia (T-ALL) in a p16-dependent manner

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In T-cell Acute Lymphocytic Leukemia (T-ALL), the inhibitors of cyclin-dependent kinases (CDK) 4 and 6, p16 and p15, are inactivated almost universally at the DNA, RNA and protein levels. This suggests that CDK-targeting may be an effective therapeutic approach for T-ALL and other cancers. In this study, we tested 3 inhibitors of CDK4, 3-aminothioacridone (3-ATA), thioacridone (TA), and oxindole, for their effects on DNA synthesis and viability in primary T-ALL. Each compound was an effective inhibitor, with overall IC_{50}s in similar ranges. In colony formation assay, leukemic cells were approximately 10-fold more sensitive to 3-ATA than normal bone marrow cells. When sorted by G1 protein status of T-ALL, p16(+), p15(+) or pRb(-) samples were significantly less sensitive to 3-ATA and TA, but not to oxindole, than p16(-), p15(-) or pRb(+) samples. There was no relationship of sensitivity with ARF expression. Despite their *in vitro* function as inhibitors of CDK4, 3-ATA did not inhibit pRb phosphorylation or cause G_1 arrest, but did cause DNA damage and result in the induction and phosphorylation of p53. We conclude that 3-ATA efficacy can be predicted by p16 status in T-ALL, but the mechanism of action may be distinct from their *in vitro* ability to regulate CDK4 kinase activity

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in childhood. It is a systemic myeloproliferative disease, with malignant cells that are thought to originate from normal lymphoid progenitor cells arrested at early stages of B- or T-

lymphocyte ontogeny. Of these patients, approximately 85% present with B-lineage ALL and 15% with T-lineage ALL. The therapeutic success in the treatment of childhood ALL is one of the crowning achievements of cancer research. With current therapies tailored to not only the disease (T-ALL vs. B-precursor ALL vs. AML for example), but also to risk of relapse, aggressive multi-modality treatments currently assure that virtually 100% of both B- and T-lineage ALL will respond favorably and achieve an initial successful induction of remission (1). Indeed, long-term event free survival for T-ALL, which has traditionally harbored a poorer prognosis than B-lineage ALL, has increased from less than 10% 20 years ago to its current rate of close to 80%.

Despite success in inducing first remission, approximately 20% of T-ALL patients relapse and die within the first five years of initial diagnosis. A thorough understanding of the molecular biology of T-ALL may serve to help develop novel therapies directed at specific molecular targets of the tumor cell. One family of specific targets are the cyclin dependent kinases (CDK). In conjunction with cyclin D, CDK4/6 hyperphosphorylate and inactivate pRb, releasing transcription factors that drive the cell through the G_1 phase of the cell cycle. CDK4/6 are negatively regulated in the cell cycle by CDK inhibitors (CDKI) such as p16 and p15. P16 is located on chromosome 9p21 and co-localizes with two other cell cycle regulators, p15 and ARF. P15 is 25Kb centromeric of p16, and is sequentially and functionally homologous to p16. Splicing of an alternative exon 1 (exon 1β, located centromeric of p16 exon 1) to the second exon of p16 yields a unique protein, ARF, which serves as a regulator of p53 by promoting MDM2 degradation. Molecular studies by our group and others (2-4) demonstrated that these three genes are inactivated by deletion in 60-70% of T-ALL patients, suggesting that they may play an important etiological role in the development of the disease. More recently, we have shown that even in T-ALL harboring intact p15 and p16 genes, other molecular alterations, such as mutations, promoter hypermethylation and translational inactivation, can render the genes non-functional (5).

As the function of CDK4 and CDK6 are uninhibited in the absence of p16 and p15, these enzymes are excellent targets for therapeutic intervention. Flavopiridol and UCN-01 are two early generation CDKIs that have shown promise as anti-cancer agents. Both were originally characterized as protein kinase inhibitors, with flavopiridol potently inhibiting virtually all CDKs, while UCN-01 is a relatively non-specific kinase inhibitor. Both compounds also have been demonstrated to be potent inhibitors of cell proliferation and both have shown some success in early clinical trials (for recent clinical reviews, see (6, 7). Compounds which selectively inhibit CDK4 may be highly efficacious against tumors inactivated for p16 and p15, the physiological inhibitors of CDK4. Utilizing the NCI drug screen panel, Kubo et al. (8) identified a number of compounds with a selectivity towards CDK4 inhibition and against p16(-) tumor *cell lines*. 3-ATA (3-aminothioacridone, NSC680434) and TA (thioacridone, NSC521164) were two of the most efficacious compounds identified, exhibiting a 28-fold or greater inhibition of CDK4 kinase activity over other CDKs. While the exact mechanism of kinase inhibition by 3-ATA and TA are unknown, they have been shown to inhibit kinase activity without blocking/inhibiting p16 or ATP binding to CDK4. 3-ATA has further been shown to be able to attenuate kainic acid-induced apoptosis in neuronal cells (9). Using high throughput screening of small molecule compound libraries, Kent et al. (10) identified oxindole as showing inhibition of CDK4/cyclin D1 over CDK2/cyclin E and CDK1/cyclin B. Oxindole inhibited the growth of pRb(+) cell lines much more effectively than the growth of a pRb(–) cell lines, with a mechanism of kinase inhibition believed to be through the competitive inhibition of ATP binding to CDK4. Oxindole has also been shown to be an effective inhibitor of tyrosine kinases (11).

In view of the very high frequency of p16/p15-inactivation in T-ALL (5), we hypothesized that T-ALL may be highly sensitive to a therapeutic strategy targeting CDK4. Using UCN-01, we have demonstrated the effectiveness of this approach. In primary T-ALL, samples with an intact p16 were approximately 10-fold more resistant to the inhibition of DNA synthesis and viability by UCN-01 than were p16(-) samples (12). These studies demonstrate the impact of p16 protein status on the effectiveness of a compound for the treatment of a primary human cancer and suggest that targeting the CDKs may be an effective approach for those tumor cells that have lost the normal regulatory mechanism through p16 inactivation. We further speculated that compounds that are more efficacious in inhibiting CDK4 over other CDKs may selectively inhibit p16/p15 inactivated primary tumors. In this study, we have investigated the ability of CDK inhibitors 3-ATA, TA and oxindole, to suppress DNA synthesis and viability of primary T-ALL cells and correlated their efficacy with p16/p15/pRb status. We demonstrate that p16(+), p15(+) or pRb(-) samples are significantly less sensitive to 3-ATA and TA, but not to oxindole, than p16(-), p15(-) or pRb(+) samples. The lack of effect on pRb phosphorylation, the failure of the cells to arrest in the predicted G_0-G_1 phase and their ability to induce DNA damage, however, suggests that the despite the
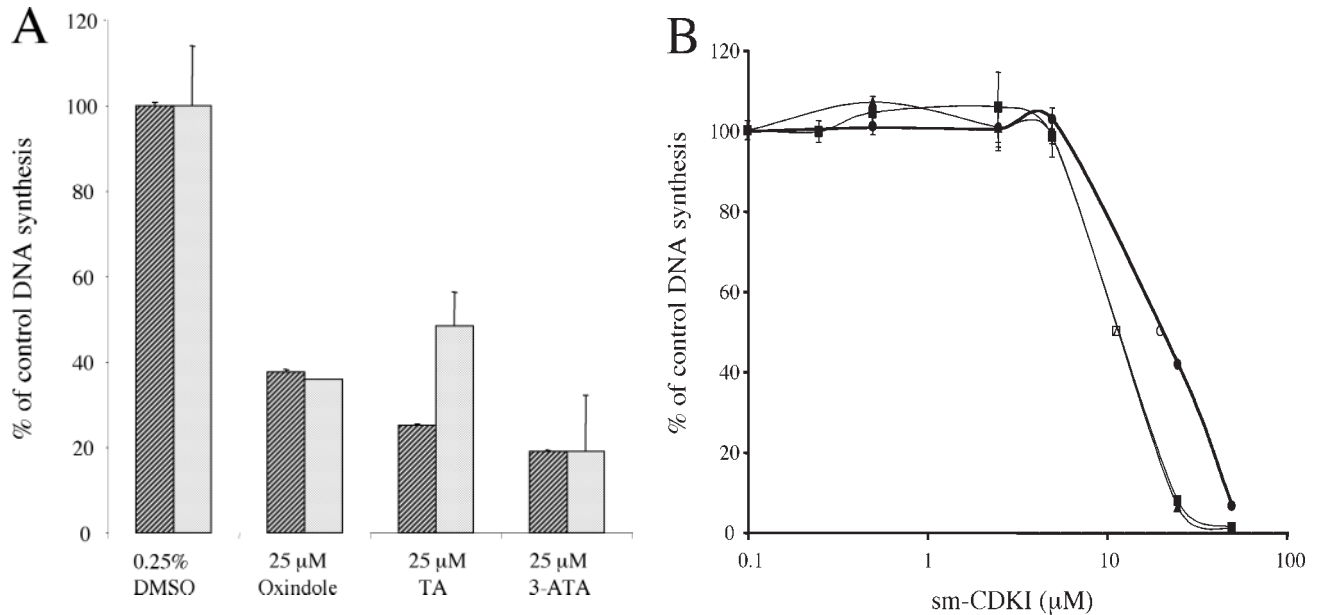
Figure 1. 3-ATA, TA and oxindole inhibit cell growth and thymidine incorporation in the T-ALL cell line CEM. A) The ability of the compounds to inhibit DNA synthesis (dark histograms) and the proliferation (light histograms) of CEM cells was investigated by culturing 5 X 10^6 CEM cells in RPMI plus 10% fetal calf serum in the presence of 25 μM Oxindole, TA or 3-ATA or 0.25% DMSO as a control for 72h. DNA synthesis was determined by thymidine incorporation. Viability was assessed by Trypan blue staining and counting. Data is plotted as percent of control. B) Dose-response analysis of inhibition of DNA synthesis in CEM was investigated by culturing 1 X 10^5 cells in a microtiter plate in the presence of varying concentrations of Oxindole (●), TA (▲) or 3-ATA (■). After 72h, cells were pulsed for 6h with 10 μCi thymidine and harvested. Percent of control (0.25% DMSO) thymidine incorporation is shown. Open symbols represent the IC50s of 18.6 μM, 14.1 μM and 13.0 μM for Oxindole, TA and 3-ATA, respectively.

