A PRECLINICAL STUDY OF FLAVOPIRIDOL IN THE TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

By

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To those whose lives have been touched by cancer; especially women who have lost their fathers. Even though our loved ones have moved on, a small part of them is still here in us.

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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Approximately 80% of children with acute lymphoblastic leukemia (ALL) will be cured; however, it is essential to study novel agents and new combinations of existing therapies for their potential use in relapsed patients. Loss of p16 function might play a part in the progression of ALL, which makes this pathway an interesting target for novel therapeutics. I have chosen to study flavopiridol (FP), a semi-synthetic flavonoid that targets the p16 pathway. FP acts as a pan-cyclin dependent kinase inhibitor with the ability to induce apoptosis and cell cycle arrest in human cancer cells. My studies have shown that at a concentration approximately equal to the IC_{50}, FP induces a transient G_1-S arrest and a low percentage of apoptosis in ALL cell lines. At approximately twice the IC_{50}, FP induces a sustained G_1-S and G_2-M arrest with a high percentage of apoptosis. My work has also shown that FP treatment decreases the phosphorylation of retinoblastoma protein on specific serine residues; an indication of a reduction in endogenous CDK activity. Further, despite a high level of binding by FP to proteins present human serum and subsequent reduction in its *in vitro* activity reported by others, I show that there is not a substantial difference in FP activity in the presence of human serum when compared to fetal bovine serum.

Based on disappointing results from early clinical studies of FP by others, I chose to test FP in combination with paclitaxel. PAC has a mechanism of action which is complementary to that of FP. PAC enhances the activity of CDK 1, inhibits microtubule depolymerization, and induces G_2-M arrest. Others have reported that FP enhances the efficacy of PAC in a sequence-dependent manner in cell types other than ALL. My results show that FP enhances the efficacy of PAC in ALL cell lines and that this enhancement is dependent on the sequence of administration. In this study I established optimal times of exposure for each of the agents when used in combination and confirmed that the enhancement of PAC activity by FP is present both in fetal bovine serum and human serum.

CHAPTER 1
BACKGROUND

General Treatment of Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer, accounting for approximately 30% of pediatric malignancies (1). With current multi-agent chemotherapy regimens, approximately 80% of patients are cured of their disease; however, relapse remains a significant clinical problem (2). Treatment of children with ALL consists of three phases: induction, consolidation and maintenance (Figure 1-1) (2, 3). The total length of treatment lasts 2-3 yrs. The purpose of the first phase of treatment, which lasts approximately one month, is to induce a complete remission or an absence of morphologically detectable leukemic blast cells in the blood or bone marrow. This is successfully achieved in 99% of patients with three or four drugs (2, 3). The consolidation phase of therapy lasts 4-8 months and is designed to reduce the number of remaining leukemic blast cells using the agents listed in Figure 1-1 (3). Maintenance therapy (1.5 to 2.5 years) consists of methotrexate and 6-mercaptopurine; in addition to vincristine and either prednisone or dexamethasone.

Patients with recurrent ALL receive more intensive therapy involving any or all of the agents previously outlined in other phases with other active agents such as ifosfamide, etoposide or teniposide often added. Stem cell transplant is also frequently performed for patients whose disease recurs during treatment or within 6 months of completing therapy. In the case of relapse outside of the bone marrow, such as leukemic blasts found in the central nervous system or testes, radiation can be administered at that site if it has not been previously administered.

There are agents which are currently used on an experimental basis in children with ALL. These include newer cytotoxic agents such as clofarabine, a nucleoside analog, agents which target tyrosine kinases and those that target histone deacetylases (2). Imatinib mesylate (Gleevec)

targets the tyrosine kinase formed by the BCR-ABL fusion protein resulting from a translocation between chromosome number 9 and chromosome number 22 (Philadelphia chromosome) as well as other tyrosine kinases. Use of this agent has induced remissions in BCR-ABL positive ALL (4-6). Other therapies under investigation include the use of RNA interference technology, gene therapy and immunotherapy (2).

Relapsed ALL: The Clinical Problem

Relapse can occur in a number of sites, including but not limited to the bone marrow, central nervous system, and testes. Survival rates after bone marrow relapse range from 5% to 57% and are especially poor for those with a relapse within 36 months of initial diagnosis (7-11). Increased dosage, the use of other existing chemotherapy agents not typically used in primary treatment (etoposide, ifosfamide, and others) and widespread use of stem cell transplantation have not significantly improved outcome for these patients. In addition, while complete remission rates for children who relapse more than 3 years after diagnosis are similar to those seen at initial diagnosis (>95%), patients who relapse less than 3 years after diagnosis often fail to attain a second remission (12).

Thus, there is a need to develop novel agents and/or new combinations of existing agents in order to improve the outcome of relapsed pediatric ALL patients. Many new agents have been developed that have novel modes of action. Some of these include the cyclin-dependent kinase inhibitors (CDKIs), examples being flavopiridol and UCN-01 (13, 14). Other drugs which are typically used in other types of cancers, such as the microtubule depolymerization inhibitors paclitaxel and docetaxel, have been used experimentally in ALL with mixed results (15-17). It becomes essential to study the biology that makes relapsed ALL different from ALL at initial diagnosis so that priority can be given to the study of the most promising agents.

Important Regulators of Cell Cycle

Gene expression profiling reveals that several key pathways are altered at the time of ALL relapse vs. at initial diagnosis, including cell cycle regulation, DNA repair and apoptosis (18). This project has focused on preclinical testing of agents that target the aberrations in cell cycle regulation present in relapsed ALL. Regulatory proteins can be broadly separated into those which regulate the transition from G_1 (Gap 1) to S (DNA synthesis) phase and from G_2 (Gap 2) to M (mitosis) phase. CDK 2, CDK 4, and CDK 6 regulate the transition from G_1 to S and CDK 1 (cdc2) regulates the transition from G_2 to M (Figure 1-2) (19). Most molecules of interest in this project function in the restriction point from G_1 to S or the point at which the cell is committed to divide with or without the presence of growth factors (20), as beyond this point the cell is less likely to respond to external stimuli such as a drug. CDK 4 and CDK 6 become functional after cyclin D1, cyclin D2, or cyclin D3 binding (19). These kinases phosphorylate retinoblastoma protein (pRb) at specific serine and/or threonine residues. This phosphorylation is normally prevented by p16 (cyclin-dependent kinase 4 inhibitor A; INK4A), which binds to CDK 4 and CDK 6 in the place of the cyclin (21). p16 is part of the INK4 family of proteins, including p15 (INK4B), p18(INK4C), and p19(INK4D), which work to inhibit CDK 4 and CDK 6, along with members of the CIP/KIP family, including p27 (cyclin dependent kinase inhibitor 1B; KIP1), p57 (cyclin dependent kinase inhibitor 1C; KIP2) and p21 (cyclin dependent kinase inhibitor 1A; WAF1/CIP1) (Figure 1-2) (22). When p16 is present, the hypophosphorylated form of pRb acts as a tumor suppressor by binding to E2F transcription factor, making E2F unable to bind to DP-1 and 2 (Figure 1-3). These molecules function as transcription factors that act in DNA synthesis and nucleotide metabolism (22).

p16 has been a major interest in this project; however, other molecules such as p15, p21, and p27 have been studied by the Hunger lab and others for their possible roles in the

progression of ALL (see below). p15 shares great homology with p16, is found within 25kb of the *p16* gene on chromosome 9 (23), and acts as a TGF-β (transforming growth factor-β) induced inhibitor of CDK 4 and CDK 6 (24). p21 and p27 regulate not only CDK 4 and CDK 6, but also CDK 2, which functions in concert with CDK 4 and CDK 6 to phosphorylate pRb (19). It is CDK 2 that actually completes the hyperphosphorylation of pRb. This inhibits the tumor suppressive nature of pRb and allows progression of the cell cycle through S-phase. p21 is activated by the p53 transcription factor (22). P53 is regulated by MDM-2 (mouse double minute 2; HDM-2 in humans) which inactivates the transcriptional activity of p53, flags it for ubiquitylation, and ensures its transport from the nucleus into the cytoplasm. An alternate reading frame of the *p16* locus produces p14 (alternate reading frame; ARF) which acts as a tumor suppressor by preventing the p53 suppression activity of MDM-2.

Cell Cycle Regulators in Cancer and ALL

Deletion of *p16* is the most common form of genetic alteration in cancer among cell cycle regulators (25). Studies have shown that greater than 30% of ALL cases have *p15* and *p16* deletions, with that percentage increasing to greater than 50% in T-cell ALL and remaining greater than 20% in B-precursor ALL (26). It has been found by the Hunger lab and others that a substantial number of patients develop *p15* and/or *p16* deletions in the bone marrow between the time of initial diagnosis and relapse of ALL (27, 28). The *p15* promoter has also been studied and has been found to undergo methylation between diagnosis and relapse, much more commonly than the *p16* promoter (29-31). This *p15* methylation takes place in CpG islands at the 5' end of the gene, which results in loss of transcription in the promoter region (32). A study from the Hunger laboratory used 18 matched specimen pairs from children with ALL at initial diagnosis and first relapse to determine if *p15/p16* deletions or hypermethylation of the *p15*

promoter occurred between diagnosis and relapse (27). Results showed that out of 14 pairs that were germline at diagnosis, three developed homozygous deletions of both *p15* and *p16* and two developed homozygous *p16* deletions and retained germline *p15* status between the time of initial diagnosis and relapse. *p15* promoter hypermethylation developed in two patients between diagnosis and relapse. Out of the eighteen total cases, seven had homozygous *p15* deletions, nine had homozygous *p16* deletions, and two of eight cases tested had *p15* promoter hypermethylation at relapse. Similar findings have been reported by Carter, et al., showing that out of a group of 25 pediatric ALL patients, at diagnosis 32% and 20% had homozygous and hemizygous *p16* (exon 2) deletions respectively (28). The incidence of homozygous *p16* deletion at relapse increased to 64%, illustrating the potential importance of the loss *p16* in the progression of ALL.

The prevalence of *p15* and *p16* alterations is much higher than the level of *p21* and *p27* alterations found in ALL (33, 34). Both p21 and p27 function to inhibit CDK 2 and CDK 4 (35-37). p21 is regulated by p53 in order to control cell growth (38). Hayette, et al. performed a study of alterations of molecules which inhibit CDKs in leukemia, using bone marrow or peripheral blood from 121 newly diagnosed ALL cases, 85 newly diagnosed acute myeloid leukemia cases, and 42 newly diagnosed B-cell chronic lymphocytic leukemia cases (34). Via Southern blot this group found that *p16* was inactivated in 25 of 38 T-cell ALL cases and 28 of 83 B-lineage ALLs. After testing 40 ALL samples with a *p16* aberration, it was found that 22 cases (55%) had biallelic *p15* deletions and 11 cases (28%) had monoallelic deletions. All cases with a *p15* deletion also had an anomaly in the *p16* gene. There were no alterations found in *p21* and monoallelic deletion of *p27* was present in 4 of 85 acute myeloid leukemia cases tested. These data show that *p21* and *p27* alterations are much less prevalent in leukemia than deletions of *p15*

and *p16*. Another study by Kawamura, et al. further illustrates this point by analyzing 71 primary T-ALL samples and 18 T-ALL cell lines for alterations in *p15, p16, p21, p53*, and *RAS* via polymerase chain reaction-single strand conformation polymorphism analysis (33). They found that none had alterations in *p21*. In contrast, 18 of 47 (38%) newly diagnosed patients had *p16* alterations and 7 of 14 (50%) patients had *p16* alterations at relapse.

Gene deletion is not the only cause of loss of functional p16. Many samples have been found to have an intact *p16* gene, but no protein expression. An interesting study by Nakamura, et al. notes that when p16 expression was investigated in childhood ALL samples via Western blot, 18 of 22 samples with an intact *p16* gene did not express p16 protein; however, protein expression was able to be induced after treatment with a demethylating agent, indicating that the loss of p16 protein expression was due to gene hypermethylation (39). Others have reported similar results in T-cell ALL and/or AML (40-42). A separate study of pediatric T-cell ALL patients reported that only 9 of the 45 samples with intact *p16* expressed p16 protein (43). This study found that p16 was altered at the DNA, RNA or protein level in 115 of the 124 (93%) samples tested and concluded that alteration in both p16 and p15 were essential to the progression of T-cell ALL. Most recently a study of adults with untreated ALL found that not one of the samples tested (n=91) expressed p16 protein (44).

A study performed by Carter, et al. on 45 patient samples via quantitative PCR techniques found that ALL patients with a hemizygous deletion of *p16* at diagnosis were 6.5 times more likely (P=0.00687) to relapse and those with a homozygous deletion had an even higher risk ratio of 11.5 (P=0.000539) (45). In contrast to the findings of Carter et al., Einsiedel et al. found that there was no association between *p16* deletions and event free survival in ALL (46). *p15* and *p16* status could be correlated to two major prognostic indicators: T-cell immunophenotype and first

remission duration. This study did not assess for hemizygous deletions, as Carter did, because of theoretic and methodologic considerations. Another group compared wildtype *p16* to hemizygous deletions and found no difference in potential for event free survival (47).

Given this information it becomes apparent that despite the fact that its prognostic value is still somewhat controversial, p16 alterations occur commonly in relapsed ALL and are often acquired during disease progression. Deletion of *p16* and hypermethylation of the *p15* promoter region occur much more frequently in ALL than alterations in other cell cycle regulatory molecules such as p21 or p27. Therefore the p15/p16 pathway is an attractive target for therapeutic intervention in relapsed ALL. Several agents exist that modulate cell cycle progression and are logical candidates to test in relapsed ALL. One such agent is flavopiridol, a description of which follows.

Flavopiridol

Flavopiridol (FP) is a semisynthetic flavonoid derived from rohitukine, an alkaloid isolated from a plant indigenous to India (48). Flavopiridol has a variety of mechanisms of action; however, most relevant to my studies is the ability of FP to decrease the activity of CDKs and induce cell cycle arrest (Figure 1-4). Cell cycle regulatory elements such as the CDK inhibitors p15 and p16 are altered in ALL between diagnosis and relapse, indicating that this loss of checkpoint control in the cell cycle could be a critical factor in the progression of the disease and an attractive target for novel therapeutic agents. FP competitively binds to the ATP binding cleft of the CDK (14) and is capable of reducing the activity of CDK 1, CDK 2, CDK 4, CDK 6, and CDK 7 with IC_{50} values in the range of 20-400 nM (49). FP also reduces the activity of CDK 9 (50-52).

FP induces cell cycle arrest at the G_1-S phase border as well as during G_2-M. Inhibition of CDK 2 and CDK 4 has been correlated to G_1 arrest in MCF-7 breast carcinoma cells (14).

Another group had similar findings using MDA-468 breast carcinoma cells that were synchronized either in G_1 phase with aphidicolin or synchronized in M phase with nocodazole (53). MDA-468 cells treated with 200 nM FP after release from aphidicolin G_1 block arrested in G_2-M after 24 hours. Cultures released from nocodazole M phase block and treated with 200 nM FP showed a G_1-S arrest when compared to control cultures not treated with FP.

The ability of FP to induce cell death has been tested *in vitro* in a variety of cancer cell types, including adult leukemia. An early study in a variety of solid tumor cell types and HL-60 leukemia cells found that FP was cytotoxic as measured by trypan blue exclusion and colony formation assays (54). Previous studies had only shown that FP was cytostatic (53). The former study also found that 90% cell death was induced 72 hours following a 24 hour exposure to 250-300 nM FP compared to 50% cell death induced immediately following the 24 hour drug exposure, thus showing that more time was needed to achieve a maximum cell death response. This group also showed that both logarithmically growing and cytostatic cell lines were affected by FP treatment. Similar results have been found by others testing non-small cell lung carcinoma cell lines (55). It was found that seven different cell lines were sensitive to FP at concentrations ranging from 100-500 nM; regardless of whether the cell lines were in logarithmic growth phase or cytostatic. This group also showed that cell cycle arrest preceded cell death in most cases and that maximal cell death occurred 72 hours post-treatment with concentrations of FP 500 nM or below. These data illustrate the cytotoxic action of FP during a prolonged exposure. This activity combined with the ability of FP to inhibit CDK activity and induce cell cycle arrest contribute to this project's focus on testing the efficacy of FP as a potential treatment for ALL.

In Vitro Testing of Flavopiridol in Combination with Other Agents: Sequence of Administration and Synergy

It has been found that administering FP with traditional antineoplastic agents can improve the efficacy of those agents and that in some cases this interaction is synergistic. The enhancement of a traditional agent by FP has been shown to be dependent on the sequence in which the drugs are given, such as the enhancement of paclitaxel (PAC) activity by FP (56). Paclitaxel (trade name Taxol) prevents microtubule depolymerization (57) and induces G2-M phase arrest (58). An example of the enhancement of PAC activity by FP can be found in work by Motwani, et al. in which MKN-74 human gastric carcinoma cells and MCF-7 human breast carcinoma cells were exposed to PAC, FP, or both agents either sequentially or simultaneously (56). When MKN-74 cells were exposed to PAC and FP for 24 hours, the level of apoptosis increased from 3 +/- 1% with FP alone to 8 +/- 1%. A significant increase was then seen when the drugs were used sequentially. MKN-74 cells were exposed to PAC for 18 hours followed by FP for 24 hours and the level of apoptosis was 40 +/- 2%; however, when using FP followed by PAC the level was 8 +/- 1%, which was not significantly different from the amount of apoptosis found after exposing the cells to FP for 24 hours followed by no drug for 18 hours. Caspase-3, the final activator of the apoptotic cascade was activated when MKN-74 and MCF-7 were treated with PAC followed by FP. Without FP, PAC only minimally activated caspase-3. If the sequence of administration was reversed, FP inhibited the function of PAC by preventing mitosis and CDK 1 activity. Similar results in regard to cytotoxicity have been achieved when using FP in conjunction with docetaxel *in vitro* and in xenograft tumor models (59).

When testing eight agents against a human non-small cell lung carcinoma cell line (A549), Bible and Kaufmann found that seven of the eight agents had synergy with FP that was sequence specific (60). These authors extensively studied the possibility that treatment with PAC and FP

could show sequence dependent synergy. Their finding that the effects of PAC were more pronounced when administered before FP treatment as opposed to after or concomitantly was particularly intriguing. A marked decrease in clonigenic cell survival over PAC alone and FP alone was seen when PAC treatment was followed by FP. Synergy was assessed through the use of combination index or CI. A CI of 1.0 indicates that the relationship between the drugs being studied is nearly additive, while a CI of <1.0 indicates synergy and a CI of >1.0 indicates antagonism (61). At the concentration at which cell proliferation was inhibited by 75% (IC_{75}) and 95% (IC_{95}), combination indices of 0.49 +/- 0.21 and 0.20 +/- 0.14 were found, respectively, indicating synergy if PAC was given before FP in the treatment sequence (60). Antagonism was found if PAC followed FP.

Others have tested many agents in conjunction with FP in myeloid leukemia cell lines. These agents have included phorbol 12-myristate 13-acetate (PMA), imatinib mesylate (Gleevec), bryostatin 1, bortezomib (Velcade) and suberoylanilide hydroxamic acid (SAHA) (62-66). All have shown promising results for the ability of FP to enhance the activity of other agents.

