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Aaron D. Schuler

Development of Sirtuin and Calmodulin-Dependent Protein Kinase Inhibitors As Anti-Cancer Therapeutics

Aaron D. Schuler

A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2006

Program Authorized to Offer Degree:
Department of Chemistry

UMI Number: 3230787

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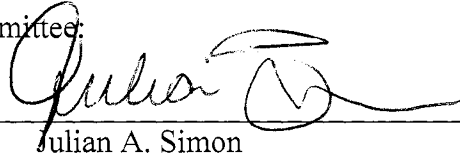

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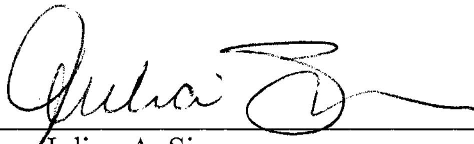

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Abstract

Development of Sirtuin and Calmodulin-Dependent Protein Kinase Inhibitors As Anti-Cancer Therapeutics

Aaron D. Schuler

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Department of Chemistry

Small molecule inhibitors of the human sirtuins and calmodulin-dependent protein kinases have shown promising anti-cancer activity in cell-based screens and animal models. We have synthesized analogues of these compounds, identifying more selective sirtuin inhibitors and more potent calmodulin-dependent protein kinase inhibitors.

The sirtuins are a family of NAD^+-dependent deacetylases that regulate cellular aging and gene silencing in simple organisms and appear to play important regulatory roles in human cells that make them attractive anti-cancer targets. We have previously identified the compound cambinol, an inhibitor of the human sirtuins SIRT1 and SIRT2, which is selectively toxic to Burkitt's lymphoma cells. In order to determine which sirtuin is the relevant target, we screened analogues of cambinol, identifying compounds that exhibited moderate selectivity for both SIRT1 and SIRT2. The compound JP136 is ten-fold more selective *in vitro* for SIRT1 over SIRT2, with respective IC_{50}'s of 13 μM and 125 μM, and it is far less potent against the Daudi Burkitt's lymphoma cell line than cambinol. Conversely, the compound ADS010, which is selective for SIRT2 in cell-based assays, is slightly more toxic to Daudi cells than cambinol. Like

cambinol, ADS010 has been found to be toxic only to B-cell lymphomas. SIRT2 appears to be the relevant target for cambinol-induced Daudi cell toxicity.

KN-62, an inhibitor of the calmodulin-dependent protein kinases (CaMKs), enhances the terminal differentiation of retinoic acid sensitive human myeloid leukemia cell lines. In an effort to identify additional CaMK inhibitors that exhibit more potent activity in triggering leukemia cell differentiation, we synthesized 45 analogues of KN-62 and determined their ability to induce HL-60 cell differentiation. Sixteen of these novel analogues exhibited significant differentiation-inducing activity, and one analogue, AS-004, was five times more potent than KN-62 in inhibiting proliferation and inducing differentiation of HL-60 cells. Such KN-62 analogues and/or related compounds may prove useful in treating promyelocytic leukemia.

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ACKNOWLEDGEMENTS

The author wishes to thank Dr. Julian Simon for allowing him to spend the past four years working in his laboratory and Drs. Antonio Bedalov and Steve Collins for their collaborative assistance in these projects. I would also like to thank LeMoyne Mueller and Jutong Si for their assistance with the research described in Chapter Two and Jeff Posakony for his chemical insight in both projects, especially the work described in Chapter One. I would like to acknowledge Birgit Heltweg for her development of the assays performed on the cambinol analogues (Chapter One). Finally, I want to thank Eric Verdin and Danesh Moazed for their gifts of plasmids used in Chapter One.

DEDICATION

To my grandfather, without whose financial support and encouragement none of this would have been possible.

Introduction

Cancer is a family of diseases characterized by uncontrolled proliferation of mutated cells. There is more than one cancer death every minute in the United States. In 2005, over 1.37 million Americans were diagnosed with cancer, and if current trends hold, more than one-third of these people will die within five years.[1] We have all lost a friend or relative to this horrible disease, and despite the best efforts of countless scientists and doctors around the world, we are still many years away from eliminating cancer as a source of pain, suffering, and death in the world.