strong correlation with p16, inhibition of DNA synthesis and growth by these compounds does not involve CDK4 inhibition, and is likely independent of their *in vitro* ability to inhibit CDK activity.

MATERIAL AND METHODS

MATERIALS

3-ATA, TA and NSC625987 were gifts from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. 3-ATA was further purchased from Alexis Biochemicals.

METHODS

Patients Population and Isolation of Primary T-ALL Cells. Heparinized bone marrow or peripheral blood samples were obtained from 26 T-ALL patients who were enrolled in Children's Oncology Group ALL biology protocol #9900 at the time of diagnosis. Mononuclear cells (MNC) were isolated from the

samples by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). The content of lymphoblasts in the samples was generally > 80%. All samples were utilized after obtaining informed consent and conform to HIPAA rules on confidentiality. Use of these samples has been approved by the UCSD committee on human subjects, IRB#021216.

Expression Analysis. For Western Blot Analysis, 25 μg of protein from whole cell lysates was denatured in sample buffer, separated on a 10% NuPage gel (Invitrogen Corp, Carlsbad, CA), transferred to an Immobilin-P membrane, blocked in 5% non-fat milk in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.05% Tween 20) and incubated for 1h at RT or overnight at 4°C with primary antibody. After TBST washing, blots were incubated for 1h at RT with 1:1000 alkaline phosphatase conjugated anti-mouse or anti-rabbit secondary antibody (KPL, Gaithersburg, MD), followed again by TBST washing, dH₂O rinsing and incubation with ECF substrate (Amersham Pharmacia
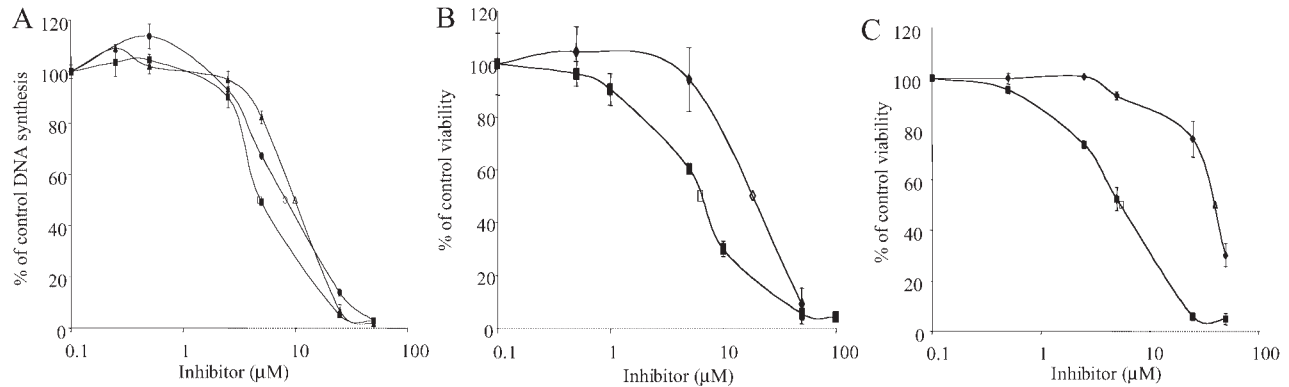
Figure 2. Sensitivity of primary T-ALL to 3-ATA, TA and oxindole. A) Inhibition of DNA synthesis in primary T-ALL was investigated by incubating 1 X 10^5 primary T-ALL cells in a microtiter plate in the presence of varying concentrations of Oxindole (●), TA (▲) or 3-ATA (■). After 72h, cells were pulsed for 6h with 10 µCi thymidine and harvested. Percent of control (0.25% DMSO) thymidine incorporation is shown. Sensitivity of a representative primary T-ALL (#051220) to all three compounds is shown. The IC50 (see Table 1) is indicated on the graph by open symbols. B-C) The effect of 3-ATA on DNA synthesis vs. viability in primary T-ALL was investigated by incubating 1 X 10^5 primary T-ALL cells in a microtiter plate in the presence of varying concentrations of 3-ATA for 24 or 72h. Viability was determined by Trypan blue exclusion and counting. DNA synthesis was measured in a parallel experiment by pulsing the cells for 6h with 10 µCi thymidine and determining incorporation. Percent of control is shown. B) 3-ATA induces a loss of viability in sample 182622, as determined by Trypan blue staining (◆), that is approximately 3-fold higher than that observed for inhibition of DNA synthesis (■) in this same sample at 72h. C) After as little as 24h, 3-ATA could induce a significant inhibition of thymidine incorporation (■). However, after only 24h, a loss of viability in sample 120101 is observed only at the very high concentrations of the drug (◆). Open symbols document the IC50s.

Biotech) for 5 min. at RT. Proteins were visualized by a STORM imager (Amersham Pharmacia Biotech). Antibodies: p16, p27 and p53 were purchased from Neomarkers Inc. (Fremont, CA) and used at 0.5µg/ml; pRb, Caspase 8, Bax, and serine 15 phosphorylated p53 were from Cell Signaling Technology (Beverly, MA) and used at 1:1000 unless otherwise indicated; β-actin (#AC15) was from Sigma-Aldridge (St. Louis, MO) and used at 1:100,000. Semi-quantitative PCR analysis of ARF was performed for 32 cycles of 30 sec each at 95ºC, 62ºC, 72ºC in 10 mM Tris-HCl, pH 9.1, 25 mM KCl, 1% DMSO, 1.5 mM $MgCl_2$, 40 µM 4dNTP, 0.2U Taq polymerase (Qiagen) and 10 pmol each sense (β238F; 5'-CTGGAGGCGGCGA GAACAT-3') and antisense (3p16; 5'-CTACGAAA GCGGGGTGGGTTGT-3') primer.