Efficacy of Flavopiridol in Clinical Trials

Based on its action as a CDK inhibitor and promising preclinical activity, FP was tested in phase I human trials. Studies designed to obtain clinical pharmacology data after giving FP as a 72 hour infusion readily achieved plasma concentrations that were comparable to that found to be effective *in vitro* (67, 68). However, most clinical trials involving cancer patients gave FP as a 72 hour infusion every 2-3 weeks and found that it had limited efficacy as a single agent (69-75). One of these trials found that FP had antitumor activity in certain patients with renal, prostate, and colon cancer, and non-Hodgkin's lymphoma (69). The two maximum tolerated doses (MTDs) found in this phase I study gave peak plasma concentrations of 271 nM and 344 nM, the

second after antidiarrheal prophylaxis. The concentrations of FP needed to inhibit cyclin dependent kinase function (200 to 400 nM) were safely achieved in this study. Despite both *in vitro* and *in vivo* data showing that FP was cytostatic and cytotoxic in non-small cell lung carcinoma cells, Shapiro and colleagues stopped a phase II study after only 20 of 45 patients projected to be in the study were treated, as no responses were observed in these individuals (72). This study also noted that a mean steady-state plasma concentration of 200±89.9nM was achieved, which was well within the FP concentration range found to be effective *in vitro*.

Questions regarding dose and length of treatment have been the main focus of many clinical trials involving FP. In addition to the traditional 72 hour infusion schedule, FP has also been tested as a 24 hour continuous infusion given every two weeks and a 1 hour bolus administered over a range of schedules. Flinn, et al. found that FP had no clinical activity in patients with fludarabine refractory chronic lymphocytic leukemia (CLL) when given as a 24 hour infusion (76). A study from the same group compared FP activity in CLL when the agent was administered as a 72 hour infusion to a 1 hour bolus and found that the 72 hour schedule did not result in any patient responses; however, the bolus dose did result in slight clinical activity (75). A separate phase I study using FP as a single agent in patients with advanced neoplasms tested FP at varying 1 hour infusion doses over 5-days, 3-days and 1-day every 3 weeks (77). During the trial, median peak total concentrations at the MTD of 1.7 μM (range 1.3 to 4.2 μM) for 5-day administration, 3.2 μM (range 1.7 to 4.8 μM) for 3-day administration, and 3.9 μM (range 1.8 to 5.1 μM) for 1-day administration were found. Twelve of the 55 patients studied had stable disease for greater than or equal to three months with a median duration of six months (range, three to eleven months). A similar study was conducted by the National Cancer Institute of Canada using FP as a bolus infusion over 3 days in patients with untreated or relapsed mantle-

cell lymphoma (78). No complete responses were observed; however, 11% of patients had a partial response and 71% had stable disease. Similar results for the 1 hour bolus have also been reported in malignant melanoma and multiple myeloma (79, 80). The only study of FP in pediatric patients also used this schedule and was performed by Whitlock, et al. in patients with solid tumors (81). No responses were observed despite achieving mean peak plasma concentrations of 3.71 and 9.11 μM after doses of 37.5 mg/m^2 and 80 mg/m^2 respectively.

Shorter infusion schedules for FP as outlined above were pursued by clinicians with the intention of increasing the peak plasma concentration of the agent. Early trials of FP given as a 72 hour infusion were based on drug activity data generated from *in vitro* studies of FP that were performed in media supplemented with fetal bovine serum (FBS). Later studies showed that FP is highly bound to human plasma proteins (68, 82). Approximately 92-95% of FP is human plasma protein bound compared to 0-37% bound in FBS (82). This difference in protein binding results in a decrease in the *in vitro* cytotoxicity of FP. Studies of primary CLL cells have shown 1 hour and 24 hour LC_{50} values in FBS of 670 nM and 120 nM respectively, compared to 3,510 nM and 470 nM in human plasma or human serum (HS) (82). Based on these data and clinical pharmacology data, a pivotal study of the use of FP as a short infusion was performed (83). FP was given as a 30 minute bolus infusion followed by a 4 hour continuous infusion in patients with CLL with the goal of achieving a peak plasma concentration of 1.5 μM. Patients were divided into cohorts, with the first receiving a 30 mg/m^2 bolus dose followed by a 30 mg/m^2 infusion. The second cohort received a 40 mg/m^2 bolus followed by a 40 mg/m^2 infusion. The maximum plasma FP concentrations achieved at these dose levels were 2,080 nM after 30 minutes and 960 nM after 4.5 hrs (84). A third cohort was given a 30 mg/m^2 bolus followed by a 50 mg/m^2 infusion. These dosages achieved peak plasma levels of 1,950 nM after 30 minutes

and 1,540 nM after 4.5 hours This study had to be temporarily discontinued, as this schedule had high clinical activity that resulted in tumor lysis so severe that one patient died (85). The group implemented procedures for monitoring patients for tumor lysis syndrome and continued the study which resulted in a 45% overall response rate in CLL patients.

Biological Correlates of Clinical Activity

Several clinical trials have included studies to determine if the same mechanisms of action for FP observed *in vitro* could be achieved *in vivo*. Previous *in vitro* studies have shown that FP inhibits CDK activity (14, 49-52, 86, 87), induces apoptosis (54, 55, 87-96), reduces the transcription and/or expression of anti-apoptotic proteins Bcl-2 (B-cell leukemia/lymphoma 2) and Mcl-1 (myeloid cell leukemia sequence 1) (49, 87, 96-100), and binds to DNA (101). In a phase I study by Thomas, et al. FP was tested as a single agent given as a 72 hour infusion every two weeks in patients with a variety of tumor types (67). Peripheral blood lymphocytes were collected during treatment and analyzed via flow cytometry for evidence of apoptosis or changes in cell cycle kinetics. No evidence of changes in these measurements was found; however, the authors noted that there were early signs of clinical activity.

During a phase I trial of FP combined with docetaxel in patients with metastatic breast cancer, Tan and colleagues examined Ki67, p53, and phosphorylated pRb in paired patient tumor and buccal mucosa samples (102). Ki67 was used as an indication of cell proliferation and phosphorylated pRb was used as an indirect measurement of CDK activity. The buccal mucosa biopsies of ten of the eleven patients enrolled in the study showed increased nuclear expression of p53 and decreased expression of phosphorylated pRb after treatment with FP as a single agent. The authors postulated that the increase in p53 expression could have been due to the ability of FP to bind to DNA (101) or the ability of FP to reduce transcription or down regulate MDM-2, based on the activity of other CDK inhibitors (50, 103). Six paired tumor samples showed no

changes in p53, Ki67, or phosphorylated pRb. The authors concluded that the biological effect of FP was achieved in the buccal mucosa; however, the treatments tested were not feasible due to dose limiting toxicities.

A similar phase I study used FP in combination with cisplatin or carboplatin in patients with advanced tumors (104). Peripheral blood mononuclear cells were analyzed before and after FP treatment and found to have increased p53 expression and increased phosphorylated STAT3 (signal transducer and activator of transcription 3) levels. Treatment had no effect on cyclin D1, phosphorylated RNA polymerase II (indicator of CDK activity), or Mcl-1. The authors felt that there was a possibility that the increased p53 and pSTAT3 levels were due binding of FP to DNA and that the lack of an effect by FP on cyclin D1 expression, the phosphorylation of RNA polymerase II, or Mcl-1 expression might have been due to the inability of FP to inhibit P-TEFb (CDK 9) *in vivo*. There was a lack of clinical activity observed during the trial and it was further postulated that the lack of an effect on Mcl-1 expression by FP could have been an explanation for this low clinical response. Alternatively, the authors could not definitively say that the same effects that were observed in non-cycling peripheral blood cells could be observed in tumor cells, as these were not tested.

Finally, a phase II trial of relapsed or refractory melanoma patients had disappointing clinical results that the authors partially attributed to a lack of biological activity *in vivo* (80). Western blot analyses very similar to those performed in the studies cited above found that only one patient out of eight tested had the expected results of decreased Mcl-1 with increased p53 expression and increased expression of phosphorylated STAT3 as a result of FP treatment. Two additional patients had decreased Mcl-1 in combination with lower levels of p53 and

phosphorylated STAT3. It was noted that the former patient progressed after one cycle of FP treatment.

All of the trials cited above used FP as either a 1, 24, or 72 hour infusion. This is contrary to the most recent use of FP as a 30 minute bolus followed by a 4 hour infusion found to be highly effective in CLL (85). Most of the *in vitro* studies to which the above authors were attempting to correlate biological activity *in vivo* were performed in FBS. As previously cited, the newer infusion schedule takes the high percentage of protein binding of FP that occurs in HS into account by achieving a higher plasma FP concentration in a shorter time than that achieved in previous trials. Studies have been conducted *in vitro* in CLL cells grown in the presence of HS that show that FP is biologically active under these conditions when used at concentrations higher than those previously utilized in FBS (100).

Clinical Trials of Paclitaxel (PAC) and Combining Flavopiridol with Paclitaxel

With the exception of recent studies in CLL, FP has had limited efficacy in clinical trials when used as a single agent. However, as outlined previously, there have been promising preclinical results showing synergy between PAC and FP. Early clinical trials have also tested FP in combination with PAC in cancer types other than ALL.

PAC has been found to be effective as a single agent in the treatment of several types of cancer including breast, ovarian, and lung cancer, and melanoma (105, 106). PAC also has considerable *in vitro* activity against ALL (107) and has been tested in both adults (15, 108) and children (16) with leukemia. Studies in adults used 3 doses of 100 minutes each repeated every three weeks (15) and a 24 hour infusion repeated every 3-4 weeks (108). A trial in pediatric leukemia patients used a 24 hour PAC infusion; achieving peak plasma concentrations of approximately 1,000 nM. Unfortunately, these studies did not report any substantial clinical responses. Minimal responses have been reported using PAC as a single agent in children with

solid tumors (109). This trial used varying doses of PAC as a 24 hour infusion repeated every three weeks. Peak plasma concentrations were dose-dependent and ranged from approximately 1,000 nM to 7,000 nM. Two out of 31 total patients treated reported significant toxicity. PAC is 89-98% bound to plasma proteins *in vivo* (110). Perhaps similar to FP, a shorter infusion schedule with the goal of obtaining a high peak PAC plasma concentration might prove beneficial in the treatment of ALL.

Promising results have been achieved when FP was combined with PAC in patients with a variety of solid tumor types (111). Clinical responses were observed in patients with esophagus, lung and prostate cancer, some of whom had progressed on PAC single agent treatment. It should be noted that the agents were given in the specific sequence of PAC followed by FP treatment.

Project Rationale

Preclinical *in vitro* studies or clinical studies using FP have never been conducted in relation to childhood ALL. Relapsed ALL patients become increasingly refractory to agents typically used in the treatment of ALL, thus creating a need to investigate new drugs. I examined FP because of the high frequency of p15/p16 abnormalities and altered expression of other cell cycle regulatory proteins in relapsed ALL. Results from preclinical studies suggest that FP can act similarly to these molecules in that it inhibits CDK activity and induces cell cycle arrest. FP can also induce apoptosis in human cancer cells. Based on these findings, I performed *in vitro* studies of FP at different times of exposure to mimic prolonged infusion and newer bolus schedules. During these studies I examined the cell death and alterations in cell cycle progression induced by FP.

Clinical responses measured during trials of FP as a single agent have shown that it has limited efficacy when used in a 72-, 24- or 1 hour dosing schedule. Recent data from studies in

CLL have suggested that prolonged FP infusions have a low overall response rate due to failure to achieve an effective free FP concentration as a result of secondary protein binding in human plasma. A shorter infusion of a higher dose of FP was found to be very promising. In order to model this new infusion strategy, this project includes experiments using cultures that were grown in medium supplemented with human serum in place of fetal bovine serum. A higher concentration of FP is also administered over a shorter period of time when compared to previous experiments.

This project has not only served as a means to determine the potential efficacy of FP when used as a single agent in ALL cell lines, but has also served as a study to determine the effects of combining FP with PAC. *In vitro* studies and clinical trials in patients with types of cancer other than ALL have shown that FP can enhance the activity of PAC. In some cases using FP in combination with PAC can have a synergistic effect on *in vitro* treatment. This enhancement is dependent on the sequence in which the drugs are administered. In the case of FP combination treatment with PAC, this sequence dependence has been reported to be due to the ability of PAC to activate CDK 1 activity coupled to the inhibitory action of FP against this same CDK (56). Because PAC is not typically used in the treatment of ALL, I first established that ALL cell lines were sensitive to PAC treatment. I also tested whether FP enhances the efficacy of PAC *in vitro* and if this enhancement was dependent on the sequence in which the two drugs were administered. This drug combination could offer a treatment regimen to children with relapsed ALL that would utilize two agents to which the patients will not have been previously exposed.

In the future, data from this project could be used to develop a clinical trial which would utilize either FP as a single agent or PAC in combination with FP, both in the schedule that I have found to be most efficacious. This project also provides data to indicate the mechanism of

action behind the efficacy of FP in ALL in order to provide a biological basis for the clinical study.

Induction: 1 month; Results in complete remission in 99% of patients

Treatments can include the following:
--L-asparaginase
--vincristine
--steroid
--anthracycline for high-risk patients (daunorubicin)

Intrathecal therapy-2 doses in the first month since diagnosis and 4-6 doses during the next 1 or 2 months. Agents utilized include
--methotrexate
--hydrocortisone and cytosine arabinoside (ara-C) added for high-risk patients

Patients with high white blood cell (WBC) count (high risk) or WBC in the cerebral spinal fluid receive radiation to the brain and possibly the spinal cord. May also administer high dose intrathecal methotrexate with leucovorin to treat side effects.

Consolidation: 4-8 months; Reduces the remaining number of leukemic blasts

Standard risk patients receive
--methotrexate
--6-mercaptopurine or 6-thioguanine
--optional: vincristine and prednisone

High risk patients receive:
--L-asparaginase, doxorubicin, etoposide, cyclophosphamide, ara-C and dexamethasone substituted for prednisone (possibly two rounds)

Maintenance: 1.5 to 2.5 yrs

--methotrexate, 6-mercaptopurine
--vincristine; prednisone or dexamethasone (every 4-8 weeks)

Figure 1-1. Treatment of Childhood Acute Lymphoblastic Leukemia (ALL): 2-3 yrs. total (3)

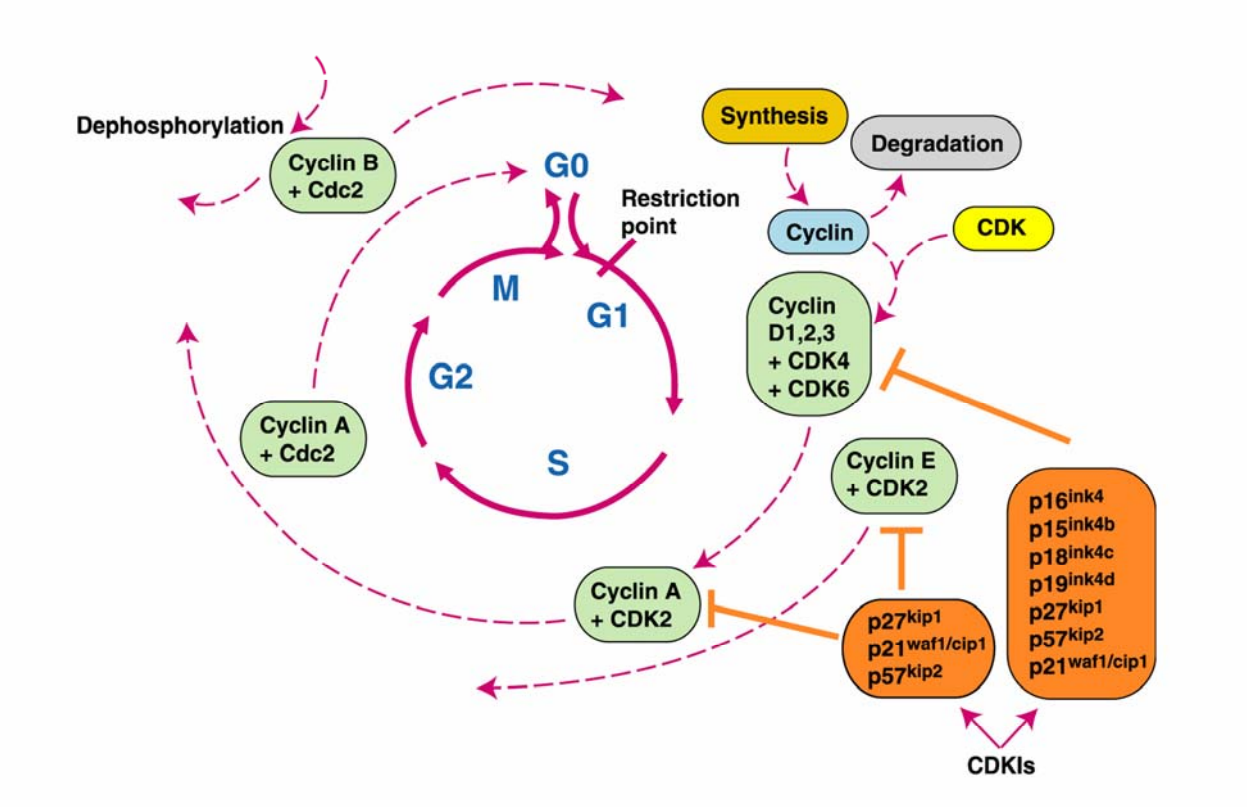


Figure 1-2. Cyclin dependent kinase (CDK) inhibitors function in the transition from G_1 (Gap 1) to S (DNA synthesis) phase of the cell cycle. p15 and p16 inhibit CDK 4 and CDK 6, as do p21 and p27. The restriction point of the cell cycle is located at the transition from G_0 to G_1 and marks the point at which the cell is no longer sensitive to external agents such as growth factors or a drug. Pursuing permission from American Association for Cancer Research: [Clinical Cancer Research] Shah MA, Schwartz GK. Cell cycle mediated drug resistance: an emerging concept in cancer therapy. Clinical Cancer Research 2001; 7:2168-2181., copyright 2001, originally published at Figure 1, p. 2169.

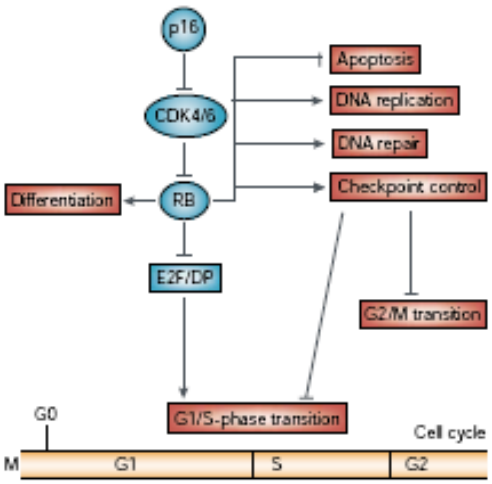


Figure 1-3. p16 works in concert with pRb to regulate the G_1-S transition. p16 inhibits the activity of CDK 4 and CDK 6, thus preventing the phosphorylation of pRb. In the hypophosphorylated state, pRb can prevent binding of E2F/DP transcription factors to genes involved in progression through the G_1-S transition. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] Classon M, Harlow E. The retinoblastoma tumour suppressor in development and cancer. Nature Reviews Cancer 2002; 2:910-917, copyright 2002, originally published as Figure 1, p.911.

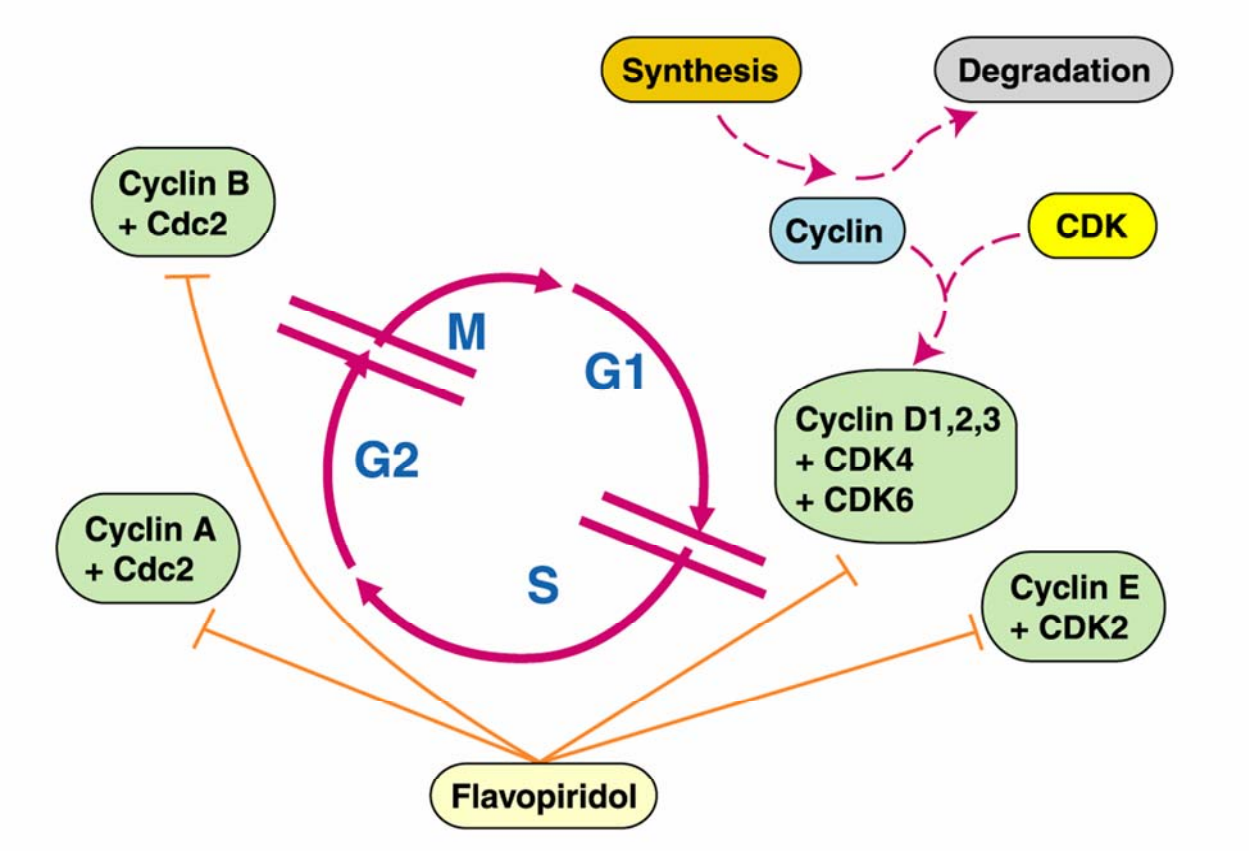


Figure 1-4. Flavopiridol is a pan-CDK inhibitor. FP inhibits CDK 4, CDK 6 and CDK 2, thus inducing a G_1-S arrest. FP can also inhibit CDK 1 (cdc2) and induce G_2-M arrest Reprinted by permission from Meniscus Ltd: [Horizons in Cancer Therapeutics: From Bench to Bedside] Shah MA, Schwartz GK. Cell cycle modulation: an emerging target for cancer therapy. Horizons in Cancer Therapeutics: From Bench to Bedside 2004; 4(3):3-21., copyright 2004, originally published as Figure 5, p.7.

CHAPTER 2
FLAVOPIRIDOL DISPLAYS PRECLINICAL ACTIVITY IN ACUTE LYMPHOBLASTIC LEUKEMIA

Introduction

One of the most commonly used methods of evaluating the potential efficacy of chemotherapeutic agents prior to their use in patients is to determine the ability of the agent to prevent growth of cancer cells *in vitro*. Results from methyl-thiazol-tetrazolium (MTT) assays have shown a correlation between *in vitro* sensitivity of leukemia cells taken from peripheral blood and bone marrow of patients and clinical outcome (112-119). Hongo et al. (112) found that using agents determined to be efficacious in MTT assays resulted in better outcome for patients with ALL or acute nonlymphoblastic leukemia when compared with patients whose treatment regimens were determined via conventional methods of the time. Approximately 82% (n=11) of patients treated with agents determined to be efficacious in MTT assays had complete or partial remissions as compared with 40% (n=15) of patients treated by conventional means. I have chosen to use a modified MTT assay as my initial means of determining the sensitivity of ALL cell lines to FP. I have expanded my studies by testing the ability of FP to induce apoptosis and cell cycle arrest in ALL cell lines, as cell proliferation assays merely measure an increase or decrease in viable cell number.

The mechanism of action of a chemotherapeutic agent is an integral part of determining how the agent will be used as well as what side effects might occur as a result of its use. Knowing the mechanism of action can also help to target cancer cells without affecting non-cancerous tissue. The ultimate fate of a cell, ie. cell death or senescence as a result of cell cycle arrest can have an effect on the progression of the cancer. Cells which senesce might have the ability to secrete signaling molecules which promote the growth of other cancer cells in the surrounding area (120). For some researchers, this possibility makes apoptosis a preferred

mechanism of action for anti-neoplastic agents. I have determined that FP has the ability to reduce ALL cell proliferation via a modified MTT assay and have further investigated the ability of FP to induce cell cycle arrest and apoptosis. Through Western blot analysis I have observed a correlation between the concentration dependent effects of FP on cell proliferation and the endogenous phosphorylation of pRb**.** Correlating these mechanisms with drug concentration and the effect that FP has on cell cycle regulatory elements will serve as important information when deciding the use of FP as a single agent or in combination with other agents in the treatment of childhood ALL.

Methods

In Vitro Drug Sensitivity Testing

In vitro drug sensitivity assays were modeled after those described originally by Pieters and colleagues (113). Cell lines were grown in RPMI 1640 (Mediatech, Inc. Herndon, VA) with 10% fetal bovine serum (FBS, Mediatech) or 10% human AB serum (HS, Mediatech) and 1% penicillin/streptomycin (Mediatech) at 37°C with 5% CO_2. Nalm-6 (B-precursor ALL) was originated by Minowada, et al. (121). Molt-4 and Jurkat (both T-cell ALL) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). RCH-ACV is a B-precursor ALL cell line provided by Dr. Seshadri (122)**.** K562 (ATCC) is a chronic myelogenous leukemia (CML) cell line commonly used for *in vitro* testing in the NCI 60 cell line test set and was included in this study as a control. Exponentially growing cell cultures were plated in flat bottomed 96-well dishes in 100 μL of cell culture medium with a dilution of drug in vehicle appropriate for each agent. Vehicles included ethanol for dexamethasone (Sigma, St. Louis, MO), water for doxorubicin (Sigma), and dimethyl sulfoxide (DMSO) for FP (Sanofi-Aventis, Bridgewater, NJ). All were further diluted in RPMI 1640. Samples for each drug concentration were tested in quadruplicate for each experiment. Also tested in parallel were appropriate

dilutions of vehicle without drug, which functioned as an untreated control for calculation of IC_{50}. For the purpose of my study IC_{50} was defined as the concentration of drug at which cell proliferation was inhibited by 50% as compared to an untreated control. Cell lines were plated at a concentration of $1X10^5$ cells/mL in 100 μL for RCH-ACV, Molt-4, and Jurkat. Nalm-6 and K562 were plated at a concentration of $5X10^4$ cells/mL in 100 μL. Different cell concentrations were used in order to maintain the cultures in log phase growth throughout the period of the experiments. Cell lines were incubated with drug for 96 hours, at which time WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) reagent (Roche, Indianapolis, IN) was directly added to each well according to manufacturer's instructions. WST-1 is a modified version of the MTT (methyl-thiazol-tetrazolium) reagent. Absorbance was measured on a Molecular Devices (Sunnyvale, CA) *Vmax* kinetic microplate reader at 450nm, subtracting a reference wavelength of 650nm. IC_{50} was calculated by plotting leukemic cell survival (LCS) against drug concentration. The drug concentration at which LCS equaled 50% was defined as the IC_{50}. LCS was calculated as follows:

$$\frac{abs_{treated} - abs_{blank}}{abs_{control} - abs_{blank}} \text{ X } 100\%. \qquad (2\text{-}1)$$

Results are the mean of at least two independent experiments.

Western Blot Analyses

ALL cell lines were tested for p16 protein expression with HeLa cells used as a positive control. HeLa extract was diluted into extract from Nalm-6 (previously found to be *p16* deleted via Southern blot (40)) in order to simulate a low level or variable amount of p16 protein expression. Protein extracts were prepared using Radio-Immunoprecipitation Assay (RIPA) Buffer (Sigma) with sodium orthovanadate (Santa Cruz, Santa Cruz, CA), phenylmethylsulfonyl fluoride (PMSF, Santa Cruz), and a protease inhibitor cocktail (Sigma). Fifty micrograms of

protein was loaded onto a 4-20% gradient polyacrylimide gel (Biorad, Hercules, CA) and subjected to sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE). Proteins were transferred to a 0.2 μM pore nitrocellulose membrane (Biorad). After transfer, the membrane was blocked with 5% dry non-fat milk in TBS with 0.1% Tween-20 (TBS-T) for one hour with gentle agitation. Following blocking, the membrane was incubated at room temperature (RT) with mouse monoclonal IgG_1 antibody to full length p16 protein (50.1, catalog number sc-9968, Santa Cruz) at a dilution of 1:375 in 5% non-fat milk for one hour. The membrane was then washed 3 X 15 minutes in TBS-T and incubated for one hour in goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase in 5% milk. Retinoblastoma protein phosphorylated on serine 795 (pp-Rb^{ser795}) and retinoblastoma protein phosphorylated on serines 807 and 811 (pp-$Rb^{ser807/811}$) were resolved via SDS-PAGE after loading 25 μg protein lysate. The proteins were transferred to a nitrocellulose membrane which was blocked as described and incubated with rabbit polyclonal antibodies to pp-Rb^{ser795} and pp-$Rb^{ser807/811}$ (product numbers 9301 and 9308, Cell Signaling Technology, Danvers, MA) 1:1000 in 5% bovine serum albumin (BSA) overnight and treated as previously described. Detection of total p-Rb expression was performed using mouse monoclonal IgG_1 antibody (IF8, catalog number sc-102, Santa Cruz) 1:200 in 1% BSA after blocking for 1 hour at RT with 1% BSA. Detection of actin (isoform non-specific) (C2, catalog number sc-8432, Santa Cruz) was used as a loading control on all membranes. After washing 3 X 15 minutes, proteins were visualized on radiographic film via ECL or ECL Plus reagent (Amersham, Piscataway, NJ). Results from Western analyses were obtained from at least two separate experiments.

Measurement of Cell Death

Two methods were utilized to detect cell death and/or apoptosis in drug treated samples. Samples were stained with Annexin V (Pharmingen, San Diego, CA) and Propidium Iodide (PI)

(Roche) as recommended by Pharmingen. Direct TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) staining was also performed according to manufacturer's instructions (Apo-Direct Kit, Pharmingen). Samples were analyzed via flow cytometry using a Becton Dickinson (San Jose, CA) FACSort flow cytometer. Percentages of cell death/apoptosis were measured by obtaining the sum of the upper right and lower right quadrants of the scatterplot generated by analysis of samples stained with AnnexinV/PI. Percentages of apoptotic cells were measured via TUNEL by obtaining the percentage of the cell population staining positive for FITC-dUTP. Results were obtained from at least three independent experiments.

Cell Cycle Analysis

To determine cell cycle kinetics as a result of FP treatment, cell lines were analyzed for DNA content using PI staining and flow cytometric analysis essentially as described by Ormerod (123). Data were generated using ModFit LT for Mac version 3.1 software (Verity Software House, Topsham, ME)**.** Results are representative of at least three independent experiments.

Results

ALL Cell Lines Used for *in Vitro* Testing Lack p16 Protein Expression

I determined p16 protein expression in the cell lines used for *in vitro* drug sensitivity testing via Western blot. Nalm-6, REH, Molt-4, and Jurkat have been reported previously to have homozygous *p16* deletions (33, 40, 124, 125), while the Hunger laboratory has found RCH-ACV to have intact *p16* via Southern blot (126). HeLa cells were used as a positive control. HeLa lysate was diluted into Nalm-6 (*p16* deleted) lysate in order to simulate 10% and 1% p16 expression. I found that none of the ALL cell lines tested expressed a detectable amount of p16 protein, including RCH-ACV (Figure 2-1a).

In Vitro Drug Sensitivity in ALL Cell Lines

I determined the sensitivity (IC_{50}) of Nalm-6, Molt-4, Jurkat, RCH-ACV and K562 to a continuous 96 hour exposure to dexamethasone (Dex) and doxorubicin (Dox), two agents commonly used in the treatment of childhood ALL, and FP. Each of the four ALL cell lines and K562 were highly resistant to dexamethasone and variably sensitive to doxorubicin (Figure 2-1b). Each of the cell lines tested showed sensitivity to FP, with IC_{50}s ranging from 99±11.5 nM in Molt-4 to 312.5±159.1 nM in K562. These values are similar to concentrations achieved *in vivo* in phase I/II trials of FP administered both as a 1 hour and a 72 hour infusion (67-69, 72, 77, 127)**.**

FP Induces Apoptosis in ALL Cell Lines

WST-1 assays measure the numbers of viable cells present following exposure to drug. Decreased numbers of viable cells could be due to apoptosis, decreased cell proliferation, or both. I performed Annexin V/PI staining and subsequent flow cytometric analysis on cell lines which were exposed to drug for 72 hours to determine whether FP induced apoptosis. First, I compared the cell death induced by FP and Dox in Nalm-6 and RCH-ACV at concentrations approximating the IC_{50} of each drug (FP:150nM; Dox:10ng/mL). At these concentrations, FP induced a substantially lower percentage of cell death than Dox in both Nalm-6 and RCH-ACV (Figure 2-2a). I then examined apoptosis induced by 300 nM FP and observed much higher rates of apoptotic cell death, 93% and 83% in Nalm-6 and RCH-ACV respectively. I expanded these studies and confirmed that FP induces apoptosis by performing parallel Annexin V/PI and TUNEL analysis in Nalm-6, RCH-ACV, Molt-4 and Jurkat following 72 hours exposure to FP at various concentrations (Figure 2-2b and 2-2c). For each cell line tested, modest levels (<25%) of apoptosis were induced by 72 hours exposure to 150 nM FP and high levels (>80%) were observed following exposure to 300 nM FP. Similar results were seen via Annexin V/PI and

TUNEL assays, confirming the apoptotic nature of the observed cell death. Taken together, these data establish that FP treatment induces modest apoptotic cell death in B-precursor and T-cell ALL lines at lower concentrations and is a potent inducer of apoptosis at higher concentrations.

FP Induces Cell Cycle Arrest in ALL Cell Lines which Correlates with Effects on pp-Rb Protein Expression

My results demonstrated that the inhibition of cell proliferation observed with WST-1 assays can only partially be attributed to apoptosis when cell lines are treated with 150 nM FP; however, 300 nM FP fully induces apoptosis. I hypothesized that the remaining inhibition at 150 nM FP could be due to cell cycle arrest. In order to test this hypothesis I performed cell cycle analysis of samples treated with 0, 50, 150 and 300 nM FP for 24 and 48 hours. Treatment with 50 nM FP did not induce arrest when compared to an untreated control in RCH-ACV (Figure 2-3a) and Nalm-6 (Figure 2-4). I observed a transient G_1-S arrest after 150 nM treatment that was present at 24 hours, but resolved by 48 hours. Sustained G_1-S and G_2-M arrest were induced after treatment with 300 nM FP; which was apparent at 24 hours and more pronounced at 48 hours. In order to address the possibility that the transient nature of the arrest induced by 150 nM treatment was due loss of drug potency over time, I performed experiments in which treated cells were exposed to FP for 24 hours, at which time the growth medium and drug were replaced (Figure 2-3b). Cultures were allowed to incubate in parallel with those established 24 hours prior for an additional 24 hours. Following incubation all cultures were evaluated for cell cycle kinetics. Data showed similar cell cycle phase distributions between cultures treated with FP for 48 hours and those which had medium and drug replaced after 24 hours.

To investigate the mechanism of the observed cell cycle arrest, I determined expression of total pRb and specific phospho-pRb forms (pp-Rb^{ser795} and pp-$Rb^{ser807/811}$) in parallel to cell cycle analysis (Figure 2-3c). Phosphorylation of pRb on ser 795 has been largely linked to CDK 4

activity and regulation of the G_1-S transition by pRb (128). Phosphorylation of ser 807/811 has also been linked to CDK 4 activity (129). Treatment of ALL cell lines with 300 nM FP resulted in a sustained decrease in pp-Rb^{ser795} and pp-$Rb^{ser807/811}$ protein expression, which correlates with the G_1-S arrest observed at this drug concentration. Total pRb protein levels indicate stable levels of total protein and a decrease in the expression of the hyperphosphorylated form of pRb (upper band) that is dependent on drug concentration and time of exposure to FP. Treatment with a low level of FP (50 nM) did not result in a decrease in the phosphorylation of pRb; however, treatment with 150 nM and 300 nM FP did result in a decrease in the expression of the phosphorylated form of pRb after 48 hours treatment.

Apoptotic Effects of FP in Human Serum

Others have shown that FP is 92-95% protein bound in human plasma and that there is a decrease in the activity of FP in CLL cells when grown in human plasma or serum vs. FBS (82). In order to determine if supplementation with human serum (HS) would have a similar effect on FP efficacy in ALL cell lines, I tested the ability of FP to induce apoptosis in Nalm-6, RCH-ACV, Jurkat, and Molt-4 grown in medium supplemented with FBS and compared this to cell death of cell lines grown in HS. In order to mimic the peak drug levels that occur with FP infusion schedules with high activity against CLL cells (30 minute bolus followed by a 4 hour infusion), I measured cell death at 4.5 hrs (Figure 2-5a). For measurement of cell death at a sub-peak level, I analyzed after 24 hours drug exposure (Figure 2-5b). Varying concentrations of FP were used in keeping with those found to be achieved in CLL patients treated with the above schedule at approximately these time points (85). After 4.5 hours, a modest percentage of cell death is induced in Nalm-6 and RCH-ACV (15-20% and 10%, respectively). In contrast, approximately 55-60% cell death is induced in Jurkat and Molt-4 at 4.5 hours. When comparing the cell death induced in cultures supplemented with FBS to that of HS, I show that the

differences in the percentage of apoptosis induced in media containing FBS vs. HS are not substantial for Nalm-6, RCH-ACV, and Molt-4; however, more cell death was induced by FP in Jurkat cells grown in media containing FBS vs. HS. The percentage of apoptosis observed after 24 hours FP treatment was higher than that at 4.5 hours in all four cell lines tested. When comparing the cell death achieved in cultures supplemented with FBS to that of HS at the 24 hour timepoint, I observed substantial differences between media containing FBS and HS for RCH-ACV at all FP concentrations tested and at the lowest FP concentration (300 nM) in Nalm-6 and Jurkat. My results show no differences between FBS and HS for Molt-4.

Discussion

The poor outcome of children with ALL who experience a bone marrow relapse despite intensive chemotherapy and/or stem cell transplant, makes it imperative to identify agents with novel mechanisms of action. Based on the frequent acquisition of *p16* deletions at relapse (27) and alterations in expression of genes that encode for cell cycle regulatory proteins at relapse (18), I performed preclinical studies of FP in ALL cell lines. My results support the use of these cell lines as a model of relapsed ALL in that none of the lines expressed p16 protein and all were resistant to dexamethasone and variably sensitive to doxorubicin, two agents commonly used in the treatment of ALL and to which relapsed patients frequently become resistant (130). I report that childhood ALL cell lines are sensitive to FP, providing a biological rationale for clinical trials of FP in relapsed ALL.