Clonogenic Assay for Normal Hematopoietic Progenitor Cells. Cultures of hematopoietic progenitor cells were performed essentially as described previously (13). Briefly, 2 x 10^5 mononuclear cells (MNC) from normal bone marrow donors were plated in 35-mm Petri dishes containing methylcellulose 0.1 µM -thioglycerol, 15% horse serum, 15% FCS, 1% BSA, 10 ng/ml PMA, and 100 units/ml recombinant human IL-2 (Immunex, Seattle, WA) and incubated at 37°C in the presence of increasing concentrations of 3-ATA. Replicates were set up for each individual data point/experiment. After 14 days, colonies were counted, and the concentration of 3-ATA resulting in 50% inhibition of colony formation in the cultures (IC50) was calculated. BFU-E (erythroid burst-forming unit) was identified as a large aggregate of more than 64 hemoglobinized cells or as clusters of three or more subcolonies consisting of eight or more

Table 1 *Summary of the IC50's of the 3 agents on thymidine incorporation and their CDKI status in primary T-ALL.*

| | | DNA Synthesis | | | 72h Viability | Expression | | | |
| | | 3-ATA | TA | Oxindole | 3-ATA | protein | mRNA | protein | protein |
	ID	IC50 (μM)	IC50 (μM)	IC50 (μM)	IC50 (μM)	p15	ARF	p16	pRb
1	201205	0.6	7.6	0.5	nd	-	-	-	+
2	120101	1.3	9.5	23.9	nd	-	-	-	nd
3	031518	1.8	8.7	nd	10.6	-	-	-	+
4	190301	2.2	6.4	25.9	nd	-	-	-	+
5	050413	2.2	10.0	3.8	nd	-	-	-	+
6	050118	3.9	12.9	15.9	40.0	-	-	-	nd
7	051220	4.8	10.0	8.1	nd	-	-	-	nd
8	080511	5.6	12.6	3.8	nd	-	-	-	+
9	031512	6.2	14.5	0.5	nd	-	+	-	+
10	102018	6.3	18.6	nd	nd	-	-	-	+
11	030807	6.3	10.9	2.5	nd	nd	nd	-	nd
12	182622	6.5	20.2	8.0	18.4	-	-	-	+
13	021920	7.4	27.8	3.9	nd	-	+	-	+
14	201525	7.5	50.0	nd	nd	-	-	-	+
15	021807	9.3	40.0	21.0	23.7	-	+	-	nd
16	221407	12.1	12.3	10.5	nd	nd	nd	-	+
17	010201	17.0	50.0	nd	>50	-	-	-	+
18	131908	4.6	11.3	5.0	nd	-	+	-	-
19	200505	4.8	39.8	3.5	nd	-	+	+	+
20	130113	16.6	35.6	50.0	10.0	-	-	-	-
21	081523	18.5	29.5	4.1	nd	-	+	+	-
22	180505	50.0	24.0	3.3	nd	-	+	+	+
23	181005	50.0	17.0	14.6	nd	-	-	-	-
24	230419	57.1	54.4	50.0	nd	-	-	-	-
25	100418	62.8	59.2	8.2	nd	+	+	-	+
26	192001	24.5	27.7	17.7	nd	na	na	na	na
	avg ± stdev	*15.0 ± 18.4 n=26	*23.9 ± 16.5 n=26	*12.9 ± 14.1 n=22					

na, not available; nd, not determined; p=0.045; Repeated Measures ANOVA at n=22

hemoglobinized cells/subcolony. The CFU-GM (granulocyte/monocyte colony-forming unit) was enumerated as a group of more than 50 granulocytic/monocytic translucent cells.

Effect of 3-ATA, TA and oxindole on DNA Synthesis and Viability. The effect of the three agents on DNA synthesis was assessed by ^{3}[H]thymidine incorporation. Primary T-ALL cells (1 x 10^5 cells) were plated in triplicate in 200 μl of complete RPMI in 96-well plates in presence of the indicated concentration of drug (see figure) or 0.125% or 0.25% DMSO (carrier) as a control. After 24 – 72h in the presence of drug at 37°C, cells were pulsed for 6h with 10 μCi ^{3}[H]thymidine and incorporation determined with the use of an automated microtiter harvester and scintillation counting. For viability assays, the experimental protocol was essentially identical except that after a 24–72h incubation in the presence of drug, cell numbers were counted by hemocytometer and viability assessed by Trypan blue exclusion.

Comet Assay for DNA Damage. The single cell gel electrophoresis (Comet) assay is a sensitive assay that allows the direct visualization of DNA fragmentation associated with DNA damage in individual cells. In cells harboring DNA damage, alkaline lysis allows the unwound, relaxed DNA to migrate out of the cell during electrophoresis which, when visualized with SYBR® Green, appear as fluorescent "tail" while normal, undamaged DNA does not migrate far from the origin. To assess DNA damage by the experimental agents in CEM or primary T-ALL samples, cells were incubated for 24h with drug before DNA was visualized with SYBR® Green using the Trevigen Comet Assay Kit (Trevigen Inc., Gaithersburg MD) according to the manufacturers directions. In order to quantify the extent of DNA damage, an entire field of cells (usually 80 or more) were counted and assigned to one of four categories depending on the extent of their comet tail. Cells that were round or egg shaped with little comet tail were considered negative for DNA damage. Cells that had moderate or very long comet tails were considered to harbor extensive DNA damage.
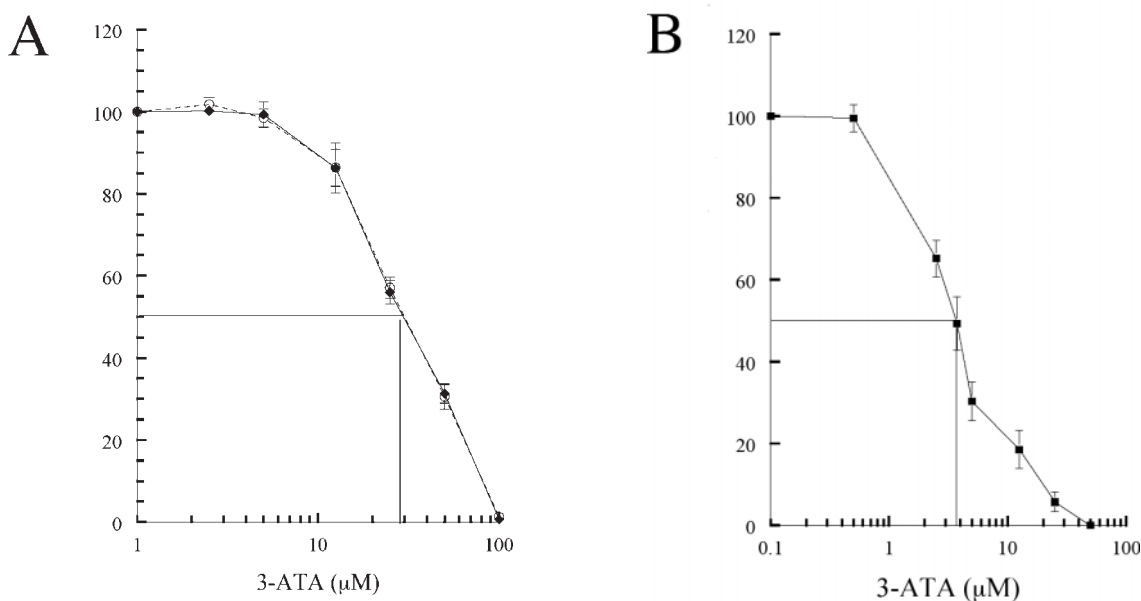
Figure 3. Effect of 3-ATA on colony formation of primary T-ALL cells. Inhibition of colony formation in MNCs of bone marrow from 4 healthy donors (A) were analyzed for normal hematopoietic CFU-GM (solid line) and BFU-E (dashed line) cells versus cells obtained from 4 T-ALL patients (B) cultured with increasing amounts of 3-ATA. The IC50 for 3-ATA, shown by the lines, was determined as the concentrations at which there was 50% inhibition of colony formation for T-ALL and for CFU-GM and BFU-E colonies, respectively. Unpaired t-test indicated that the IC50 for T-ALL (3.1 ± 0.3) is significantly different from those for CFU-GM (28.8 ± 4.1) and BFU-E (29.1± 5.1) from normal marrow (both P < 0.001).

FACS Analysis. Primary T-ALL cells at 1×10^6 cells/ml were incubated in complete media plus drug for 24-72h. For cell cycle analysis, an aliquot was removed, PBS washed and fixed in 60% ethanol at $4^{\circ}C$. Samples were washed with 1X PBS/1% BSA, then resuspended in 1X PBS/1% BSA/0.1% Tween 20, with 20 µg/ml RNAse A and 50 µg/ml Propidium Iodide (PI) for 1h, and analyzed by flow cytometry on a BD Biosciences FACSCalibur (Mansfield, MA).