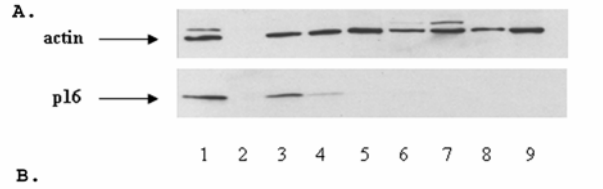
I found that FP was active in a concentration dependent manner against ALL cell lines. At a concentration approximating the IC_{50} determined in WST-1 assays (150 nM), FP induced transient cell cycle arrest with a limited percentage of apoptosis. At approximately twice this concentration, FP was a potent inducer of cell death. This information provides a dual mechanistic explanation for the decrease in viable cell number that I observed in WST-1 assays.

CDKs phosphorylate pRb and therefore regulate its ability to sequester transcription factors involved in cell proliferation during the G_1-S phase transition. Phosphorylation of pRb on approximately 16 different serine and/or threonine residues can be attributed to the activity of specific CDKs (131). My Western blot analysis shows that expression of pp-Rb^{ser795} and pp-$Rb^{ser807/811}$ is reduced after 24 and 48 hours treatment with 300nM FP. These data correlate with the sustained G_1-S arrest observed after 300 nM FP treatment and show that FP treatment results in a reduction of endogenous CDK activity. Evaluation of total pRb protein expression showed consistent expression across all treatment levels, indicating that the decrease in phospho-specific pRb was not due to loss of total pRb. Treatment with 150 and 300 nM FP resulted in a decrease in the hyperphosphorylated form of total protein after 48 hours drug exposure. This decrease further illustrates the reduction in CDK activity as a result of FP treatment in ALL cell lines. This also suggests a mechanism for the transient G_1-S arrest observed after 150 nM FP treatment and the G_2-M arrest I observed after 300 nM treatment. The apoptosis and cell cycle arrest induced by FP treatment in ALL cell lines provide two potential modes of treatment in ALL. FP could be used as a single agent to induce a cytotoxic effect or be utilized in combination with another chemotherapeutic agent that would complement the ability of FP to induce cell cycle arrest. My observation that FP inhibits CDK activity and induces cell cycle arrest in ALL cells suggests that there may be schedule dependent differences in activity if FP is combined with other agents, particularly those with cell cycle specific activity.

Initial phase I/II trials of FP in many human cancers were disappointing. While *in vitro* studies showed that FP was efficaceous against a diverse variety of tumors at concentrations of 100-300 nM, no significant clinical activity was seen with prolonged infusion regimens, despite achievement of similar FP concentrations *in vivo* (69-73). Shinn and colleagues hypothesized

that the disparity between *in vitro* and *in vivo* activity might be due to differences in binding of FP to plasma proteins present in FBS used *in vitro* vs. those present in human plasma (82). They confirmed that for CLL cells the FP IC_{50} was significantly (approximately 10-fold) higher *in vitro* when experiments were performed in human plasma rather than bovine serum. This suggested that infusion schedules that produced high peak FP concentrations might be more effective than prolonged lower dose infusion schedules. Early phase clinical trials confirmed this hypothesis in CLL using a 30 minute bolus dose followed by a 4 hour infusion (85). Expanded studies are ongoing. Based on these observations, I also examined the relative efficacy of FP *in vitro* in experiments using human serum compared to bovine serum. In my experiments I observed fewer differences between ALL cell line sensitivity to FP in HS vs. FBS than observed by Shinn and colleagues. Importantly, despite some differences among the cell lines tested, substantial amounts of apoptosis were induced by FP in all cell lines under conditions that are very similar to what might be observed clinically, particularly at the 1000 nM and 2000 nM levels at 4.5 hours and 300 nM level at 24 hours. These data suggest that the newer FP infusion schedules found to be very promising in CLL should be utilized to test FP against relapsed ALL.

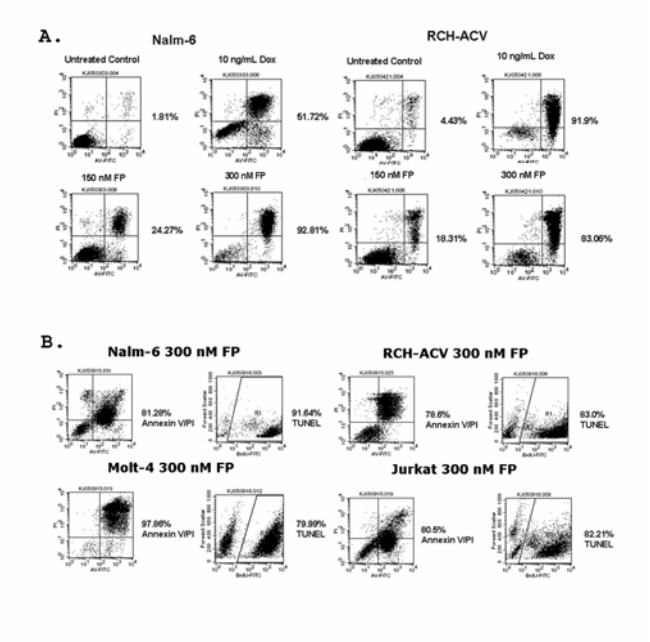
A.



B.

	Dexamethasone (ug/mL)	Doxorubicin (ng/mL)	Flavopiridol (nM)
RCH-ACV	>10	13±6.6	131.3±33.8
Nalm-6	>10	10.6±2.9	142.5±46.0
Molt-4	>10	9.7±3.8	99±11.5
Jurkat	>10	77.3±42.1	300±35.4
K562	>10	39.2±26.7	312.5±159.1

Figure 2-1. Fifty percent inhibitory concentration (IC_{50}) determinations via WST-1 in cell lines that lack p16 protein expression. A) Western blot for p16 protein expression; Lane 1) 100% HeLa (positive control), 2) empty lane, 3)10% HeLa, 4) 1% HeLa, 5) Nalm-6, 6) RCH-ACV, 7) REH, 8) Molt-4, 9) Jurkat B) IC50 values±1SD for Dex, Dox and FP after 96 hours drug exposure measured via WST-1 cell proliferation assays.



C.

	Cell death measured via AnnexinV/PI (%)	Apoptosis measured via TUNEL (%)
Nalm-6		
Untreated	2.47	0.51
150 nM FP	19.72	22.22
300 nM FP	81.45	89.02
RCH-ACV		
Untreated	5.94	0.58
150 nM FP	23.46	22.33
300 nM FP	78.16	80.04
Molt-4		
Untreated	7.91	1.81
150 nM FP	31.16	24.23
300 nM FP	98.16	81.07
Jurkat		
Untreated	4.98	0.54
150 nM FP	11.12	3.07
300 nM FP	79.28	82.03

Figure 2-2. Flavopiridol induces apoptosis in ALL cell lines in a concentration dependent manner. A) Scatter plots from flow cytometric analysis of Annexin V/PI stained samples of Nalm-6 and RCH-ACV comparing cell death induced by 72 hours exposure to 150 nM and 300 nM FP to the cell death induced by 72 hours exposure to 10 ng/mL Dox, an agent known to induce apoptosis. Percentage to right of each plot represents the sum of the lower right quadrant (cells in the early stage of apoptosis) and upper right quadrant (late stage apoptosis) of each plot B) Scatterplots generated using two different staining methods after 72 hours continuous exposure to 300 nM FP show similar results; AnnexinV/PI (left) and TUNEL staining (right) C) Comparison of results from AnnexinV/PI to TUNEL in cell lines treated with 0, 150, and 300 nM FP respectively.

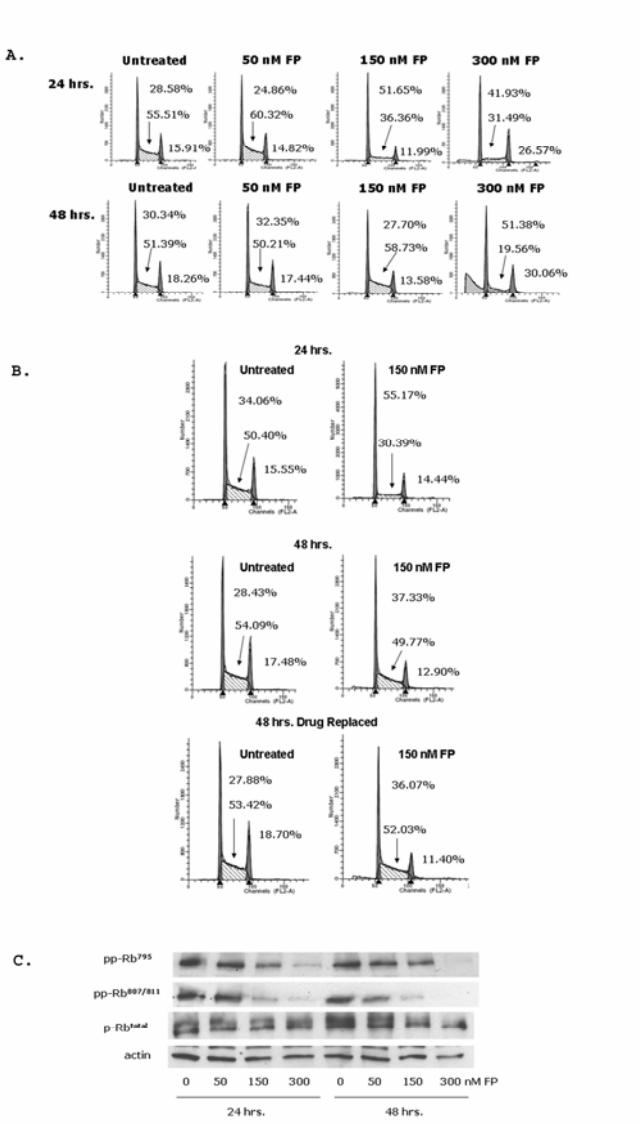


Figure 2-3. Flavopiridol induces G_1-S and G_2-M (Gap 2-mitotic) arrest in RCH-ACV with reduced phosphorylation of pRb A) Cell cycle data after 24 and 48 hours exposure to 0, 50, 150 and 300 nM FP. Treatment with 50 nM FP has no effect on cell cycle kinetics when compared to untreated control. Data show a transient G_1-S arrest after 24 hours exposure to 150 nM FP and a sustained G_1-S arrest after 24 and 48 hours treatment with 300 nM FP. Also shown is a sustained G_2-M arrest following 300 nM FP exposure. B) Release from G_1-S arrest post-24 hours exposure is not due to loss of drug potency. RCH-ACV cells were treated with FP for 24 hours, at which time medium and drug were freshly replaced. Samples collected 24 hours after replacement (48 hours total time of drug exposure) show cell cycle kinetics that are comparable to samples without drug replacement. C) Western blot showing a sustained decrease in expression of pp-$Rb^{ser\ 795}$ and pp-$Rb^{ser807/811}$ after exposure to 300 nM FP. Total p-Rb expression remains constant with a decrease in the hyperphosphorylated form (upper band) after 150 and 300 nM treatment.

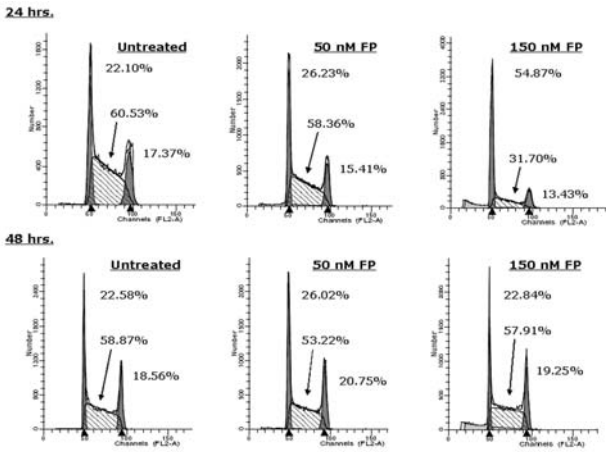


Figure 2-4. Flavopiridol induces transient G_1-S arrest in Nalm-6. Cell cycle data after 24 and 48 hours exposure to 0, 50, and 150 nM FP. Treatment with 50 nM FP has no effect on cell cycle kinetics when compared to untreated control. Data show a G_1-S arrest after 24 hours treatment with 150 nM. Cell cycle kinetics return to baseline after 48 hours treatment.

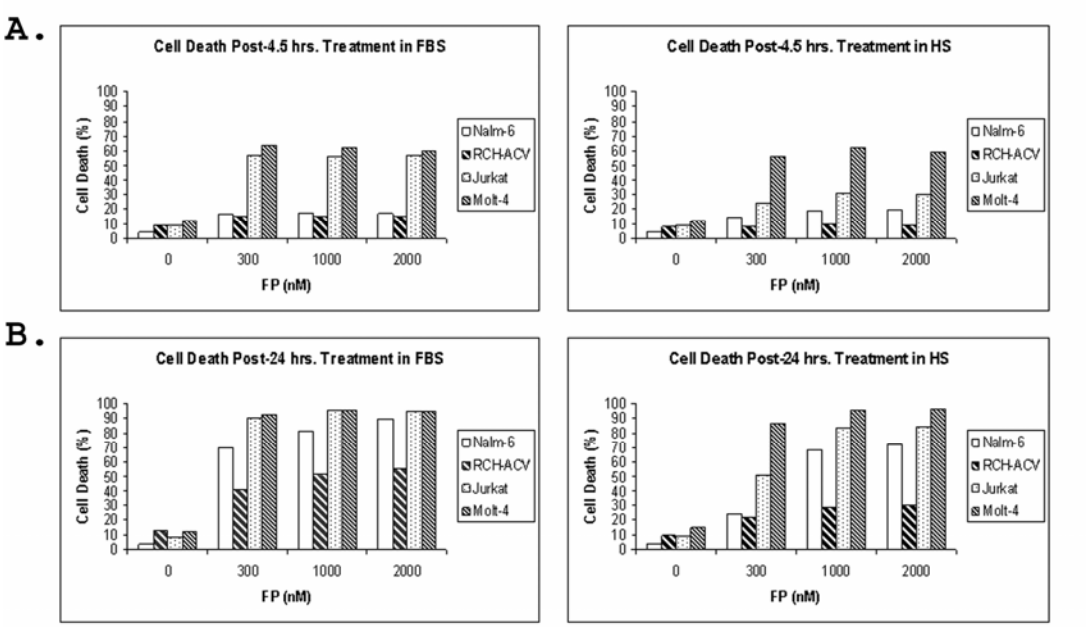


Figure 2-5. Efficacy of FP in human serum. Cell lines were exposed to clinically achievable concentrations of FP for 4.5 hours and 24 hours in medium supplemented with FBS or HS. A) More cell death was induced in Jurkat cells treated for 4.5 hours and supplemented with FBS than those in HS; however, the cell death induced in the remaining cell lines was approximately equal between the two types of sera. B) After 24 hours treatment of RCH-ACV, more cell death was induced by FP treatment at all concentrations tested in cells supplemented with FBS than those supplemented with HS. Differences in cell death between the two types of sera were also observed in Nalm-6 and Jurkat after treatment with 300 nM FP. No differences between sera were observed in Molt-4.

CHAPTER 3
PRECLINICAL STUDIES OF FLAVOPIRIDOL COMBINED WITH PACLITAXEL IN ACUTE LYMPHOBLASTIC LEUKEMIA

Introduction

Clinical trials of FP involving cancer types other than ALL have shown that FP has limited efficacy when used as a single agent. As outlined in Chapter 1, several *in vitro* studies have shown that the efficacy of traditional chemotherapy agents can be increased when used in combination with FP. One such traditional agent is paclitaxel (PAC). Few clinical trials involving FP combination therapy have been performed; however, promising results have been obtained in a variety of solid tumor patients using FP in combination with PAC (111). PAC has shown *in vitro* toxicity in leukemia cell lines; however, has had limited efficacy in clinical trials in children and adults with leukemia (15, 16). PAC and FP represent two drugs to which ALL patients will not have been previously exposed. This fact as well as *in vitro* data showing synergy between FP and PAC in other cell types lead me to question if PAC/FP combination therapy could hold promise in the treatment of ALL.

It has been previously found that the interaction between PAC and FP is dependent on the sequence in which the two drugs are administered *in vitro*. Combination therapy is most efficacious when PAC precedes FP in the treatment sequence (PAC→FP), as opposed to the reverse or concurrent therapy (56, 60). I have confirmed these findings in ALL cell lines as well as determined optimal treatment duration for each agent prior to testing combination therapy. In an effort to maintain the clinical relevance of my findings I have also taken the currently accepted *in vivo* infusion schedule for each drug into account when designing my experiments. The recommended schedule for PAC administration is either 3 hour or 24 hour infusion (110). I have tested 6 and 24 hours exposure to PAC. Early trials involving FP used a 72 hour continuous infusion schedule. My experiments reflect this, as in experiments in which PAC was combined

with FP, cell lines were exposed to FP for 72 hours. Later studies by others have shown that FP is highly protein bound and that supplementing cell culture medium with human serum (HS) in place of fetal bovine serum (FBS) decreases the sensitivity of CLL cells to FP (82). In order to address this I have conducted experiments in media supplemented with both FBS and HS.

Methods

Materials

ALL cell lines were obtained and cultured as described previously (132). PAC (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in RPMI 1640 prior to each experiment. FP (Sanofi-Aventis, Bridgewater, NJ) was dissolved in DMSO and further diluted in RPMI 1640 no more than 30 days prior to each experiment.

Single Agent *In Vitro* Sensitivity Assays

Nalm-6 and RCH-ACV were exposed to 0-300 nM FP in RPMI 1640 with 10% fetal bovine serum (FBS; Mediatech, Inc. Herndon, VA) continuously for 72 hours. Cell death was measured every 24 hours via flow cytometric analysis of Annexin V (Pharmingen, San Diego, CA)/Propidium Iodide (PI, Roche, Indianapolis, IN) as described previously (132). Treatment duration and sensitivity to PAC were determined by exposing Nalm-6 and RCH-ACV to 0-100 nM PAC for 6 hours and 24 hours in parallel followed by cell death measurements every 24 hours for a total of 72 hrs (Figure 3-1). In a separate experiment, Nalm-6 was exposed to 0-1,000 nM PAC in RPMI 1640 supplemented with 10% human serum (HS; Mediatech) for a period of 6 hours in parallel to samples treated in media supplemented with 10% FBS with cell death measured every 24 hours. In order to determine the effect of HS on untreated cell proliferation and viability, growth curves were generated using trypan blue staining (Sigma) of samples grown with HS compared to FBS.

Drug Combination Studies

FP was combined with PAC using a drug concentration ratio of 1:10 (PAC:FP) in Nalm-6 and RCH-ACV. Due to greater sensitivity to PAC, Molt-4 and Jurkat were treated at a ratio of 1:20 to allow for a lower concentration of PAC to be utilized. Cell lines were exposed to PAC in cell culture medium supplemented with 10% FBS for approximately six hours, washed, and then treated with FP for an additional 72 hours. Treatment duration was selected based on PAC and FP single agent experiments. Single drug controls for PAC consisted of six hours incubation with PAC followed by incubation in drug free medium for approximately 72 hours. Cell lines used for single drug treatment with FP were incubated in drug free RPMI 1640 for approximately six hours; after which the cell lines were incubated with FP for an additional 72 hours. At the completion of the 72 hour incubation, cell death was evaluated for all samples. In order to confirm that results similar to that found in medium supplemented with FBS could be achieved in cultures supplemented with HS, Nalm-6 was exposed to PAC for 6 hours followed by FP for 72 hours at a concentration ratio of 1:3. Control samples were treated and cell death was evaluated in the same manner as described for combination studies in FBS.