RESULTS

We first examined the ability of 3-ATA, TA and oxindole to inhibit DNA synthesis in the T-ALL cell line CEM, which was previously demonstrated by our group to be sensitive to the non-specific kinase inhibitor UCN-01 (12). At 25µM, all three agents were effective inhibitors of DNA synthesis, with 80%, 75% and 62% inhibition by 3-ATA, TA and oxindole, respectively (Figure 1A). Dose-response analysis showed that all three compounds had similar average IC50s (Figure 1B), with a mean IC50 of 13 µM for 3-ATA (11.4 µM and 14.5 µM in two experiments), of 14.1 µM for TA (11.5 µM and 16.8 µM) and of 18.6

µM for Oxindole (20 µM and 17.1 µM). In addition to inhibiting DNA synthesis, the compounds suppressed the growth of CEM cells to comparable extents as that observed for inhibition of DNA synthesis (Figure 1A).

The efficacy of these agents on the inhibition of DNA synthesis was next examined in primary T-ALL samples. The three compounds showed a dose dependent inhibition of DNA synthesis with IC50s ranging from 0.6 µM to greater than 50 µM (Table 1), with average IC50s of 15.0 ± 18.4 for 3-ATA (n=26), 23.9 ± 16.5 for TA (n=26), and 12.9 ± 14.1 for oxindole (n=22). A representative sample is shown in Fig 2A. Despite similar overall IC50s, individual T-ALL samples often showed very different sensitivities to each of the compounds (Table 1). For example, though sample 051220 was sensitive to all 3 agents in the low µM range, sample 180505 had a low IC50 to oxindole only. Sample 120101, on the other hand, had a very low IC50 for 3-ATA relative to TA and oxindole, while sample 230419 was very insensitive to all three compounds. None of the samples had a low IC50 for TA exclusively. We sought to determine if the differences observed with a given patient sample to the 3 agents represented different efficacies of the compounds. Of the 26 primary patient samples in this study, 22 were analyzed for
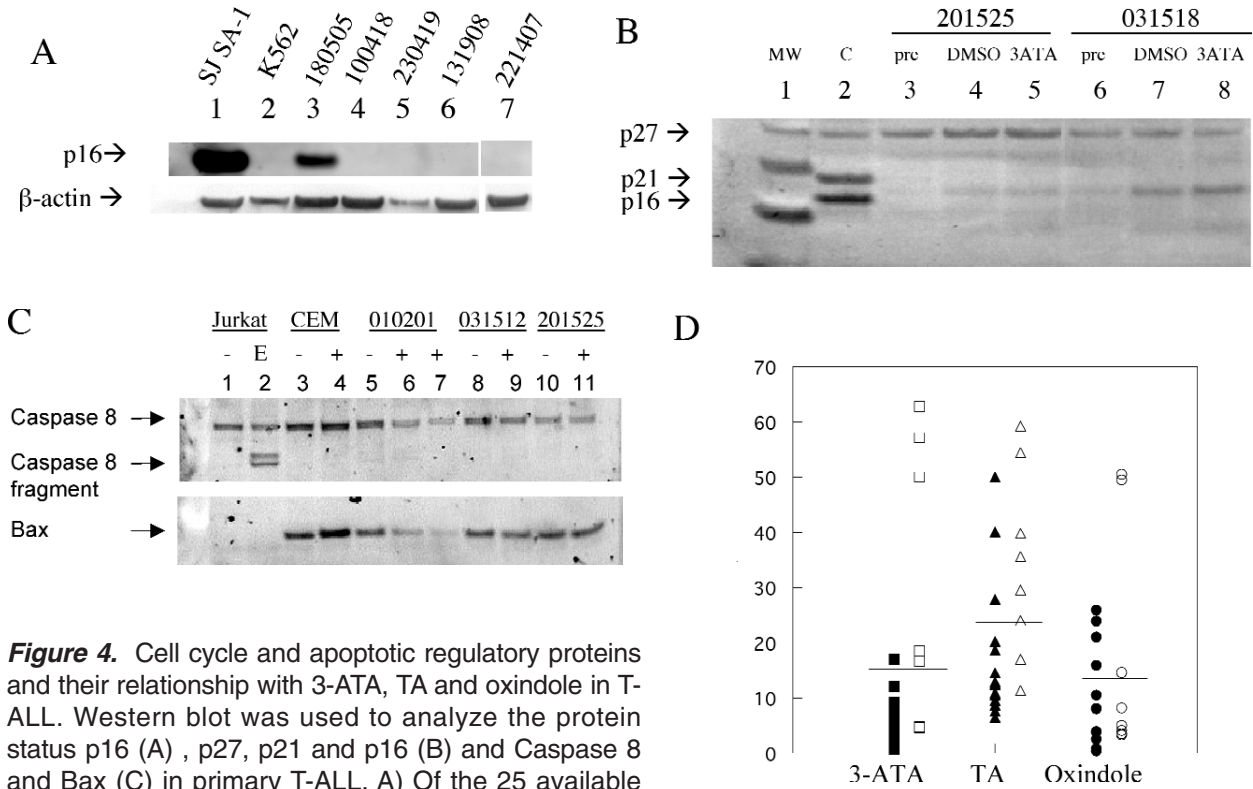
Figure 4. Cell cycle and apoptotic regulatory proteins and their relationship with 3-ATA, TA and oxindole in T-ALL. Western blot was used to analyze the protein status p16 (A) , p27, p21 and p16 (B) and Caspase 8 and Bax (C) in primary T-ALL. A) Of the 25 available samples, only 3 were positive for p16, as seen in Lane 3 with sample #180505. Extracts from the osteosarcoma cell line SJ-SA 1 (p16+, p27+ and p21+) are shown as positive controls in (A) and (C), the leukemia cell line K562 is shown as a negative control for p16 in (A). Sample IDs are as in Table 1. B) p16, p21 and p27 expression is measured in protein extracts from the pre-treated (pre) samples as well as after a 24h incubation with carrier (DMSO), 6.25 µM 3-ATA (sample 201525) or 12.5 µM 3-ATA (sample 031518). Neither p16 nor p21 is expressed in either of these samples, while p27 is expressed in both. 3-ATA did not change the expression profiles of any of the three genes in either of the samples. A non-reproducible artifact band between p16 and p21, whose identify is unknown, can be sometimes observed, as in sample 031518. C) Caspase 8 and Bax protein expression was analyzed in CEM and primary T-ALL cells before and after incubation with 3-ATA. Jurkat cells untreated (lane 1) or treated for 6h with 10 µg/ml etoposide (lane 2) is shown as a control for caspase 8 activation. CEM cells incubated for 22h with 0.125% DMSO (lane 3) or 25 µM 3-ATA (lane 4), T-ALL patient 010201 untreated (lane 5) or incubated for 22h (lane 6) or 72h (lane 7) with 5 µM 3-ATA, T-ALL patient 031512 incubated for 22h with 0.125% DMSO (lane 8) or 12.5 µM 3-ATA (lane 9), and T-ALL patient 201525 incubated for 22h with 0.125% DMSO (lane 10) or 6.25 µM 3-ATA (lane 11), are shown. Neither caspase 8 activation nor alterations in Bax expression were observed after 3-ATA treatment. D) The IC_{50}s of 3-ATA, TA and oxindole were plotted relative to G_1 pathway status. Open symbols represent samples harboring a putative refractory G_1 pathway (p16 and/or p15 positive and/or negative for pRb) and the closed symbols represent those samples harboring a putative sensitive G_1 regulatory pathway (p16 and/or p15 negative and/or with an positive pRb). A highly significant association is observed with 3-ATA (P=0.006) and TA (p=0.02) and putative refractoriness, while no statistical association was observed with oxindole (P=0.55). Statistical analysis by Fisher's Exact Test.

sensitivity to all three compounds (Table 1). We thus used the data on these 22 patients' samples to perform a repeated measure ANOVA analysis. This test compares data matched across rows, and thus weighs the fact that each patient sample is analyzed for sensitivity to all three compounds. When ANOVA is calculated in this

fashion, a small but statistically significant difference is observed (p=0.045), suggesting the compounds may have slightly different efficacies despite similar overall average IC50s.

In spite of their aggressive behavior *in vivo*, primary T-ALL cells proliferate poorly in liquid culture.

Therefore, to test for an effect on T-ALL cell proliferation, we examined the ability of 3-ATA to inhibit colony formation of normal hematopoietic progenitors versus T-ALL cells, using a clonogenic assay (13). 3-ATA inhibited colony formation of normal hematopoietic progenitor cells in 5 normal bone marrow samples from healthy donors with IC50's of $28.8 \pm 4.1 \mu M$ and $29.1 \pm 5.1 \mu M$ for CFU-GM and BFU-E, respectively (Fig. 3A). In contrast, the IC50 of 4 primary T-ALL samples was only $3.8 \pm 6.5 \mu M$ (p<0.001; Fig 3B). Thus, as we observed with CEM cells, 3-ATA has the capacity to inhibit cell growth as well as to inhibit DNA synthesis.