Treatment Sequence

Nalm-6 and RCH-ACV were used to determine the optimal treatment sequence. One 1:10 combination was chosen from the drug combination studies in FBS in order to confirm that PAC followed by FP (20nM PAC→200nM FP) was indeed the most efficacious treatment sequence. Briefly, for the samples in which FP treatment followed PAC treatment (PAC→FP), cell lines were cultured in RPMI 1640 with and without 20 nM PAC for 6 hours, then washed and transferred to RPMI 1640 with and without 200 nM FP. In sample sets in which FP treatment preceded PAC treatment (FP→PAC), cell lines were treated similarly, with the sequence of drug exposure reversed. Cell death measurements were taken immediately after FP treatment for

PAC→FP and its controls. For FP→PAC and its controls, cell death was determined approximately 18 hours following completion of PAC treatment. Concurrent exposure experiments were performed separately; during which cell lines were treated with both agents for a total of 72 hours. Cell death was measured at 24 hour intervals via flow cytometry as previously described.

Statistical Analysis

All experiments, excluding concurrent exposure to PAC and FP, were performed with three independent replicates. A mixed model was used to analyze each experiment. The replicates for each experiment were considered a random factor. If there was a significant interaction ($p<0.05$) between the variables in each experiment, mainly cell line, treatment, time of exposure, and drug concentration depending on the type of experiment, then Least Squares Means of the treatment combinations were compared using a Student's t-test or F test.

Results

Single Agent FP Treatment

In order to determine the time of exposure to FP that resulted in the maximum cell death response in ALL cell lines, I incubated Nalm-6 and RCH-ACV with 0-300 nM FP with cell death measured at 24, 48, and 72 hours. There was a significant concentration dependent response in both cell lines ($p<0.0001$; Figure 3-2 a). I compared the cell death induced after 24 hours treatment to the cell death induced after 72 hours. There were no significant differences in cell death based on time of exposure at the 50 nM concentration in either Nalm-6 or RCH-ACV. In Nalm-6, there was a significant difference in cell death between 24 and 72 hours exposure to 300 nM FP ($p<0.0001$), while in RCH-ACV these differences were observed between 24 and 72 hours exposure to 150 nM ($p=0.0034$) as well as between 24 and 72 hours exposure to 300 nM FP ($p<0.0001$).

Single Agent PAC Treatment

I exposed Nalm-6 and RCH-ACV to PAC for 6 or 24 hours and measured apoptosis at 24, 48, and 72 hours following initial exposure. I observed concentration dependent cell killing in Nalm-6 and RCH-ACV samples treated for both 6 and 24 hours with a greater percentage of cell death observed after 24 hours vs. 6 hours drug exposure (Figure 3-2 b). Further, I show that cell lines treated for 6 hours then transferred to drug-free medium show a greater gradation in response between 10 nM PAC and 100 nM PAC than those treated for 24 hours, particularly in Nalm-6. Cell death peaked 48 hours post-treatment and remained consistent with no substantial change at 72 hours post-treatment in both cell lines. Statistical analysis showed that time of exposure (6 or 24 hours) to PAC was a significant factor in the percentage of cell death observed in both Nalm-6 ($p=0.0001$) and RCH-ACV ($p=0.0138$). The post-treatment time at which cell death measurements were taken was also a significant factor in both Nalm-6 ($p<0.0001$) and RCH-ACV ($p=0.0008$). The interaction between the factors of time of exposure and sample time was significant in Nalm-6 ($p=0.0427$); however, this interaction was not significant in RCH-ACV ($p=0.6812$). My results indicate that even though a 24 hour drug exposure time resulted in a greater percentage of cell death, a shorter exposure period of 6 hours still resulted in a substantial amount of cell death. Importantly, incubation for 48-72 hours post-treatment was needed in order to achieve a maximum response.

Combination Treatment with FP and PAC

Based on my single agent studies I treated four cell lines with PAC for 6 hours; then transferred the cultures to media containing FP for 72 hours and measured cell death at the end of this period. Nalm-6 and RCH-ACV were treated with a drug concentration ratio of 1:10 (PAC:FP) and Molt-4 and Jurkat at a ratio of 1:20. I observed a concentration dependent cell death response for both the single agent treatments as well as each combination (Figure 3-3). My

results demonstrate that when PAC is combined with FP the cell death that results is significantly higher than when either of the two agents is utilized by itself. I found a statistically significant difference ($p<0.05$ or $p<0.0001$) between single agent and combination treatment in the range of 10-30 nM PAC and 100-300 nM FP in Nalm-6. In RCH-ACV these differences were present for treatments in the range of 15-25 nM PAC and 100-250 nM FP. Significant differences ($p<0.0001$) were present at 5-10 nM PAC and 100-150 nM FP in Molt-4 and Jurkat.

Determination of Optimal Schedule for PAC+FP

I tested PAC→FP, the reverse sequence, and each of the single agent controls to confirm that the former was the most effective treatment sequence. The most promising dose level (20 nM PAC→200 nM FP) was selected for more detailed analysis. Combining PAC with FP in the sequence PAC→FP results in significantly greater cell death in Nalm-6 than FP→PAC or single agent treatment (Figure 3-4 a). The cell death resulting from the PAC→FP was significantly higher than the other treatments with p values ranging from $p<0.0001$ to $p=0.001$. I observed a similar response in RCH-ACV with p values ranging from $p<0.0001$ to $p<0.01$ (Figure 3-5).

I also examined concurrent exposure to 20 nM PAC and 200 nM FP and observed no enhancement (Figure 3-4 b) in activity. Indeed, the cell death induced after treatment with both agents is less than that observed with FP alone across three days of testing.

Activity of PAC in Human Serum

Studies have found that 89-98% of PAC is protein bound in human serum (110). This prompted us to investigate how the efficacy of PAC in ALL cell lines would be affected if culture medium was supplemented with human AB serum (HS) in place of fetal bovine serum (FBS). I show that Nalm-6 cells exposed to 10, 100, and 1,000 nM PAC for 6 hours in media supplemented with HS underwent significantly less apoptosis than observed when experiments were performed using media supplemented with FBS (Figure 3-6). These differences in PAC

sensitivity were not due to intrinsic differences in cell growth in media supplemented with FBS vs. HS (Figure 3-6 d).

Combination Studies in Human Serum

Shinn and colleagues have reported that 63-100% of FP is free (non-protein bound) in FBS, as compared to only 4.7-7.9% free in human plasma *in vitro* (82). Based on this information and the high level of protein binding by PAC to plasma proteins, I treated Nalm-6 with PAC combined with FP at a concentration ratio (PAC:FP) of 1:3 in medium supplemented with HS in order to confirm that the enhancement of PAC activity by FP that I observed in FBS could also be achieved under these culture conditions (Figure 3-7). Higher concentrations of PAC were used in order to compensate for the lower sensitivity of ALL cell lines to PAC in the presence of HS. I show that FP significantly enhances the efficacy of PAC at all concentrations tested ($p<0.0001$). Combination treatment was significantly different from FP single agent treatment at the lowest concentration tested ($p<0.05$); however, significant differences did not exist at higher concentrations of FP most probably due to a high percentage of cell death induced from single agent FP treatment using this prolonged exposure schedule.

Discussion

There is a significant need to identify novel agents and new combination treatments for relapsed ALL. Others have shown that PAC and FP have limited efficacy when utilized as single agents in leukemia patients (16, 75, 133); however, together these drugs have mechanisms of action that complement each other and might effectively target aberrations in cell cycle regulation that are commonly present in ALL cells at relapse. My studies demonstrate that ALL cell lines are sensitive to both PAC and FP *in vitro*, and that FP enhances the efficacy of PAC in a sequence specific manner.

My observation that the cell death induced after treatment with 1,000 nM PAC in HS is comparable to cell death achieved by treatment with 100 nM PAC in FBS is consistent with prior studies by others showing a high degree of PAC binding by proteins present in HS. In contrast to data of Shinn and colleagues in CLL (82), my previous studies showed relatively little difference between the *in vitro* activity of FP when experiments are performed in HS vs. FBS. My current observations confirm that despite a decrease in sensitivity to PAC in the presence of HS, FP enhances the efficacy of PAC under these treatment conditions.

The time of actual drug exposure utilized for PAC treatment (6 hours) in my experiments was in keeping with the recommended clinical administration of 3 or 24 hours infusion (110). My findings show that 48-72 hours of incubation are needed to achieve a maximum cell death response to PAC treatment. I also found that the amount of cell death induced by FP in the concentration ranges studied was dependent upon the duration of exposure, with 72 hours inducing a peak response. Based on these two factors, I studied a 72 hour FP exposure in the combination studies. This schedule of FP exposure is different than newer FP dosing strategies that administer a 30 minute FP bolus followed by a 4 hour infusion to produce much high peak FP concentrations than those achieved with other infusion schedules, which have yielded promising early results in patients with refractory chronic lymphocytic leukemia (CLL) (83). In other studies, I have found that shorter *in vitro* exposure of the same ALL cell lines, cultured in media containing either FBS or HS, to high FP concentrations similar to those attained in CLL clinical trials induced substantial amounts of apoptosis. Based on the results attained in the current studies, I anticipate that administration of PAC prior to the FP bolus should enhance ALL cell death and suggest that this combination should be investigated in clinical trials for relapsed ALL.

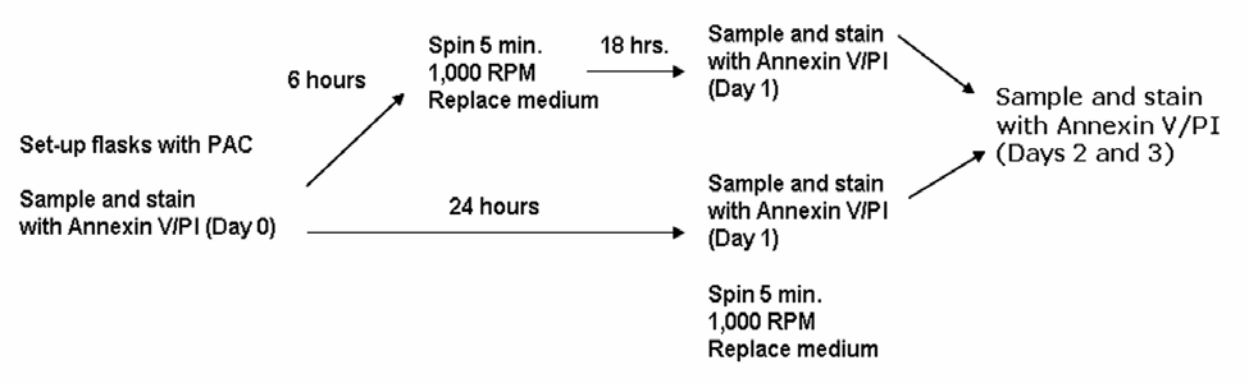


Figure 3-1. Experimental design for PAC single agent treatment.

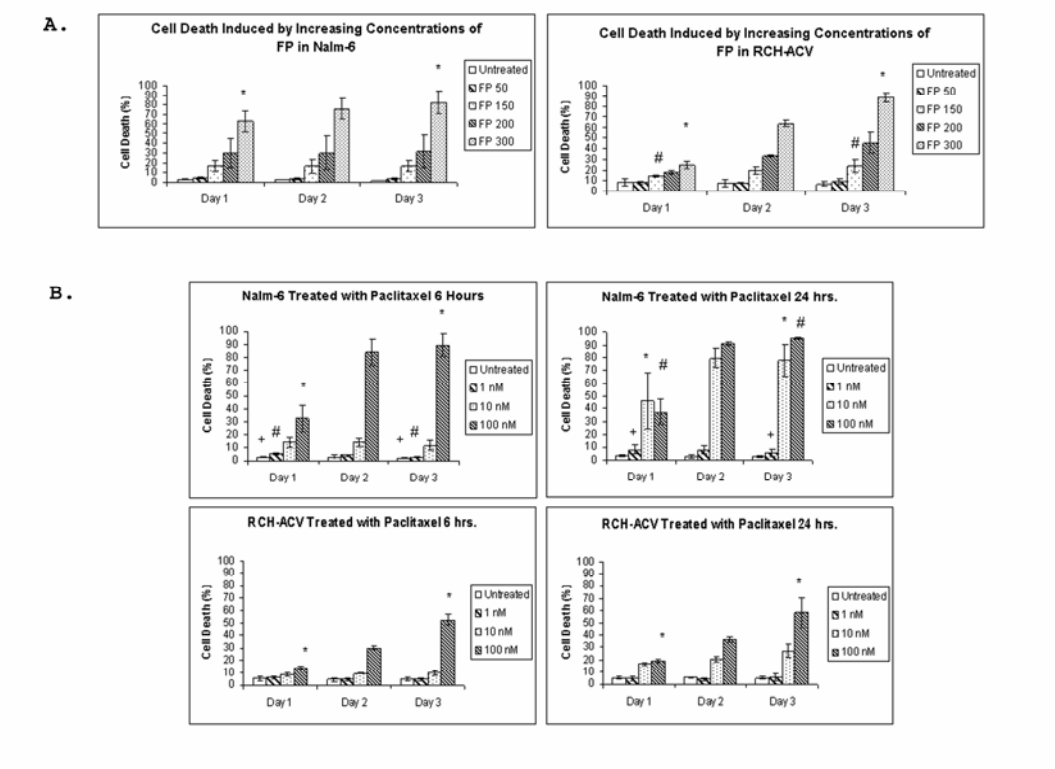


Figure 3-2. Cell death induced by treatment with FP or PAC in Nalm-6 and RCH-ACV. A) Percent cell death induced after 72 hours treatment with 0, 50, 150, 200, and 300 nM FP measured every 24 hours via flow cytometric analysis of Annexin V/PI stained samples of Nalm-6. Results are the mean of three independent experiments±1SD. Significant differences in time of exposure at identical drug concentrations are indicated; *$p<0.0001$ and #$p=0.0034$. B) Cell death induced after 6 hours or 24 hours treatment with 0, 1.0, 10, 100 nM PAC measured every 24 hours thereafter for a total of 72 hours. Results are the mean of three independent experiments±1SD. Significant differences at identical concentrations between the 24 and 72 hour sample times are indicated; p values ranged from 0.01 to <0.0001 for all symbols.

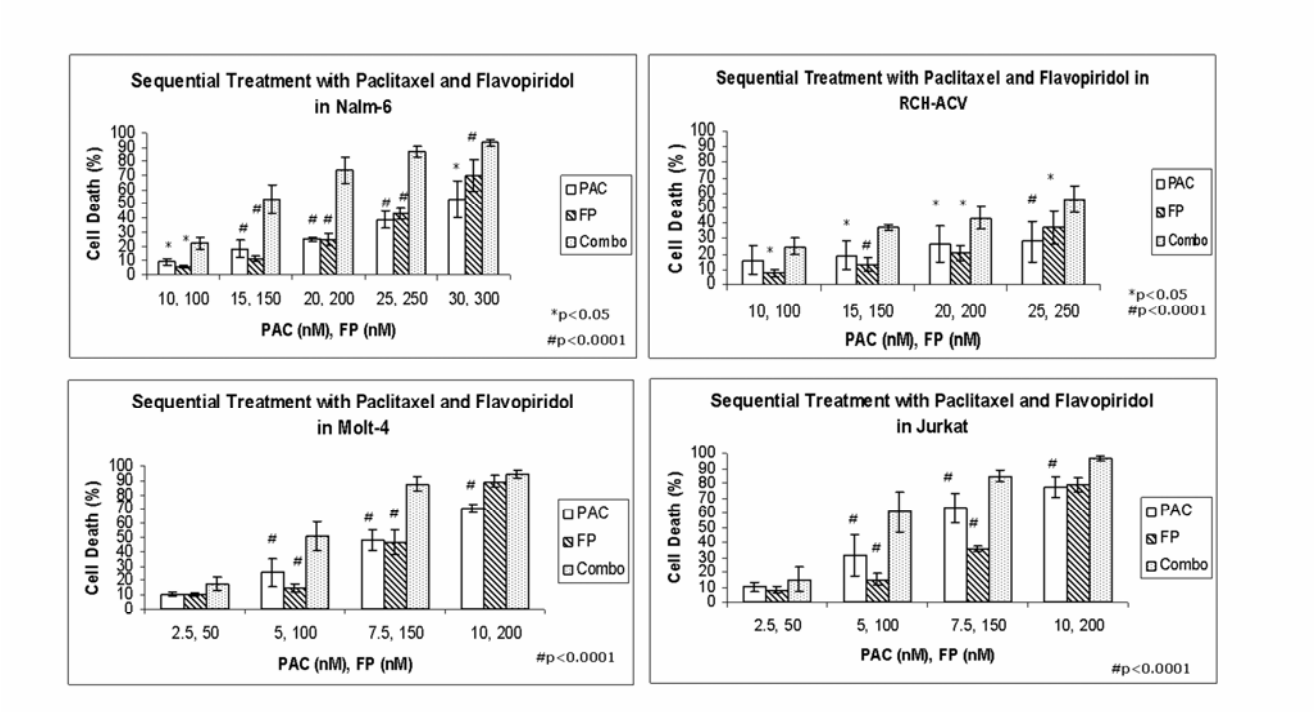
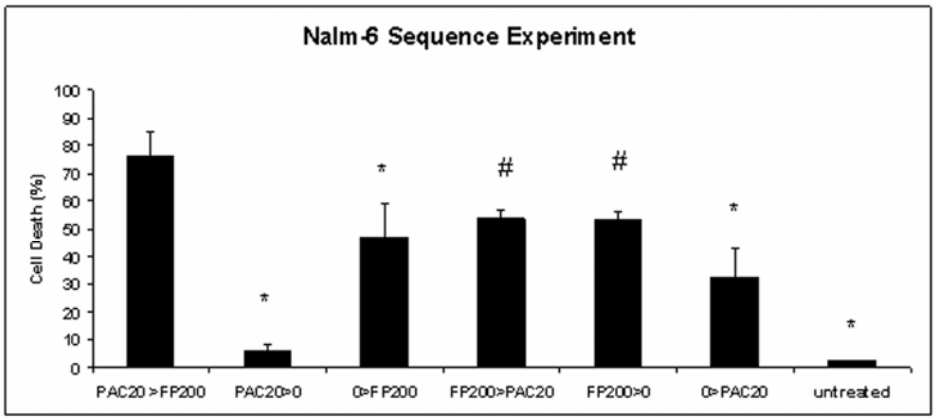


Figure 3-3. Flavopiridol enhances the efficacy of PAC in ALL cell lines. FP was combined with PAC in the sequence PAC→FP at a concentration ratio of 1:10 in Nalm-6 and RCH-ACV and 1:20 in Molt-4 and Jurkat. Cell lines were treated with PAC for 6 hours immediately followed by FP for 72 hours. Cell death was measured at the conclusion of treatment via flow cytometric analysis of Annexin V/PI stained samples. Results represent averages from at least three independent experiments±1SD.

A.



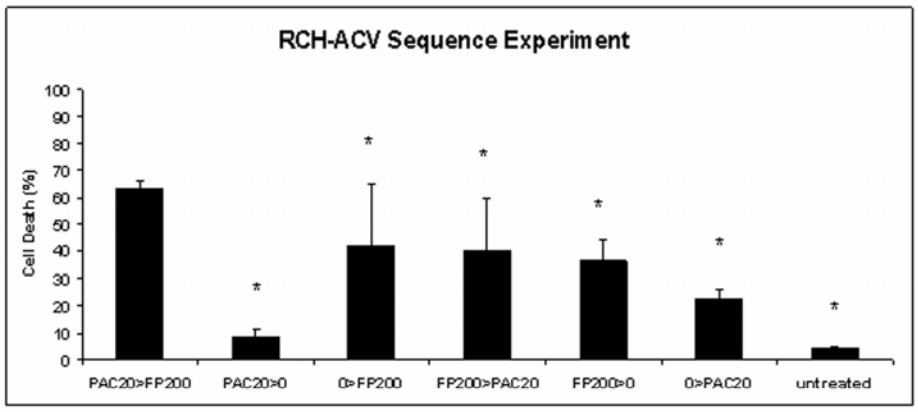
B.

Nalm-6 Concurrent Exposure

Treatment	Day 0	Day 1	Day 2	Day 3
untreated	3.19	4.13	2.68	1.64
PAC 20+FP 200	3.19	31	56.53	53.63
PAC 20	3.19	44.33	40.59	41.58
FP 200	3.19	55.46	65.77	67.45

Figure 3-4. PAC→FP is a more efficacious treatment sequence than FP→PAC or concurrent exposure in Nalm-6. A) Nalm-6 was treated for 6 hours with 20 nM PAC followed by 200 nM FP for 72 hours or the reverse sequence. Both sequences included appropriate single agent controls and an untreated control. Cell death was measured via flow cytometric analysis of Annexin V/PI stained samples immediately following FP treatment for PAC→FP and approximately 18 hours after PAC treatment for FP→PAC. Results are the mean of three independent experiments±1SD. Statistical significance was measured by comparing PAC20→FP200 to the remaining treatment sequences. *$p \leq 0.0001$; #$p = 0.001$. B) Concurrent exposure to PAC and FP in Nalm-6. Cell lines were simultaneously exposed to 20 nM PAC and 200 nM FP for a total of 72 hours with cell death measured every 24 hours.

A.



B.

RCH-ACV Concurrent Exposure

Treatment	Day 0	Day 1	Day 2	Day 3
untreated	4.5	4.12	3.97	3.25
PAC 20+FP 200	4.5	15.27	28.76	28.23
PAC 20	4.5	16.61	32.12	44.14
FP 200	4.5	15.23	18.7	19.52

Figure 3-5. PAC→FP is a more efficacious treatment sequence than FP→PAC or concurrent exposure in RCH-ACV. A) RCH-ACV was treated for 6 hours with 20 nM PAC followed by 200 nM FP for 72 hours or the reverse sequence. Both sequences included appropriate single agent controls and an untreated control. Cell death was measured via flow cytometric analysis of Annexin V/PI stained samples immediately following FP treatment for PAC→FP and approximately 18 hours after PAC treatment for FP→PAC. Results are the mean of three independent experiments±1SD. Statistical significance was measured comparing PAC20→FP200 to the remaining treatment sequences. *$p \leq 0.01$. B) Concurrent exposure to PAC and FP in RCH-ACV. Cell lines were simultaneously exposed to 20 nM PAC and 200 nM FP for a total of 72 hours with cell death measured every 24 hours

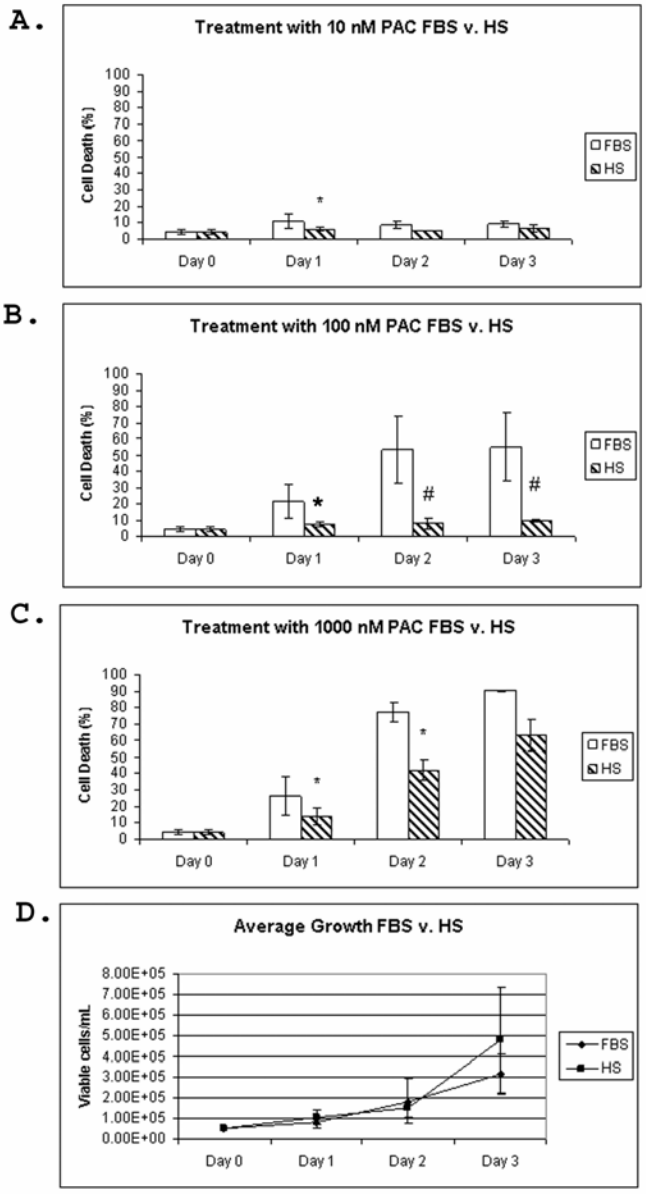


Figure 3-6. Efficacy of PAC in Nalm-6 in the presence of human serum. Cell death resulting from 6 hours exposure to PAC in the concentrations shown in medium supplemented with HS compared to that in FBS measured every 24 hours for a total of 72 hours. a) 10 nM PAC b) 100 nM PAC c) 1000 nM PAC d) growth curve from untreated cell cultures comparing HS and FBS supplements. All results are the mean of three independent experiments±1SD; *$p<0.05$, #$p\leq0.0002$.

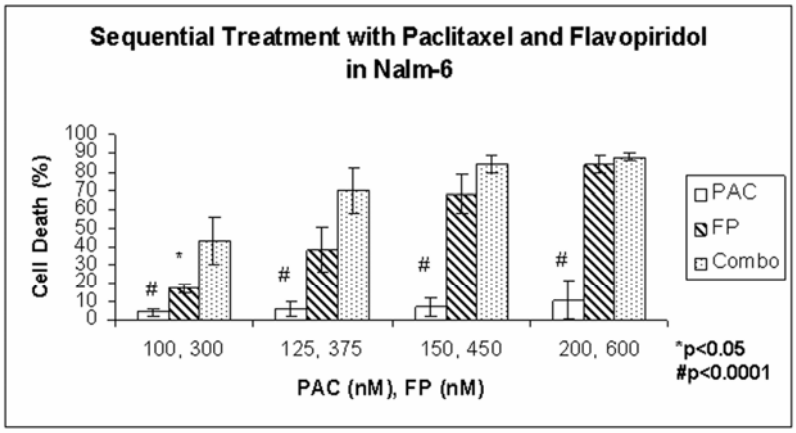


Figure 3-7. Flavopiridol enhances the efficacy of PAC in human serum. PAC was combined with FP at a concentration ratio of 1:3 in Nalm-6 cultured in the presence of HS. Nalm-6 was treated and sampled as in previous assays in FBS. Results are the mean of three independent experiments±1SD.

CHAPTER 4
CONCLUSIONS AND DISCUSSION

FP Single Agent Studies

Establishing an *in Vitro* Treatment Model of ALL

In this project I have focused on determining the potential efficacy of FP both as a single agent and in combination with PAC in ALL cell lines. I tested these agents based on their mechanisms of action. FP could target defects in cell cycle regulation produced by mutations in human cancer, such as p16; which is frequently altered at the gene and/or protein expression level in relapsed ALL (124). The action of PAC as an inducer of CDK1 and microtubule depolymerization inhibitor complements this activity. Following the first chapter of background, the second chapter of this dissertation details the results that I obtained when testing FP as a single agent. As part of my initial studies, I established that the cell lines to be used for sensitivity testing were an accurate model of relapsed ALL by determining their level of p16 expression and their sensitivity to dexamethasone and doxorubicin, agents typically used to treat ALL (3). I found that despite the fact that one of the cell lines had an intact *p16* gene, none of the cell lines expressed p16 (Figure 2-1). I also showed that the cell lines were highly resistant to dexamethasone and variably sensitive to doxorubicin; findings not unlike what would be obtained in a patient (2). Importantly, the cell lines were sensitive to FP at concentrations similar to those found to be effective *in vitro* by others.

Drug Sensitivity Testing via Cell Proliferation Assays

Results for drug sensitivity were obtained using WST-1; a modified version of the MTT cell proliferation assay. As introduced in the second chapter, *in vitro* sensitivity to chemotherapeutic agents measured via this type of assay has been found to correlate to *in vivo* efficacy (112-119). In order to use these assays, I first needed to establish that the cell lines could

be maintained untreated in log phase growth over the standard time course of four days. I found that using a cell concentration of $1X10^4$ cells/well in 96 well plates gave the best exponential growth over time (Figure 4-1 a, b). Nalm-6 was later established at a lower concentration due its lower doubling time (Chapters 2 and 3). In addition, as part of my preliminary studies I showed that measuring cell proliferation over time via a cell proliferation assay resulted in the same type of exponential growth curve as when the number of viable cells over time was measured by trypan blue exclusion (Figure 4-1 c); thus validating the assay. Cell proliferation assays were used to generate growth curves to establish the IC_{50} concentrations reported in Chapter 2 (Figure 2-1). Examples of these growth curves may be found in Figure 4-2.

The Mechanism of Cell Death Induced by FP in ALL Cell Lines

Also in Chapter 2, I established that FP induces apoptosis in ALL cell lines. FP induced less apoptosis at its IC_{50} than a similar concentration of doxorubicin, a known cytotoxic agent (Figure 2-2). I also showed that FP treatment resulted in apoptosis consistently across four ALL cell lines (Figure 2-2). Results were obtained after 72 hours treatment to reflect treatment strategies current at the time. These measurements were taken using flow cytometric analysis of both Annexin V/PI and TUNEL stained samples in order to confirm that the level of cell death measured using Annexin V/PI assays was consistent with another method.

Based on the ability of FP to induce cell cycle arrest in cancer cell types other than ALL (14, 53) and the apparent disparity in cell death at the IC_{50}s for doxorubicin and FP, I conducted a study of cell cycle kinetics after treatment with both low and high concentrations of FP. I found that at the IC_{50}, FP induced a transient G_1-S block that appeared after 24 hours treatment; with cell cycle kinetics returning to baseline by 48 hours treatment (Figure 2-3). This arrest gave an explanation for the decrease in cell proliferation observed at this FP concentration despite a lack of apoptosis. Treatment with a concentration of FP twice the IC_{50} resulted in a sustained G_1-S

and G_2-M arrest over the course of 48 hours. Drug replacement studies showed that the transient nature of the G_1-S arrest at the IC_{50} was not due to loss of drug potency over time. Further, I established that FP prevents the phosphorylation of specific serine residues on pRb, an indication of a decrease in endogenous CDK activity.

FP Activity in Human Serum

Recent findings have shown that FP is highly protein bound in human plasma and that this low drug availability can decrease *in vitro* efficacy (82). Researchers hypothesized that this could explain the disappointing results obtained in clinical trials using FP as a 72, 24 or 1 hour infusion, despite obtaining plasma concentrations similar to that found to be effective *in vitro* (20-400 nM) (69-81, 124). As explained in Chapters 2 and 3, Byrd and colleagues from Ohio State University (OSU) designed a clinical trial for patients with refractory CLL with the goal of obtaining a plasma FP concentration of 1.5 μM after a 30 minute bolus dose followed by a 4 hour infusion (83). The trial was quite successful despite issues with tumor lysis syndrome, with an overall response rate of 45%. In order to determine if protein binding would have an effect on the cell death induced by FP treatment in ALL cell lines, I tested *in vitro* sensitivity in the presence of HS and compared it to FBS. Cell death measurements were taken after 4.5 hours and 24 hours continuous exposure to mimic peak and trough concentrations in the OSU infusion schedule (Figure 2-5). I found that despite the high level of protein binding in human serum reported by others, it is possible to achieve a high percentage of cell death in ALL cell lines *in vitro* with concentrations that mimic those expected to be produced by the treatment schedule utilized by Byrd and colleagues.

PAC+FP Combination Studies

Note about Statistical Analysis

Chapter 3 details my studies of FP in combination with PAC. To make meaningful comparisons between treatments, statistical analysis was applied to the data. The analyses of each data set followed the same basic pattern; beginning with a mixed model analysis of variance (ANOVA) followed by post-tests to determine if there were significant differences between treatments, times of exposure, or treatment sequences depending on the variables of the individual experiment. ANOVA was chosen as the method of analysis based on the fact that each type of experiment involved multiple comparisons. If a Student's t-test had been applied to each individual measurement within each data set, the probability of obtaining significant p values would have been artificially high (134).

By definition ANOVA compares the actual results to the data that would have been obtained if the null hypothesis were correct. ANOVA was used to test the significance of the interaction between the variables for each experiment. If the null hypothesis were correct, there would be no interaction between any of the variables in the experiment. Each p value given from the mixed model analysis assigns a percentage to the probability that the interaction present was due to chance. If significant interactions between variables were present, results based on one variable could not be analyzed for significance without taking the other variables into account. The "mixed model" designation to the ANOVA simply states that there were fixed and random factors in each of the experiments. The variables tested in each experiment were defined as being fixed effects and the number of replicates was taken as a random effect. The mixed model analyses are given in tables which are discussed throughout the text that follows.

If the interaction between the factors in each experiment was significant, then the Least Squares Means of the replicates from each experiment were compared using a Student's t-test or an F-test. These analyses are also included in the discussion that follows.

Enhancement of PAC Activity by FP

PAC and FP have complementary modes of action in that PAC enhances CDK 1 activity while FP is a pan-CDK inhibitor. This is the hypothesis behind why the enhancement of PAC by FP is dependent on the order in which the drugs are administered (56). As summarized in Chapter 3, others have shown that FP can enhance the activity of PAC *in vitro* in cancer types other than ALL (56, 60). Promising results have also been obtained during a clinical trial using patients with various types of cancer (111). I chose to test PAC in combination with FP due to the aberrations in cell cycle regulatory proteins frequently found in relapsed ALL patients and because this treatment regimen would offer a new possibility to children with relapsed ALL using two agents to which they would not have been previously exposed.

In order to conduct these experiments I first needed to establish whether ALL cell lines were sensitive to PAC and determine a treatment schedule for each of the drugs as single agents. After testing FP in two cell lines over 72 hours, I was able to determine that the time of exposure had a significant effect on the percentage of cell death induced by FP. Statistical analysis was conducted by first determining if there was a significant interaction between the factors of FP concentration and time of exposure (Tables 4-1 and 4-2). When it was determined that a significant interaction existed, the data were further analyzed by determining if there were significant differences in cell death at different times of exposure for a given FP concentration (Tables 4-3 and 4-4). With the results given in Chapter 3 (Figure 3-2), I was able to conclude that 72 hours FP treatment gave a peak cell death response.

Through single agent PAC studies I established that ALL cell lines were sensitive to this agent. I chose to measure cell death as a result of 6 hours and 24 hours treatment based on earlier pharmacokinetic studies by others (110, 135, 136)**.** Following drug exposure, apoptosis was measured every 24 hours for a total of 72 hours. Statistical analysis of my data showed that there was a significant interaction between PAC concentration, time of exposure, and sample time in Nalm-6 but not RCH-ACV (Tables 4-5 and 4-6). I chose to use a 6 hour exposure to PAC for my combination studies, as this resulted in a better range of choices for drug concentrations to test given the gradation in response between 10 nM and 100 nM when compared to 1.0 and 10 nM after 24 hours exposure. Also, as detailed in Chapter 3, my data showed that 48-72 hours incubation were required to achieve maximum cell death after treatment (Figure 3-2). Statistical analysis of PAC single agent treatment may be reviewed in Tables 4-7 and 4-8.

Methods of Determining Synergy

I combined FP with PAC to determine if the combination would increase the efficacy of the single agents. Synergy can be defined as when the effect of a combination of agents is greater than the sum of the effects of each of the single agents (137)**.** There are various methods to determine if the effect of treatment with multiple agents is synergistic, including isobologram analysis, fractional effect, and median-effect analysis (138). Isobologram analysis begins by measuring the dose of each drug required to produce the same effect, e.g. 50% cell death. These doses are plotted against each other and a line is drawn connecting the two doses (Figure 4-3) (139). The line is said to represent the doses of the two drugs which are equipotent. If a dose combination produces the designated effect and is plotted far below the line, this combination is considered synergistic, e.g. point Q in Figure 4-3. If the drug combination is plotted far above the line, it is considered to be antagonistic (point R). Points very close to the line represent additivity (point P). The isolobolgram method requires a large number of measurements, applies only if the

drugs have similar modes of action (mutually exclusive) and can only be used for combinations of two drugs (138).

The fractional product method is very intuitive in that one simply multiplies the percentages represented by the unaffected fraction (e.g. percent viable cells post-treatment) for each single agent (138). If the combination of drugs results in a percentage that is equal to the product of the two single agents, then the two agents are additive. The requirements of this method are that the drugs must have different modes of action (mutually non-exclusive) and that the dose-effect curves for the agents are hyperbolic.

The most common method in current literature used to evaluate for synergy between agents is based on the median-effect principle authored by Chou and Talalay (61). One uses median-effect analysis to determine a Combination Index (CI) value for each drug combination. According to median effect analysis, if CI=1.0 this indicates an additive relationship between the two drugs. If CI<1.0, synergy is present and if CI>1.0 antagonism is indicated. This method has the advantage of allowing the researcher to evaluate a minimal number of drug concentrations and determine the relationship between greater than two drugs if desired. In addition, one is not limited to evaluating agents with only the same or different modes of action. Both mutually exclusive and mutually non-exclusive agents can be analyzed.

To understand median-effect analysis, let us first examine the median effect principle. This principle is based on the IC_{50} for each individual agent. Consider statement 4-1:

$$\frac{(fa)_{1,2}}{(fu)_{1,2}} = \frac{(fa)_1}{(fu)_1} + \frac{(fa)_2}{(fu)_2} = \frac{(D)_1}{(Dm)_1} + \frac{(D)_2}{(Dm)_2} \quad . \tag{4-1}$$

The term $(fa)_X$ is the fraction affected by drug (percent cell death after treatment with drug), *fu* is the fraction unaffected by drug (percent viable cells), D is the dose of a single drug in the

combination and D_m is the IC_{50} for that drug if it were used as a single agent. If drugs 1 and 2 were combined at their IC_{50}s,

$$\frac{(fa)_1}{(fu)_1}+\frac{(fa)_2}{(fu)_2}=0.5+0.5=1.0. \quad (4\text{-}2)$$

Now consider two examples from PAC combined with FP in Nalm-6 at a ratio of 1:10. In the first, 10 nM PAC was combined with 100 nM FP. The IC_{50} for PAC was 28.95 nM based on the single agent controls in the experiment and the IC_{50} for FP was 279.1 nM. Thus, according to statement 4-1:

$$\frac{(D)_1}{(Dm)_1}+\frac{(D)_2}{(Dm)_2}=\frac{10}{28.95}+\frac{100}{279.1}=0.703 \quad (4\text{-}3)$$

In the second example, 15 nM PAC was combined with 150 nM FP with the same IC_{50}s as in the first example for a sum equaling 1.055. Comparatively, the sum in the first example is 30% below 1.0, whereas the sum in the second example is 6% above 1.0. Through the use of the median-effect principle, one can conclude that the combination in the first example is synergistic and the second combination is additive, providing that both agents have the same mode of action.