Cancer cells that express p16 and p15 may likely be refractory to agents that mimic the functions of these proteins, such as small molecule cyclin dependent kinase inhibitors (sm-CDKI), as they are capable of proliferation despite expression of these cell cycle regulatory genes. Furthermore, the action of both the endogenous p16 and sm-CDKI should be dependent on the presence of an intact and functional pRb, the substrate of CDK4 activity. We thus sought to determine if the expression of p16, p15 and pRb in these samples correlated with their sensitivity to these compounds (Table 1). Western blot analysis of p16 (Fig 4A) showed that 22 of 25 (88%) samples lacked detectable p16 protein (Table 1), while 22 of 23 (96%) lacked p15 (Table 1). These rates of inactivation are consistent with the 93% and 99% rates of inactivation of p16 and p15, respectively, in T-ALL we have previously reported (5). In a western blot analysis of total pRb (Fig. 5A), no pRb protein was observed in 5 of 20 samples investigated (Table 1). When the IC50s of 3-ATA, TA and oxindole were plotted for primary T-ALL according to their p16, p15 and pRb status (Fig 4D), a significant correlation between CDKI/pRb status and sensitivity to 3-ATA and TA becomes apparent. For 3-ATA, six of 8 samples that were p16(+), p15(+) or pRb(-) had IC50s greater than the mean IC50 of 15.0 μM, with 4 of the 6 samples more than 2-fold above the mean. In contrast, only 1 of the 17 p16(-), p15(-) or pRb(+) samples was above the mean (p=0.001, Fisher's Exact Test). A similar result was observed for TA, where again 6 of 8 p16(+), p15(+) or pRb(-) samples had IC50s above the mean of 23.9 μM while only 4 of 17 p16(-), p15(-) or pRb(+) samples were above the mean (p=0.028, Fisher's Exact Test). In contrast to 3-ATA and TA, the distribution of samples above and below the mean IC50 of 12.9 μM for oxindole was similar in both groups with 4 of 13 p16(-), p15(-) or pRb(+) samples and 3 of 8 p16(+), p15(+) or pRb(-) samples above the mean (p=1.00, Fisher's Exact Test). Analysis of the average IC50s of each group confirmed a highly significant correlation between CDKI/pRb status and sensitivity to 3ATA and

TA. The average IC50 of 3-ATA for p16(+), p15(+) or pRb(-) samples ($33.1 \pm 24.3 \mu M$, n=8) is 5-times greater than it is for p16(-), p15(-) or pRb(+) samples ($5.9 \pm 4.2 \mu M$, n=17; p=0.006, Mann-Whitney Test). For TA, less than a 2-fold difference in sensitivity was observed, though the differences are again statistically significant ($33.9 \pm 17.0 \mu M$ (n=8) vs. $18.9 \pm 14.4 \mu M$ (n=17), p=0.02). In contrast, there was no association of CDKI/pRb status with oxindole sensitivity ($17.3 \pm 20.5 \mu M$ (n=8) vs. $9.9 \pm 8.9 \mu M$ (n=13), p=0.55).

Based on its reported function as an inhibitor of CDK4 and CDK6 (8), 3-ATA is expected to block DNA synthesis by inhibition of pRb phosphorylation. We thus sought to confirm that in samples with an intact pRb and sensitivity to 3-ATA, the drug is indeed acting through CDK inhibition by looking at pRb phosphorylation at Ser780 and Ser795, two sites known to be targets of CDK4 – catalyzed phosphorylation. However, there was no decrease in total pRb or pRb phosphorylation at either of the serine's after an overnight incubation with 3-ATA (Fig 5B), despite the fact that both of the samples shown in Fig 5B have low IC50's for the 3-ATA (see Table I) and, therefore, a decrease in pRb phosphorylation would have been expected. Similarly, we investigated pRb phosphorylation in CEM cells after an overnight treatment with 25 μM of each of the three agents used here. Despite the suppression of DNA synthesis and proliferation by each of these agents (Fig 1A), no notable decrease in pRb phosphorylation at Ser780 or Ser795 was observed and the total levels of pRb remained unchanged (Fig 5C). The treatment of CEM cells with 3-ATA at time points as short as one hour and as long as 72 hours also failed to inhibit pRb phosphorylation (data not shown). We further considered the possibility that alternative phosphorylation sites may be influenced by 3-ATA, such as Ser 807/811, which lies in the cAbl binding site and Ser608, a site for which the responsible kinase has not been reported. However, 3-ATA did not affect the phosphorylation status of these sites either (Fig 5C).

Phosphorylation of pRb is an early step in S-phase progression, with CDK4 directed phosphorylation of pRb driving the cell into the G_1 phase of the cell cycle. Consistent with this, ectopic expression of p16 arrests cells in the G_1 phase (14). As we observed no decrease in pRb phosphorylation, we sought to determine whether growth arrest by 3-ATA was arresting cells in the G_1 phase as hypothesized. Two primary T-ALL samples, sensitive to 3-ATA as determined by thymidine incorporation studies, were treated for 72h with 3-ATA and analyzed by PI staining and flow cytometry. In contrast to the expected G_1 accumulation, the distribution of cells through the cell cycle remained essentially unchanged after 3-ATA treatment in both
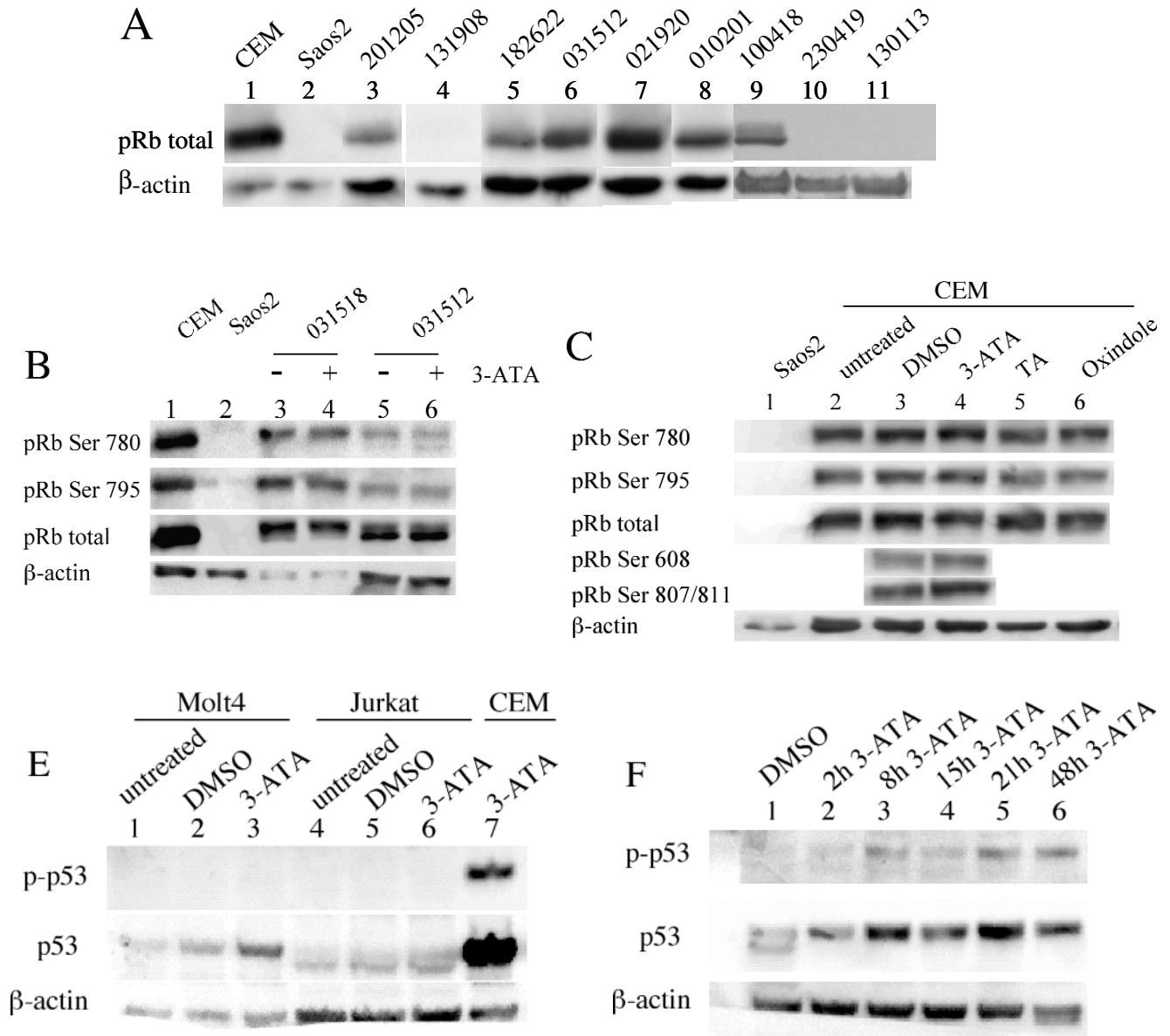
Figure 5. pRb and p53 analysis in primary and T-ALL cell lines treated with 3-ATA, TA and oxindole. A) Western blot analysis of total pRb in primary T-ALL is shown. Samples in lanes 4, 10 and 11 lacked pRb expression. Extract from Saos2 cells are included as a negative control for pRb, whereas in CEM cells are used as positive controls. B) The two primary T-ALL patients shown were treated with 12.5 μM 3-ATA overnight and pRb phosphorylation measured at two known CDK4 phosphorylation sites, Ser 795 and Ser780. No change in the amount of total pRb could be detected, and no decrease in pRb phosphorylation at either of these sites was seen in either T-ALL. CEM cells are shown as a positive controls. C) CEM cells were incubated overnight in the presence of 25μM of each of the three compounds, with the carrier DMSO or were untreated. No change in the amount of pRb at any of the phosphorylation sites with any of the agents was observed. D) T-ALL cell lines Molt4, Jurkat and CEM were either untreated or incubated overnight with either 10 μM (Molt 4 and Jurkat) or 25 μM (CEM) 3-ATA. Extracts were then probed for total p53 or Serine 15 phosphorylated p53. In both Molt4 and CEM, 3-ATA induced an increase in p53 expression. In CEM cells, 3-ATA further resulted in the phosphorylation of p53 at Serine 15 (See panel F for untreated control comparisons of CEM). Jurkat cells are negative for p53 protein. E) Time dependent increase in both the amount of total and serine 15 phosphorylated p53 in CEM cells treated with 25 μM 3-ATA.
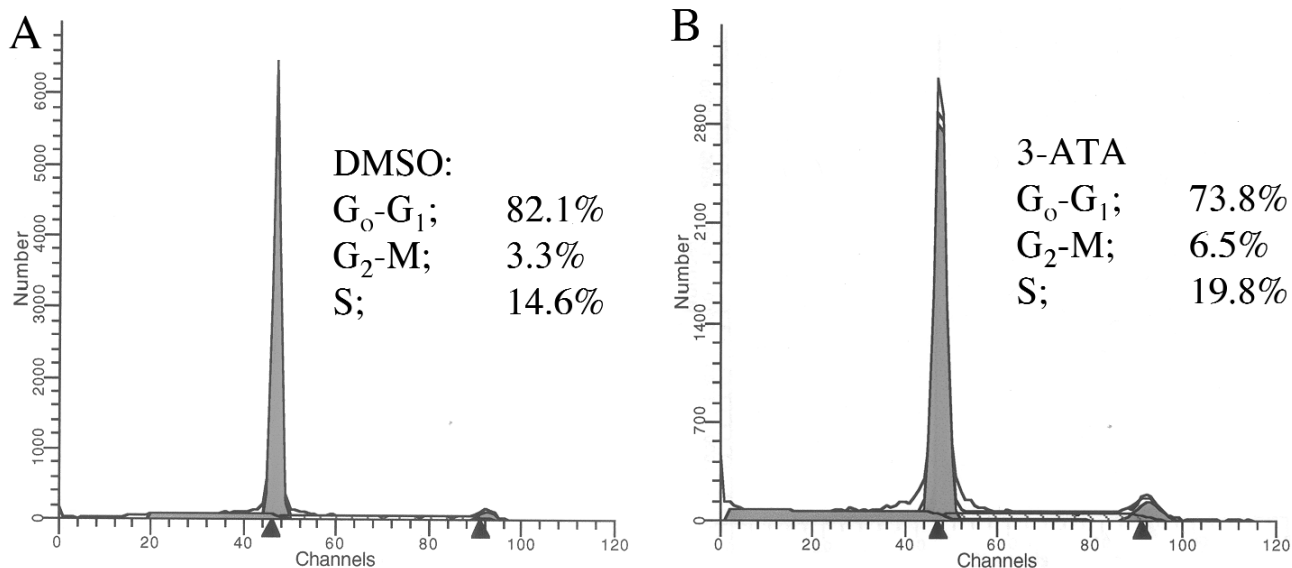
Figure 6. Effect of 3-ATA on cell cycle distribution in primary T-ALL. Primary T-ALL cells from patient 221407 were incubated for 72h with 10 µM 3-ATA or carrier, collected and analyzed by flow cytometry for cell cycle distribution. After treatment with 3-ATA (B), there is essentially no change in the distribution of cells through the cell cycle from those treated with carrier (DMSO), panel A.

primary T-ALL (Fig 6A-B) and in the CEM cell line (data not shown).

We observed that 1 of the 3 p16 positive and 2 of 5 pRb negative samples were quite resistant to 3-ATA. Sample 200505, however, which was p16 positive and sample 131908, which was pRb negative, were very sensitive to 3-ATA. Furthermore, this latter sample was also very sensitive to oxindole, a compound whose sensitivity was characterized as dependent on an intact pRb (10). The lack of a decrease in pRb phosphorylation at CDK4 sites in either primary T-ALL samples or the T-ALL cell line CEM, as well as the failure of the drug to cause arrest cells in the G_0-G_1 phase, led us to investigate alternative mechanisms by which these compounds may function. UCN-01, for example, in addition to inhibiting CDKs directly, has been shown to induce the expression of p21 and p27 (15). We have observed that most primary T-ALL samples have undetectable levels of p21 protein despite expressing p21 transcript while p27 is readily detectable at both the mRNA and protein levels in most T-ALL samples (unpublished data). We considered that the induction of either of these genes may act as an alternative mechanism of 3-ATA action. Using both the CEM cell line and primary T-ALL, we measured p21 and p27 protein levels after a 24h exposure to 3-ATA, a time point when significant inhibition of DNA synthesis was observed (for example, see Fig. 2C). P21 protein levels remained undetectable even after 3-ATA treatment in

both the cell line (data not shown) and in 3-ATA sensitive primary samples (Fig 4B). P27 levels, though detectable in both the cell line and the primary sample, also did not change after 3-ATA treatment (Fig 4B).