From this information one understands how CI values based on 1.0 were derived. From statement 1, median effect analysis defines CI as:

$$\mathrm{CI}=\frac{(D)_1}{(Dx)_1}+\frac{(D)_2}{(Dx)_2}. \quad (4\text{-}4)$$

D is the dose of each drug used in the combination and *Dx* is the dose of each single agent that would be required to induce the same percentage of cell death caused by the drug combination. In the case of two or more agents having different modes of action, Equation 4-4 is modified to:

$$\mathrm{CI}=\frac{(D)_1}{(Dx)_1}+\frac{(D)_2}{(Dx)_2}+\frac{(D)_1(D)_2}{(Dx)_1(Dx)_2}. \quad (4\text{-}5)$$

FP Combined with PAC

Combination studies were initiated by first establishing a concentration ratio for PAC and FP. Under median-effect analysis, it is suggested that agents are combined using a set drug concentration ratio (137), in this case PAC:FP. Several ratios were tested in Nalm-6 including 1:5, 1:10, 1:12, and 1:15 (Figure 4-4). I found that FP enhanced the activity of PAC most dramatically when the two agents were used in the ratios of 1:10 and 1:12. I performed median-effect analysis to generate Combination Index (CI) values for the four ratios tested. A CI<1.0 indicated synergy, while CI>1.0 indicated antagonism and CI=1.0 indicated an additive relationship between PAC and FP (61). Table 4-9 shows CI values at the 50% effective dose for the drug combination (ED_{50}), ED_{75}, and ED_{90} for each ratio. When evaluating combination data using median-effect analysis, two sets of CI values are generated based on the modes of action of the two drugs tested: mutually exclusive and mutually non-exclusive CI values. I chose to utilize the mutually non-exclusive CI values, as PAC and FP have different modes of action. Though the CI results were <1.0 when PAC was combined with FP at a ratio of 1:12, the actual cell death measurements were more compelling when the two agents were combined at a ratio of 1:10 (see Figure 4-4). This ratio also allowed for the use of lower concentrations of both agents.

Nalm-6 and RCH-ACV were tested using a ratio of 1:10. When testing Molt-4 and Jurkat, I used a ratio of 1:20 to account for the fact that these cell lines were exquisitely sensitive to PAC. This allowed for the use of a lower PAC concentration. Results from cell death measurements shown in Chapter 3 indicated a significant difference in the cell death induced by single agent controls when compared to each combination in all four cell lines tested (Figure 3-3). A mixed model analysis similar to what was employed for FP and PAC single agent studies was utilized to evaluate the overall significance of the results (Tables 4-10 and 4-11). Nalm-6 and RCH-ACV were evaluated under the same analysis, as these cell lines were treated with the

same concentration ratio (1:10). Molt-4 and Jurkat were subjected to a separate analysis, as both were treated with a drug concentration ratio of 1:20. It was found that there was a significant interaction between the factors cell line, treatment (PAC, FP, or PAC→FP) and drug concentration in all four cell lines tested. Based on the overall significance of the results, the cell death induced by each single agent control was compared to its respective combination. The results for which there were significant differences are reported in Tables 4-12 and 4-13. Representative CI values for the ED_{50}, ED_{75}, and ED_{90} for each cell line are reported in Table 4-14. The data ranged from being slightly synergistic (Nalm-6 ED_{90} CI=0.939) to antagonistic (Jurkat ED_{50} CI=1.59), with half of the drug combinations showing near additivity to slight antagonism.

The CI values reported in this chapter represent those obtained based on PAC and FP having different modes of action and clearly show that the degree of synergy between PAC and FP is very slight where it is present. In Chapter 3 I show that FP enhances PAC activity and vise versa by measuring the simple effects of the drugs. In order for this enhancement to be considered synergistic via median-effect analysis, the differences between single agent treatment and combination therapy would need to be several orders of magnitude higher than the data that I obtained (61)**.** This can be explained by the median-effect plot which graphs log (D) vs. log $\frac{fa}{fu}$ and ultimately connects to the median-effect equation from which all of the above equations are derived. Stated more simply, analysis of combined drug effects through median-effect analysis requires log order differences between single agent and combination treatment. For example, if instead of obtaining the data reported in Chapter 3 at a drug concentration ratio of 1:10, I had found similar results using a ratio of 1:100 or 1:1000, my CI values would have been much lower and therefore more synergistic.

Sequence Dependent Enhancement

Results reported previously by others show that enhancement of PAC activity by FP is dependent on the sequence in which the agents are administered (56). In order to confirm this in ALL cell lines, I chose the most promising treatment from my combination studies to determine if PAC→FP, FP→PAC or concurrent exposure would result in the highest percentage of cell death. As reported in Chapter 3, the percentage of cell death resulting from standard treatment (PAC→FP) was compared to the reverse sequence and single agent controls. An ANOVA was utilized to analyze the statistical significance of treatment sequence prior to comparing the individual effects of treatment. A one-way analysis of variance was used for Nalm-6 and a weighted one-way analysis was used for RCH-ACV (Tables 4-15 and 4-16) to make this determination. The weighted analysis was used due to an inconsistent sample size for some of the treatment conditions for this cell line. The term "one-way" connotes that the experiments were categorized in one way: by treatment sequence instead of by treatment sequence and cell line. Treatment sequence was a significant factor in the percentage of cell death resulting from the various treatments tested. Statistics were not applied to the data from concurrent exposure experiments, as these data were generated from one experiment. I confirmed that PAC→FP was the most efficacious treatment sequence in Nalm-6 (Figure 3-4 a). The statistical analysis comparing 20 nM PAC→200 nM FP to the reverse sequence and single agent controls can be found in Table 4-17. I also confirmed the proper treatment sequence in RCH-ACV (Figure 3-5 a, Table 4-18). I showed in Figure 3-4 b and Figure 3-5 b that concurrent exposure is not a feasible option for this drug combination, as it resulted in less cell death than the sum of the two single agents.

Drug Sensitivity in Human Serum

Due to the reported difference in binding by FP to human plasma proteins vs. proteins in FBS and the resulting decrease in sensitivity in CLL cells, I compared the sensitivity of ALL cell lines to FP in FBS and HS and found that there was not a substantial difference in sensitivity between the two sera (Figure 2-5). PAC is also highly plasma protein bound and I report in Chapter 3 that there is a 10-fold decrease in the sensitivity of ALL cell lines to PAC in the presence of HS when compared to FBS (Figure 3-6). Statistical analysis showed that the type of serum used had a significant impact on the results ($p=0.0370$; Table 4-19). Statistical comparisons between the two types of sera at specific PAC concentrations were performed to supplement the data shown in Chapter 3 (Table 4-20). I reported that the difference in sensitivity between FBS and HS was not due to a significant difference in cell proliferation in the two sera. The statistical analysis on which this conclusion was based is reported in Table 4-21.

Based on these results I wanted to confirm that the enhancement in PAC activity by FP that I had observed in FBS could also be achieved in the presence of HS. I chose to use a concentration ratio of 1:3 based on preliminary experiments using a variety of ratios (Figure 4-5). This concentration ratio allowed for the use of higher concentrations of PAC to compensate for lower sensitivity in HS. However, these concentrations are still substantially lower than the plasma concentrations reported during clinical trials of PAC in children with leukemia or solid tumors (16, 109)**.** I found that FP enhances the efficacy of PAC in a manner similar to the enhancement found in FBS using a ratio of 1:10 or 1:20. Statistical analysis showed that despite a lack of significance in the interaction between treatment and drug concentration, there was an enhancement of PAC activity by FP (Tables 4-22 and 4-23).

Placing Perspective on this Project

Potential Side Effects of Single Agent and Combination Therapy

As previously discussed in Chapters 2 and 3, severe tumor lysis syndrome (TLS) resulted during the initial clinical studies of the currently used administration schedule for FP (83). Though this was a clear sign of the efficacy of FP in CLL, the toxicity that resulted caused the death of one patient enrolled in the study. Steps have since been taken to prevent TLS through the use of prophylactic therapy prior to the administration of FP and monitoring of patients while on therapy. An algorithm for monitoring for hyperkalemia has also been instituted as part of the study (140). According to official monitoring criteria from the NCI, the level of hyperkalemia that has resulted has been low in both inpatients and outpatients; however, pre-treatment, potassium chelation therapy, and dialysis have still been required in some cases.

Concern might be raised about the potential side effects of combining PAC with FP. Any dose limiting toxicities reported during trials of PAC in patients with leukemia have occurred at concentrations in the micromolar range; much higher than the concentrations used in my experiments. It should also be noted that these concentrations were achieved after a 24 hour infusion. I am proposing a shorter infusion time for PAC in my combination studies.

Where Does FP Fit into the Treatment Scheme of ALL?

When a novel agent becomes available as a possible addition to the regimen used to treat ALL, researchers and clinicians must determine for what stage of therapy the new agent is best suited. Because childhood ALL has such a high cure rate, it is difficult to measure a significant improvement as a result of the addition of a new agent to the initial stages of therapy. Some feel that novel agents should replace current therapies with the goal of decreasing toxic side effects rather than increasing the cure rate; particularly when the drug is a targeted agent that would be used in a subgroup that already has a positive prognosis (141). While it might be somewhat

beyond the scope of this dissertation, considering whether FP would have a place in the treatment of ALL is relevant to proposing a clinical trial. Some might question whether a pan-CDK inhibitor has a place in an age of cancer drug discovery characterized by targeted therapies. FP has been used with success in trials of CLL patients. Others have shown that the biological mechanism behind the capability of FP to kill CLL cells is its ability to decrease the transcription and protein expression levels of short-lived anti-apoptotic molecules such as Bcl-2 and Mcl-1 (100). These molecules were targeted based on the need of CLL cells, which are non-cycling, to express them continuously in order to remain in a state of senescence.

While ALL is characterized by many types of chromosomal translocations and other genetic aberrations, there is not one specific molecule that can be targeted across subgroups of patients, such as the BCR-ABL tyrosine kinase produced by the 9;22 translocation that has made imatinib mesylate (Gleevec) so successful in patients with chronic myelogenous leukemia. As previously discussed in Chapters 2 and 3, more modern studies using microarray technology have found significant differences in the expression of genes that regulate cell cycle, DNA repair and apoptosis between the times of diagnosis and relapse in ALL; however, more work is necessary to discern a clear pattern in gene expression that would reveal which aberrations lead to relapse (18). Several studies remain that show that p16 is altered at the gene and/or protein expression level in up to 50% of ALL cases. Because FP can function in the p16 pathway and there is a lack of available targets that affect a comparable percentage of ALL patients, the fact that FP is not a precisely targeted therapy should not be a hindrance to its possible use in ALL.

Future Directions

This work represents an initial study of the efficacy of FP as a single agent and combined with PAC in ALL cell lines. It might be beneficial to expand these studies into patient samples, in order to determine if the treatments would be efficacious in samples which are far less

removed from a patient than immortalized cell lines. I would also propose testing the biological basis of the apoptosis induced by FP as a single agent by determining the activation of caspases and downregulation of antiapoptotic molecules such as Bcl-2 and Mcl-1 as result of treatment. Importantly, and perhaps in contrast to the work performed by others, I would only propose the further biological studies after FP had been successfully tested in a patient population. The studies of FP contained herein, perhaps with the addition of single agent studies in patient samples, provide a biological justification for a clinical trial of FP in children with ALL. I have shown that FP can induce apoptosis and cell cycle arrest, both mechanisms that inhibit proliferation of cancer cells. If FP was found to be successful in treating children with ALL, then further studies into its mechanism of action *in vivo* would be warranted. These studies could hold the possibility of assisting researchers in discovery of new targets for more potent agents on the horizon. Also, this information would provide a basis for using FP in combination with other agents such as PAC.

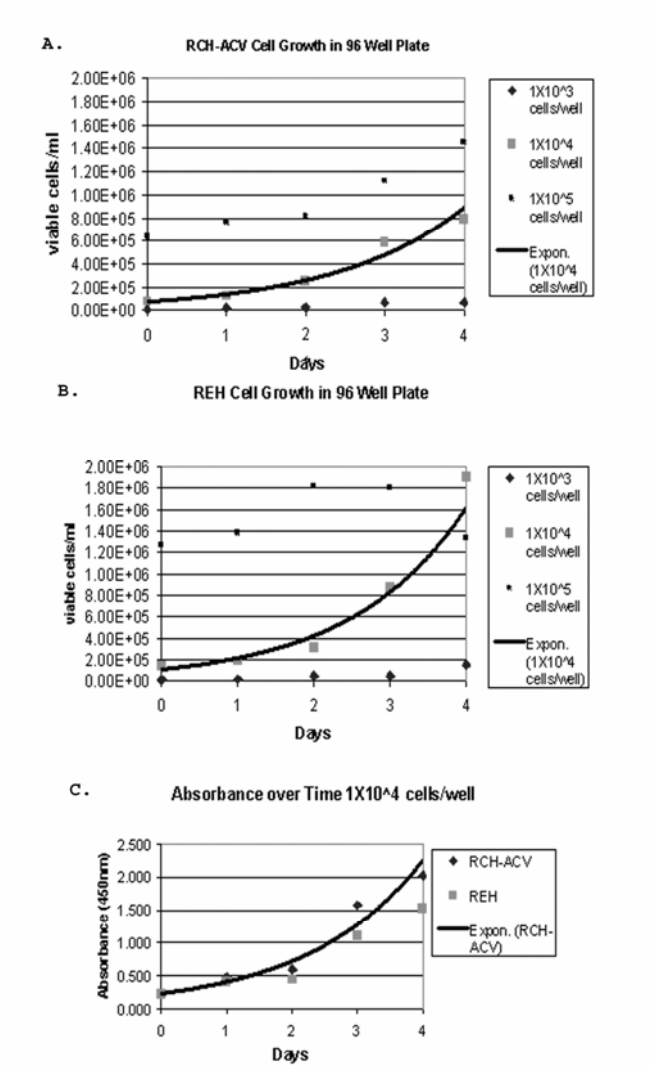


Figure 4-1. Growth curves used to establish cell concentration for proliferation assays. Trypan blue exclusion and WST-1 were utilized to measure the number of viable cells per well in a 96-well plate over a 4 day time period. a)growth curves generated from RCH-ACV; b)REH; c)growth curves generated using WST-1 for comparison to trypan blue exclusion.

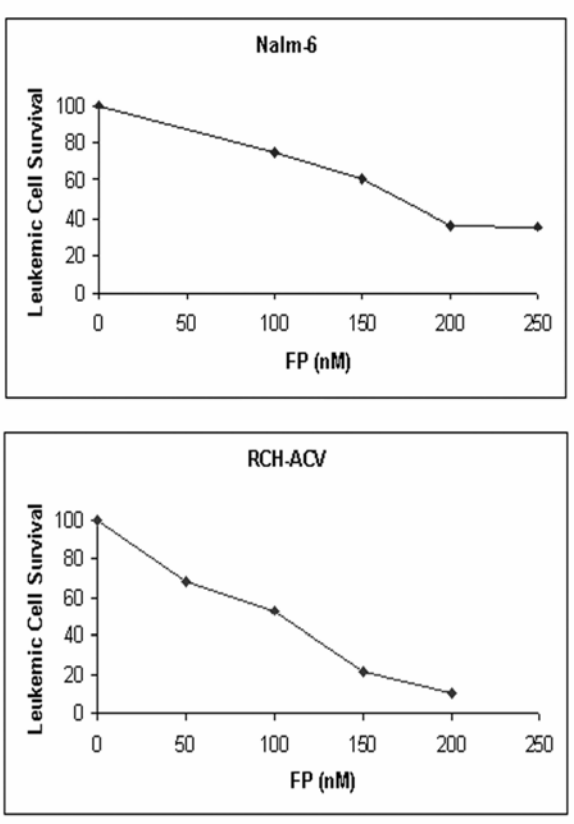


Figure 4-2. Representative dose-response curves generated from cell proliferation assays.

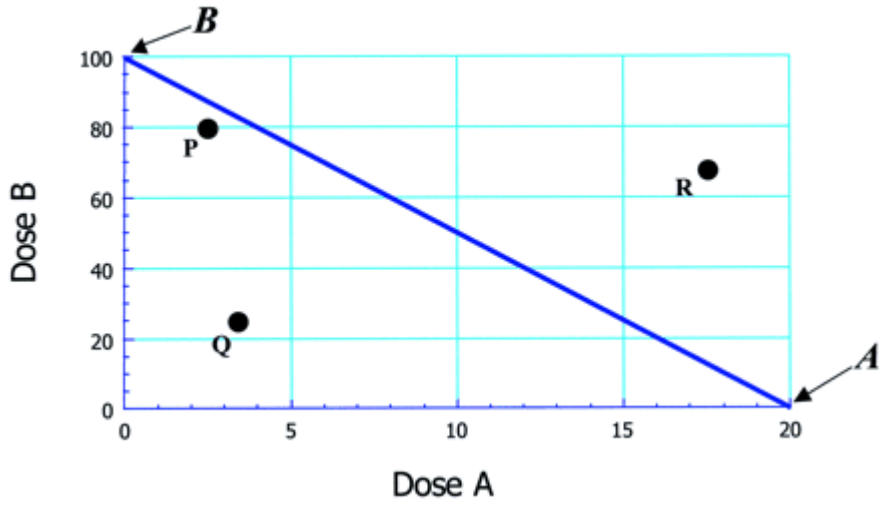


Figure 4-3. Illustration of isobologram analysis of combined drug effects. Given the combination of drugs A and B at equipotent concentrations, possible responses from the drug mixture are shown. Point "Q" represents a synergistic effect, point "P" an additive effect and point "R" represents antagonism Reprinted by permission from American Society for Pharmacology and Experimental Therapeutics: [Journal of Pharmacology and Experimental Therapeutics] Tallarida RJ. Drug synergism: its detection and applications. Journal of Pharmacology and Experimental Therapeutics 2001; 298(3):865-872, copyright 2001, originally published as Figure 1, p.866.

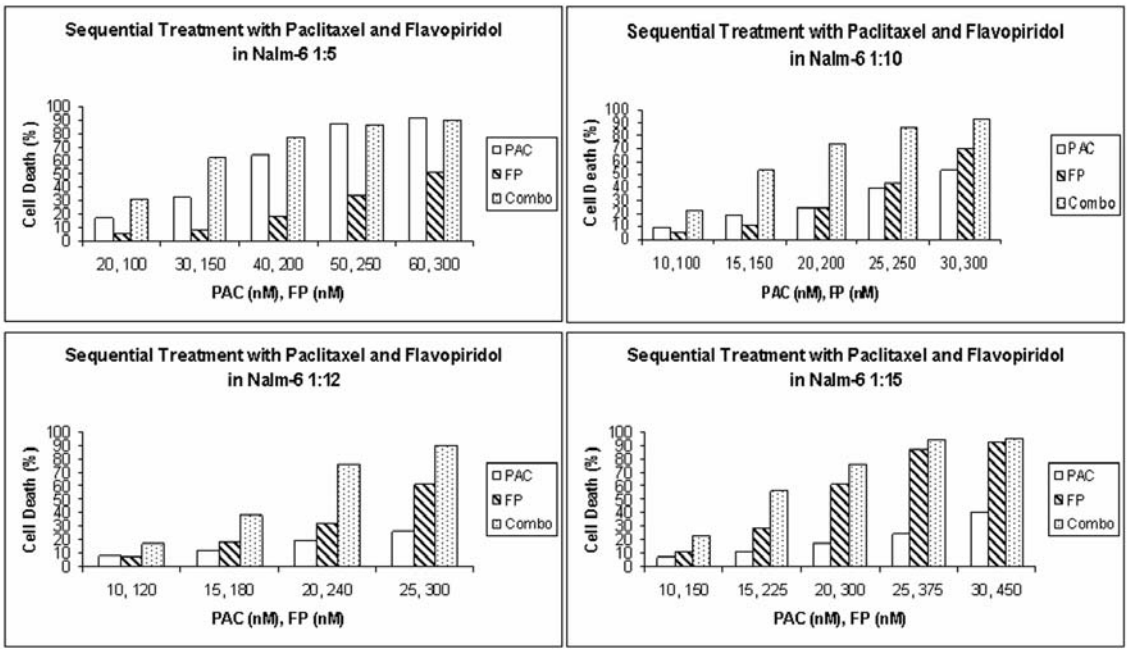


Figure 4-4. Preliminary combination data at a variety of ratios in Nalm-6. Cell death measurements after treatment with PAC for 6 hours followed by FP for 72 hours at concentration ratios (PAC:FP) of 1:5, 1:10, 1:12, and 1:15.