Both flavopiridol and UCN-01 cause cell death in addition to inhibition of DNA synthesis and growth arrest. We thus considered whether 3-ATA may be influencing cell growth by actions on the cell death pathway. Using Trypan blue exclusion, we observed a dose-dependent loss of viability after a 72h treatment with 3-ATA in all 6 of the primary T-ALL samples tested (Table 1), examples of which are shown in Fig. 2B and 2C. The IC50 for loss of viability is in general higher than that required for inhibition of thymidine incorporation. In 5 of the 6 primary T-ALL samples, IC50s ranged from almost 3-times higher than that observed for inhibition of DNA synthesis (i.e., sample 182622, Table 1, Fig. 2B) to greater than 10-fold (i.e., sample 050118, Table 1). In the sixth sample, the IC50 for loss of viability was similar to that for inhibition of DNA synthesis (sample 130103, Table 1). The difference in sensitivity of T-ALL to inhibition of DNA synthesis versus loss of viability is also observed when the cells are incubated for just 24h in the presence of drug. At this time point, significant inhibition of thymidine incorporation in primary T-ALL samples by 3-ATA was observed, while viability was affected only at high concentrations (Fig. 2C).
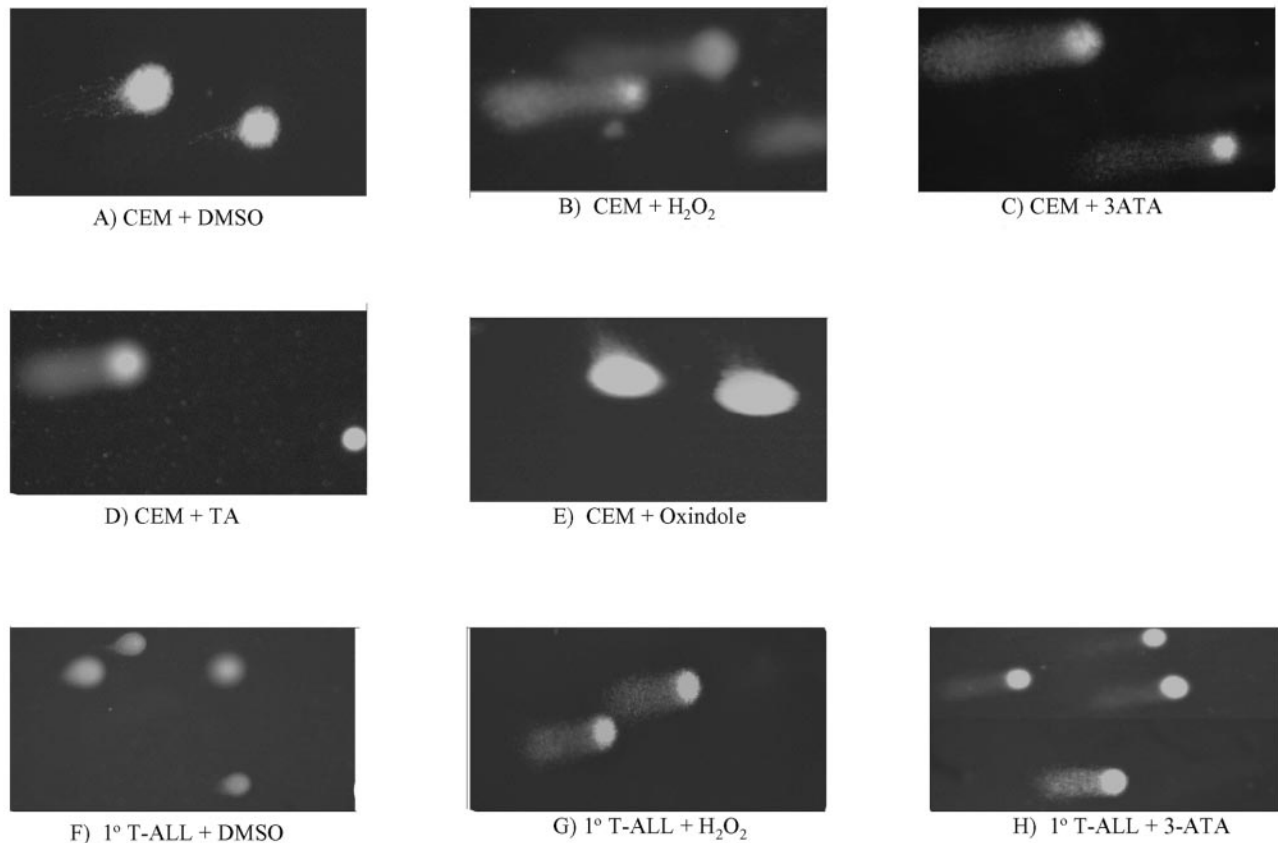
The ARF gene is composed of the second exon of

Figure 7. Induction of DNA damage in T-ALL cell lines and primary T-ALL by 3-ATA. CEM cells or cells from a primary T-ALL patient were incubated overnight with either carrier (0.125% DMSO; A and F), or with experimental agent (25 μM 3-ATA (C), 12.5 μM 3-ATA (H), 25 μM TA (D) or 25 μM Oxindole (E)). As a positive control, cells were incubated for 20 min with 100 μM H_2O_2 (B and G). DNA damage is characterized by the appearance of a long "comet" tail. 3-ATA induced extensive DNA damage in both the CEM cell line as well as the primary T-ALL patient sample. To a lesser extent, TA also induced DNA damage while no DNA damage was apparent with Oxindole treatment. Absolute numbers and statistical analysis of comet tail positives vs. negatives for each condition are summarized in Table 2.

p16 uniquely spliced onto an alternative first exon located between p15 exon 2 and p16 exon 1. Consistent with this localization, it is also frequently inactivated in T-ALL (4, 5). We considered the possibility of a relationship with ARF expression and sensitivity to 3-ATA. As to be expected from the high rate of p16 inactivation, ARF expression was observed in only 8 of the 23 T-ALL samples (Table 1). There was no relationship between the expression of ARF and sensitivity to 3-ATA, TA or oxindole. The average IC50 of 3-ATA for ARF(+) samples (20.5 ± 22.9 μM, n=8) was not statistically different than it is for the ARF(-) samples (12.2 ± 17.5 μM, n=15; p=0.15, Mann-Whitney Test). Similarly, there was no significant difference in TA sensitivity between ARF(+) samples (30.8 ± 15.5 μM, n=8) and ARF(-) samples (21.6 ± 17.1 μM, n=15; p=0.10, Mann-

Whitney Test) nor in sensitivity to oxindole and ARF(+) samples (6.2 ± 6.4 μM, n=8) and ARF(-) samples (18.6 ± 17.5 μM, n=11; p=0.09, Mann-Whitney Test).

Activation of p53 is an early first step in the activation of the apoptotic pathway. 3ATA treatment of the T-ALL cell lines CEM and MOLT4 resulted in an increase in the levels of p53 (Fig. 5E) that was both time (Fig. 5F) and dose-dependent (data not shown). In the CEM cell line, which contains a mutant p53, 3-ATA could also induce the phosphorylation of p53 (Fig. 5E-F) as well as resulted in the appearance of an ~ 200Kda, non-MDM2 immunoreactive protein (data not shown). The activation of p53 led us to consider other components of the apoptotic pathway. Bax is a pro-apoptotic, p53 trans-regulated protein. However, neither a 24h to 72h incubation with 5-25 μM 3-ATA

Table 2. *Quantitation of cells exhibiting DNA damage as measured by comet assay*

A. CEM

Tail length	0.125% DMSO	100 µM H_2O_2	25 µM 3-ATA	TA 25 µM	25 µM Oxindole
None or small	93 (95%)	48 (53%)	7 (7%)	76 (86%)	97 (97%)
Moderate or long	5 (5%)	42 (47%)	93 (93%)	12 (14%)	3 (3%)
Representative Figure	7A	7B	7C	7D	7E

DMSO vs. 3-ATA; p< 0.001, Fishers Exact Test *DMSO vs. TA; p= 0.07, Fishers Exact Test* *DMSO vs. Oxindole; p< 0.50, Fishers Exact Test*

B. Primary T-ALL

Tail length	0.125%DMSO	100 µM H_2O_2	12.5 µM 3-ATA
None or small	60 (100%)	77 (50%)	40 (40%)
Moderate or long	0 (0%)	76 (50%)	59 (60%)
Representative Figure	7F	7G	7H

DMSO vs. 3-ATA; p<0.001, Fishers Exact Test

CEM (**A**) and primary T-ALL (**B**) cells were incubated with carrier (DMSO), or 25 µM 3-ATA, TA or Oxindole for CEM or 12.5 µM 3-ATA for primary T-ALL overnight and then assessed by comet assay for the presence of DNA damage as described in the Materials and Methods. As a positive control, samples were incubated with 100 µM H_2O_2 for 20 minutes prior to assay. The number of cells exhibiting each of 4 lengths of comet tails are shown. Representative cells are shown in the indicated figure. For statistical purposes, no comet tails and small comet tails were regarded as "negative" (no DNA damage) and compared to moderate and long comet tails (significant DNA damage).

resulted in the induction of Bax (Fig 4C) nor induced the formation of Bax dimers (data not shown) in either CEM or primary T-ALL cells. Activation of caspase 8 is a early mediator of the apoptotic response. As shown in Fig 4C, etoposide treatment of Jurkat cells clearly results in the activation of caspase 8 within 6h, the earliest time point investigated. However, neither 3-ATA treatment of CEM, Molt4 nor Jurkat T-ALL cell lines, nor a 24 to 72h treatment of primary T-ALL samples of varying sensitivity to 3-ATA, resulted in the activation of caspase 8.