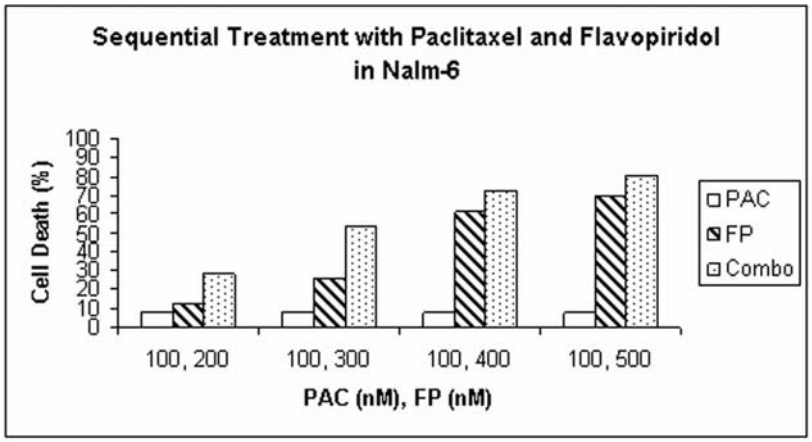


Figure 4-5. Preliminary combination data at a variety of ratios in the presence of human serum. Cell death measurements after treatment with PAC for 6 hours followed by FP for 72 hours at concentration ratios (PAC:FP) of 1:2, 1:3, 1:4, and 1:5. The 1:3 ratio was chosen for further study.

Table 4-1. Mixed model analysis for FP treatment duration in Nalm-6

	Numerator DF	Denominator DF	F value	P value
FP Concentration	4	10	20.04	<0.0001
Day	2	20	25.93	<0.0001
Day*FP concentration	8	20	26.25	<0.0001

Table 4-2. Mixed model analysis for FP treatment duration in RCH-ACV

	Numerator DF	Denominator DF	F value	P value
FP concentration	4	10	7.90	0.0038
Day	2	20	67.28	<0.0001
Day*FP concentration	8	20	19.42	<0.0001

Table 4-3. Differences between treatment duration at a given FP concentration in Nalm-6

Sample time- FP concentration (nM)	Sample time- FP concentration (nM)	Difference in mean cell death	T value	P value
Day 1 FP 150	Day 2 FP 150	- 0.3033	- 0.26	0.7978
Day 1 FP 150	Day 3 FP 150	- 0.6033	- 0.52	0.6113
Day 1 FP 200	Day 2 FP 200	- 0.2533	- 0.22	0.8305
Day 1 FP 200	Day 3 FP 200	- 1.6367	- 1.40	0.1766
Day 1 FP 300	Day 2 FP 300	-11.8500	-10.14	<0.0001
Day 1 FP 300	Day 3 FP 300	-18.5167	-15.85	<0.0001
Day 1 FP 50	Day 2 FP 50	0.4200	0.36	0.7230
Day 1 FP 50	Day 3 FP 50	0.8667	0.74	0.4669
Day 2 FP 150	Day 3 FP 150	- 0.3000	- 0.26	0.8000
Day 2 FP 200	Day 3 FP 200	- 1.3833	- 1.18	0.2503
Day 2 FP 300	Day 3 FP 300	- 6.6667	- 5.71	<0.0001
Day 2 FP 50	Day 3 FP 50	0.4467	0.38	0.7063

Table 4-4. Differences between treatment duration at a given FP concentration in RCH-ACV

Sample time-FP concentration (nM)	Sample time-FP concentration (nM)	Difference in mean cell death	T value	P value
Day 1 FP 150	Day 2 FP 150	- 6.5633	- 1.64	0.1162
Day 1 FP 150	Day 3 FP 150	-13.2733	- 3.32	0.0034
Day 1 FP 200	Day 2 FP 200	-22.0367	- 5.51	<0.0001
Day 1 FP 300	Day 2 FP 300	-38.4000	- 9.61	<0.0001
Day 1 FP 300	Day 3 FP 300	-56.9200	-14.24	<0.0001
Day 1 FP 50	Day 2 FP 50	0.8600	0.22	0.8318
Day 1 FP 50	Day 3 FP 50	- 1.7233	- 0.43	0.6709
Day 2 FP 150	Day 3 FP 150	- 6.7100	- 1.68	0.1087
Day 2 FP 300	Day 3 FP 300	-18.5200	- 4.63	0.0002
Day 2 FP 50	Day 3 FP 50	- 2.5833	- 0.65	0.5254

Table 4-5. Mixed model analysis of PAC single agent treatment in Nalm-6

	Numerator DF	Denominator DF	F value	P value
PAC concentration	3	14	174.68	<0.0001
Time of exposure (6 hours or 24 hours)	1	14	27.77	0.0001
PAC*Time	3	14	10.52	0.0007
Sample time (Day)	2	32	15.42	<0.0001
Day*PAC	6	32	31.63	<0.0001
Day*Time	2	32	3.48	0.0427
Day*PAC*Time	6	32	3.16	0.0150

Table 4-6. Mixed model analysis of PAC single agent treatment in RCH-ACV

	Numerator DF	Denominator DF	F value	P value
PAC concentration	3	14	108.48	<0.0001
Time of Exposure (6 hours or 24 hours)	1	14	7.91	0.0138
PAC*Time	3	14	5.90	0.0081
Sample Time (Day)	2	32	9.08	0.0008
Day*PAC	6	32	6.12	0.0002
Day*Time	2	32	0.39	0.6812
Day*PAC*Time	6	32	0.71	0.6454

Table 4-7. Differences in cell death based on incubation time after 6 or 24 hours PAC treatment in Nalm-6

Day	PAC (nM)	Time (hours)	Day	P value	Difference in mean cell death
Day 1	0	6	Day 2	0.1876	0.4799
Day 1	0	24	Day 2	0.0318	0.8074
Day 1	1	6	Day 2	0.1835	0.7225
Day 1	1	24	Day 2	0.7622	0.2860
Day 1	10	6	Day 2	0.9098	- 0.1913
Day 1	10	24	Day 2	<0.0001	-36.8805
Day 1	100	6	Day 2	<0.0001	-52.0495
Day 1	100	24	Day 2	<0.0001	-54.6743
Day 1	0	6	Day 3	0.0133	0.8677
Day 2	0	6	Day 3	0.2114	0.3879
Day 1	0	24	Day 3	0.0884	0.6504
Day 2	0	24	Day 3	0.6296	- 0.1571
Day 1	1	6	Day 3	0.0015	1.6311
Day 2	1	6	Day 3	0.0433	0.9085
Day 1	1	24	Day 3	0.0059	2.3689
Day 2	1	24	Day 3	0.0125	2.0829
Day 1	10	6	Day 3	0.1085	2.4893
Day 2	10	6	Day 3	0.0871	2.6806
Day 1	10	24	Day 3	<0.0001	-34.7488
Day 2	10	24	Day 3	0.8238	2.1317
Day 1	100	6	Day 3	<0.0001	-57.5053
Day 2	100	6	Day 3	0.6047	- 5.4558
Day 1	100	24	Day 3	<0.0001	-59.4446
Day 2	100	24	Day 3	0.6758	- 4.7703

Table 4-8. Differences in cell death based on incubation time after 6 or 24 hours PAC treatment in RCH-ACV

Day	PAC (nM)	Time (hours)	Day	P value	Difference in mean cell death
Day 1	0	6	Day 2	0.4185	0.9311
Day 1	0	24	Day 2	0.8931	- 0.1687
Day 1	1	6	Day 2	0.3051	1.3073
Day 1	1	24	Day 2	0.0582	1.7955
Day 1	10	6	Day 2	0.6557	- 0.9633
Day 1	10	24	Day 2	0.3989	- 3.9142
Day 1	100	6	Day 2	0.0065	-14.7008
Day 1	100	24	Day 2	0.0196	-16.2206
Day 1	0	6	Day 3	0.795	0.3189
Day 2	0	6	Day 3	0.5811	- 0.6121
Day 1	0	24	Day 3	0.9794	0.0317
Day 2	0	24	Day 3	0.8727	0.2003
Day 1	1	6	Day 3	0.4016	1.0914
Day 2	1	6	Day 3	0.8488	- 0.2160
Day 1	1	24	Day 3	0.436	- 1.0129
Day 2	1	24	Day 3	0.0096	- 2.8084
Day 1	10	6	Day 3	0.5508	- 1.3161
Day 2	10	6	Day 3	0.8795	- 0.3527
Day 1	10	24	Day 3	0.0812	- 9.3543
Day 2	10	24	Day 3	0.3514	- 5.4401
Day 1	100	6	Day 3	<0.0001	-37.3090
Day 2	100	6	Day 3	0.0265	-22.6082
Day 1	100	24	Day 3	0.0001	-37.2492
Day 2	100	24	Day 3	0.0724	-21.0286

Table 4-9. Combination Index (CI) values for drug combination studies using a variety of ratios

Nalm-6 PAC:FP	ED_{50}	ED_{75}	ED_{90}
1:5	1.53	1.65	1.80
1:10	1.25	1.08	0.939
1:12	1.14	0.885	0.715
1:15	1.39	1.26	1.16

Table 4-10. Mixed model analysis of Nalm-6 and RCH-ACV combination data

	Numerator DF	Denominator DF	F value	P value
Cell Line	1	2	20.83	0.0448
Treatment	2	61	150.97	<0.0001
Cell Line*Treatment	2	61	14.43	<0.0001
Drug concentration	4	61	114.93	<0.0001
Treatment*Concentration	8	61	5.30	<0.0001
Cell Line*Treatment*Concentration	9	61	3.61	0.0012

Table 4-11. Mixed model analysis of Molt-4 and Jurkat combination data

	Numerator DF	Denominator DF	F value	P value
Cell line	1	2	0.03	0.8727
Treatment	2	56	188.13	<0.0001
Cell line*Treatment	2	56	9.70	0.0002
Concentration	4	56	1002.86	<0.0001
Treatment*Concentration	8	56	36.00	<0.0001
Cell line*Treatment*Concentration	12	56	2.32	0.0174

Table 4-12. Significant differences in treatment for a given cell line and drug concentration

Cell line	Combo treatment PAC (nM):FP (nM)	Single agent control	SE	T value	P value
Nalm-6	10:100	100 nM FP	5.8083	2.93	0.0048
Nalm-6	10:100	10 nM PAC	5.8083	2.24	0.0286
Nalm-6	15:150	150 nM FP	5.8083	7.27	<0.0001
Nalm-6	15:150	15 nM PAC	5.8083	6.08	<0.0001
Nalm-6	20:200	200 nM FP	5.8083	8.49	<0.0001
Nalm-6	20:200	20 nM PAC	5.8083	8.41	<0.0001
Nalm-6	25:250	250 nM FP	5.8083	7.52	<0.0001
Nalm-6	25:250	25 nM PAC	5.8083	8.24	<0.0001
Nalm-6	30:300	300 nM FP	5.8083	3.97	0.0002
Nalm-6	30:300	30 nM PAC	5.8083	6.86	<0.0001
RCH-ACV	10:100	100 nM FP	5.0301	3.43	0.0011
RCH-ACV	15:150	150 nM FP	5.0301	4.86	<0.0001
RCH-ACV	15:150	15 nM PAC	5.0301	3.67	0.0005
RCH-ACV	20:200	200 nM FP	5.0301	4.57	<0.0001
RCH-ACV	20:200	20 nM PAC	5.0301	3.36	0.0014
RCH-ACV	25:250	250 nM FP	5.0301	3.63	0.0006
RCH-ACV	25:250	25 nM PAC	5.0301	5.45	<0.0001

Table 4-13. Significant differences in treatment for a given cell line and drug concentration

Cell line	Combo treatment PAC (nM):FP (nM)	Single agent control	SE	T value	P value
Jurkat	5:100	100 nM FP	3.6222	12.58	<0.0001
Jurkat	5:100	5 nM PAC	3.6222	8.03	<0.0001
Jurkat	7.5:150	150 nM FP	3.6222	13.55	<0.0001
Jurkat	7.5:150	7.5 nM PAC	3.6222	5.99	<0.0001
Jurkat	10:200	200 nM FP	3.6222	4.87	<0.0001
Jurkat	10:200	10 nM PAC	3.6222	5.32	<0.0001
Molt-4	5:100	100 nM FP	3.6222	9.99	<0.0001
Molt-4	5:100	5 nM PAC	3.6222	7.08	<0.0001
Molt-4	7.5:150	150 nM FP	3.6222	11.21	<0.0001
Molt-4	7.5:150	7.5 nM PAC	3.6222	10.71	<0.0001
Molt-4	10:200	10 nM PAC	3.6222	6.67	<0.0001

Table 4-14. Combination Index (CI) values for drug combination studies

	ED_{50}	ED_{75}	ED_{90}
Nalm-6	1.25	1.08	0.939
RCH-ACV	1.18	1.15	1.15
Molt-4	1.58	1.34	1.14
Jurkat	1.59	1.33	1.11

Table 4-15. One-Way Analysis of Variance of treatment sequence in Nalm-6

	Numerator DF	Denominator DF	F value	P value
Treatment Sequence	6	14	46.29	<0.0001

Table 4-16. Weighted One-Way Analysis of Variance of treatment sequence in RCH-ACV

	Numerator DF	Denominator DF	F value	P value
Treatment Sequence	6	14	16.86	<0.0001

Table 4-17. Significant differences between standard treatment sequence, reverse treatment sequence, and single agent controls in Nalm-6

Treatment 1	Treatment 2	Treatment 1 mean- Treatment 2 mean	SE	T value	P value
0→FP 200	PAC 20→FP 200	-29.3867	5.6021	-5.25	0.0001
0→PAC 20	PAC 20→FP 200	-44.0467	5.6021	-7.86	<0.0001
FP 200→PAC 20	PAC 20→FP 200	-22.9500	5.6021	-4.10	0.0011
FP 200→0	PAC 20→FP 200	-23.4267	5.6021	-4.18	0.0009
PAC 20→FP 200	PAC 20→0	70.6333	5.6021	12.61	<0.0001
PAC 20→FP 200	untreated	74.0600	5.6021	13.22	<0.0001

Table 4-18. Significant differences between standard treatment sequence, reverse treatment sequence, and single agent controls in RCH-ACV

Treatment 1	Treatment 2	Treatment 1 mean- Treatment 2 mean	SE	T value	P value
0→FP 200	PAC 20→FP 200	-20.7433	6.8754	-3.02	0.0092
0→PAC 20	PAC 20→FP 200	-40.4933	6.8754	-5.89	<0.0001
FP 200→0	PAC 20→FP 200	-31.6603	7.3598	-4.30	0.0007
FP 200→PAC 20	PAC 20→FP 200	-30.9227	7.4244	-4.16	0.0010
PAC20→0	PAC20→FP 200	-54.2467	6.8754	-7.89	<0.0001
PAC 20→FP 200	untreated	58.4167	6.8754	8.50	<0.0001

Table 4-19. Mixed model analysis for comparison of cell death induced by PAC in FBS vs. HS

	Numerator DF	Denominator DF	F value	P value
Type of serum	1	2	25.57	0.0370
PAC (nM)	4	14	62.75	<0.0001
Type*PAC	4	14	5.08	0.0097
Day	3	54	69.74	<0.0001
Day*Type	3	54	8.75	<0.0001
Day*PAC	12	54	18.85	<0.0001
Day*Type*PAC	12	54	1.69	0.0941

Table 4-20. Comparison of cell death induced by PAC in FBS vs. HS

Day	Type of serum	PAC concentration (nM)	Day	Type of serum	PAC concentration (nM)	P value	Difference in mean cell death
0	FBS	0	0	HS	0	1	-1.49E-14
0	FBS	1	0	HS	1	1	6.59E-15
0	FBS	10	0	HS	10	1	-2.94E-14
0	FBS	100	0	HS	100	1	-2.20E-14
0	FBS	1000	0	HS	1000	1	1.69E-17
1	FBS	0	1	HS	0	0.5418	0.6095
1	FBS	1	1	HS	1	0.3828	1.076
1	FBS	10	1	HS	10	0.0363	4.455
1	FBS	100	1	HS	100	0.0002	12.94
1	FBS	1000	1	HS	1000	0.0235	11.24
2	FBS	0	2	HS	0	0.2391	1.029
2	FBS	1	2	HS	1	0.2649	1.540
2	FBS	10	2	HS	10	0.0578	3.416
2	FBS	100	2	HS	100	<0.0001	42.72
2	FBS	1000	2	HS	1000	0.0223	35.55
3	FBS	0	3	HS	0	0.9267	-0.1275
3	FBS	1	3	HS	1	0.9722	-0.0537
3	FBS	10	3	HS	10	0.2053	2.605
3	FBS	100	3	HS	100	<0.0001	42.38
3	FBS	1000	3	HS	1000	0.1712	27.57

Table 4-21. Mixed model analysis of cell viability FBS vs. HS

	Numerator DF	Denominator DF	F value	P value
Day	3	12	16.07	0.0002
Type of serum	1	2	1.09	0.4055
Day*Type	3	12	1.27	0.3294

Table 4-22. Mixed model analysis of combination studies in human serum

	Numerator DF	Denominator DF	F value	P value
Treatment	2	16	151.97	<0.0001
Drug concentration	3	6	12.12	0.0059
Treatment*Concentration	6	16	1.25	0.3349

Table 4-23. Significant differences in treatment for combination studies in human serum

Treatment 1	Treatment 2	P value	Difference in mean cell death
300 nM FP	100 nM PAC, 300 nM FP	0.0112	-24.4877
100 nM PAC, 300 nM FP	100 nM PAC	<0.0001	37.8793
375 nM FP	125 nM PAC, 375 nM FP	0.061	-32.0078
125 nM PAC, 375 nM FP	125 nM PAC	<0.0001	63.9835
450 nM	150 nM PAC, 450 nM FP	0.4802	-16.7949
150 nM PAC, 450 nM FP	PAC 150	<0.0001	78.2333
600 nM FP	200 nM PAC, 600 nM FP	0.8746	- 4.2426
200 nM PAC	200 nM PAC, 600 nM FP	<0.0001	23.6096

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BIOGRAPHICAL SKETCH

Kelly Marie Jackman was born in December 1976 in Jacksonville, Florida where she was raised and educated until moving to Gainesville to pursue her graduate study. She graduated from Mandarin High School in 1995 and earned a B.S. in biology from Jacksonville University in 1999. Kelly attended St. Vincent's School of Medical Technology; becoming a board certified and licensed Medical Technologist (MT) in late 2000. She then worked for a short time as an MT before enrolling in the Interdisciplinary Program in Biomedical Sciences in the College of Medicine at the University of Florida in 2001. Her graduate research comprised laboratory study of a novel chemotherapy agent for treatment of children with acute lymphoblastic leukemia. Kelly hopes to use her education to contribute to the positive transformation of therapies made available to cancer patients.