Activation of p53 is also associated with the occurrence of DNA damage. Thus the ability of 3-ATA to induce DNA damage on CEM and primary T-ALLs was investigated. As shown in Fig 7, CEM cells incubated for 24h with 0.125% DMSO are essentially circular with very little comet tail (Fig 7A), while 100µM H_2O_2 treatment, serving as a positive control, induced long and profound comet tails (Fig 7B), indicative of a significant amount of DNA damage. A similar though not as pronounced pattern was also observed with a 24h treatment of CEM cells with 25 µM 3-ATA (Fig 7C) and TA (Fig 7D), while 25 µM oxindole treated cells were not noticeably different than DMSO controls (Fig 7E). Analogous results were obtained in three other studies of CEM cells treated with 20 to 25 µM 3ATA. We next sought to determine if 3-ATA also induced DNA damage in primary T-ALL cells. As with CEM cells, DMSO treatment of patient T-ALL cells yielded minimal comet tail (Fig 7F) while pronounced comet tails were observed with 100µM H_2O_2 treatment as a positive control (Fig 7G) and with

a 24h treatment of 12.5 µM 3-ATA (Fig 7H). By categorizing cells based on the length of their comet tails (see Methods), 3-ATA was determined to be a significant factor in the induction of DNA damage in both CEM and primary T-ALL (see Table 2).

DISCUSSION

In this report we have documented the sensitivity of primary T-ALL to three *in vitro* inhibitors of CDK4/6, 3-ATA, TA and oxindole, with inhibition of DNA synthesis and loss of viability. The three compounds had similar overall IC50s. However, when sorted by G_1 protein status, p16(-), p15(-) or pRb(+) samples were significantly more sensitive to 3-ATA and TA than p16(+), p15(+) or pRb(-) samples. No differential sensitivity to oxindole was observed and no relationship of ARF status and sensitivity to any of the compounds was observed. While these findings are consistent with the *in vitro* characterization of these compounds as CDK4/6 inhibitors, treatment of primary T-ALL or cell lines with 3-ATA did not lead to inhibition of pRb phosphorylation or arrest cells in the G_0- G_1 phase. 3-ATA was further shown to induce p53 expression and phosphorylation and cause DNA damage, but did not activate the pro-apoptotic proteins Bax or Caspase 8. Taken together, 3-ATA and TA can be effective inhibitors of DNA synthesis in primary T-ALL in a p16-dependent manner, but their primary mechanism is independent of their *in vitro* ability to inhibit CDKs.

T-ALL has a very high frequency of p16 inactivation and as such is an ideal disease for therapeutic agents targeting CDK4/6. We hypothesized that primary tumors with p16 inactivation are likely to be sensitive to agents that mimic the action of p16. On the other hand, samples expressing p16 are much more likely to be refractory to these agents as they are able to proliferate despite expression of this cell cycle regulatory gene. Previously, we have reported that UCN-01, a non-specific inhibitor of CDKs, was efficacious in inhibiting DNA synthesis and viability in primary T-ALL, and that this activity was dependent on p16 protein status (12). In this study, we observed a similar finding in that sensitivity of primary T-ALL to 3-ATA and TA correlated significantly with p16, p15 and pRb status.

Studies examining the effects of p16 transfection on cell cycle and pRb phosphorylation have shown that cell lines lacking p16 are growth inhibited by p16 transfections. These cell lines demonstrate decreases in pRb hyperphosphorylation and G_1 arrest, an observation consistent with CDK4 inhibition; cell lines expressing p16 did not arrest and did not show changes in pRb hyperphosphorylation (14, 16, 17). The sm-CDKI flavopiridol has also been demonstrated to exert a similar effect. Flavopiridol treatment of the p16(-) breast cancer cell line MCF-7 induced a decrease in pRb hyperphosphorylation and G_1 arrest (18). In melanoma, p16(-) cells lines were more sensitive to flavopiridol than p16(+) cell lines (19). As mentioned previously, we observed that UCN-01 was an effective inhibitor of DNA synthesis in primary T-ALL, and that samples lacking p16 were significantly more sensitive than those expressing p16 (12); cell cycle status and pRb phosphorylation status were not investigated in that study. In the present analysis, we observed that sensitivity to 3-ATA and TA also correlated with p16/p15/pRb status. However, no inhibition of pRb phosphorylation was found in treated primary samples or cell lines, and 3-ATA failed to arrest cells in G_0 - G_1. The lack of a detectable change in pRb phosphorylation did not appear to be due to a transient decrease in pRb phosphorylation as no changes in pRb phosphorylation were observed when 3-ATA treated CEM cells were sampled at multiple time points between 2h and 72h (data not shown). It is notable that the original characterization of 3-ATA as a CDKI used a purified GST-pRb system to characterize the ability of 3-ATA and TA to inhibit kinase activity (8). As pRb is a well known target of CDK4 activity, it is reasonable to assume that because 3-ATA inhibits pRb phosphorylation *in vitro*, pRb is also the likely target *in vivo*. However, it is also possible that p130 and p107, which are pRb homolog pocket proteins sharing sequence and functional homology with pRb, are the *in vivo* target of 3-ATA directed kinase inhibition. Like pRb, p130 and p107 regulate the cell cycle and are substrates of CDK4-directed kinases (20, 21). In primary mouse embryonic fibroblasts, it has been shown that pRb alone is insufficient to block DNA synthesis, and that p107 or p130 must be present for p16 to exert its inhibitory actions (22). In the leukemic cell line U937, the transfection of p16 did not inhibit pRb phosphorylation, but rather inhibited the phosphorylation of p130 (23). Thus the regulation of the cell cycle by p16 and *in vitro* kinase inhibitors such as 3-ATA may not involve pRb only, but also p130 and/or p107. A role for p130 and p107 may also explain the ability of these compounds to inhibit DNA synthesis in some pRb-negative samples.

3-ATA could also induce cell death in primary T-ALL cells. As the IC50s for inhibition of DNA synthesis and the loss of viability were generally different, the two events are likely to occur through distinct mechanisms. An investigation of several pro-apoptotic proteins revealed that while 3-ATA could cause DNA damage and the phosphorylation and induction of p53, no changes in the level of Bax or Caspase 8 were observed. However, these are just two of many proteins involved in cell death. Flavopiridol can induce caspase 8 activation in some tumor cell lines, but can also induce apoptosis in cells lacking caspase 8 (25). Similarly, flavopiridol can induce cell death in both a p53 dependent as well as independent manner (26). Recent studies have suggested that the mechanism of flavopiridol action is through the repression of the anti-apoptotic protein MCL-1 (27, 28) and the activation of E2F-1 (24, 28). The effect of 3-ATA on these pathways has not been explored.

Currently, there are several phase I and phase II clinical trials of Flavopiridol and UCN-01 as single agents or in combination with chemotherapeutics such as paclitaxel and cisplatin in different tumor types. Though not designed to measure efficacy and tumor regression, some clinical benefit was noted in single agent phase I trials of flavopiridol including some disease stabilization in renal cancer (29), stage IV non small cell lung cancer (30), gastric carcinoma (31) and melanoma (32). A partial response in a melanoma patient and a complete response in a lymphoma patient was observed with UCN-01 (33). In combination studies, *In vitro* pretreatment of lung cancer cells with cisplatin renders the cells sensitive to flavopiridol (34) and UCN-01 (35). Pretreatment of leukemia cells with flavopiridol renders them sensitive to Ara-C (36). Similarly, the combination of cisplatin followed by UCN-01 was synergistic in non small cell lung cancer cells (35). Clinical trials of combination of flavopiridol and UCN-01 with cisplatin, gemcitabine and taxol, for example, are underway, with encouraging results

beginning to emerge (37). In light of data presented in this study with 3-ATA and a previous study by our group with UCN-10 (12) and other studies using flavopiridol (18, 19) that the efficacy of some compounds can be correlated with p16/pRb protein status, these proteins may serve as important molecular determinants of clinical responsiveness. In this regard, it would be interesting to examine the relationship between clinical response and p16/pRb status of patient's tumor in the ongoing clinical trials of flavopiridol and UCN-01 and future trials of various kinase inhibitors that have been developed (7, 38). It is noteworthy that melanoma, colon cancer and lymphoma, where some early success of flavopiridol and UCN-01 have been reported, have high rates of p16 inactivation.

In summary, we have documented the sensitivity of primary T-ALL to inhibition of DNA synthesis and loss of viability by 3-ATA and TA and have shown they harbor a dependence on p16/pRb status. In T-ALL, traditional chemotherapeutics appear to have reached a plateau, and further decreasing the current relapse rate of about 20% will likely need to involve the introduction of novel chemotherapeutic directions and agents, with molecular profiling expected to be very important. In this regard, we have shown that T-ALL cells harbor a sensitivity to certain inhibitors of DNA synthesis and growth, that is dependent on their p16/pRb status, suggesting that molecular profiling may be a promising adjunct to the chemotherapeutics of T-ALL.

ACKNOWLEDGEMENTS

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