My research is a small step toward the overall goal of eradicating cancer from our lives. One of the major difficulties that we currently face in cancer treatment is that the drugs available for treating the disease can cause as much harm as the disease itself. Most current chemotherapy agents simply kill rapidly proliferating cells. Since cancer cells are generally rapidly proliferating, they are preferentially killed over the senescent, normal cells, but many healthy cells are killed as well. I have worked to develop drugs that selectively kill cancer cells by exploiting their genetic differences from normal cells. My efforts have produced compounds that hold promise as potential treatments for non-Hodgkin's lymphoma and acute promyelocytic leukemia.

Several years ago we identified the compound cambinol as an inhibitor of a class of enzymes called the sirtuins, which regulate gene expression in simple organisms and appear to be involved in cancer and aging in humans.[2] I synthesized a library of cambinol analogues and found several that are selective for particular human sirtuins. The compound that selectively targets the enzyme SIRT2 is also selectively toxic to Burkitt's lymphoma cells, showing promise as a potential lead compound for the treatment of certain non-Hodgkin's lymphoma.

The compound KN-62, an inhibitor of the Ca^{++}/calmodulin-dependent protein kinases (CaMKs), was shown by Steve Collins and his research group to induce the differentiation of several acute promyelocytic leukemia cell lines, including HL-60 and NB4. I generated a library of KN-62 analogues and identified several compounds that induce differentiation at lower concentrations than KN-62. As inhibitors of calmodulin-dependent protein kinase IIγ, these compounds show promise as lead compounds for the treatment of this disease.

However, it must be remembered that non-Hodgkin's lymphoma and acute promyelocytic leukemia accounted for less than 5% of all cancer deaths in 2005.[1] There is still much work to be done, and many more drugs to be discovered. The progress I have made is dwarfed by the pervasiveness of cancer in our society. We are still many years from ending cancer as a source of pain and suffering in our society, but perhaps this research has taken us a few steps closer.

Chapter One: Selective Sirtuin Inhibitors Exhibit Variable Toxicity Against Daudi Cells[3]

Introduction

The sirtuins are a family of NAD^+-dependent protein deacetylases with sequence homology to *SIR2* (*S*ilent *I*nformation *R*egulator), the gene encoding Sir2p, a histone deacetylase involved in silencing at the telomeres, mating-type loci, and ribosomal DNA (rDNA) in *Saccharomyces cerevisiae*.[4] Sir2p is the only protein that localizes to all three loci,[5] and since it silences at both the telomeres and rDNA, it has been proposed that Sir2p may play a role in yeast lifespan regulation.[6] Indeed, yeast cells subjected to caloric restriction undergo a Sir2p-dependent extension of lifespan.[7] Sir2p has also been shown to regulate the segregation of oxidatively damaged proteins during cytokinesis, suggesting that it may contribute to the fitness of daughter cells.[8]

Sirtuins also appear to be involved in the aging of animals. Increasing the dosage of the sirtuin *sir-2.1* in *C. elegans* extends the lifespan of that organism by up to 50%,[9] and deletion of *D. melanogaster* $sir2^+$ appears to cause a shortened lifespan.[10] In mammals the sirtuins have been linked not only to aging, but cancer as well. There are seven sirtuins in human cells, four of which have been shown to have *in vitro* NAD^+-dependent protein deacetylase activity: SIRT1, SIRT2, SIRT3, and SIRT5.[11] Of the human enzymes, SIRT1 has been the most

thoroughly investigated. Localizing to the nucleus, SIRT1 deacetylates several key proteins involved in cell cycle regulation, including p53,[12,13] Ku70,[14] and the forkhead transcription factors.[15] Mice homozygous for the deletion of SIR2α, the murine SIRT1 homologue, exhibit a high rate of postnatal mortality and sterility.[16] The primarily cytoplasmic enzyme SIRT2 is a tubulin deacetylase[17] and regulator of mitotic exit and possibly cytokinesis.[18] During the G_2/M transition and mitosis, SIRT2 relocalizes to the nucleus where it deacetylates lysine 16 on histone 4.[19] SIRT3 is a mitochondrial protein that may regulate apoptotic proteins and reduce the rate of oncogenesis.[11] It appears to be linked to survival in old age[20,21] and temperature regulation in fatty tissues.[22] Also important for aging, SIRT6 plays a key role in base excision repair and in maintaining genomic stability.[23] Required for cell viability in mammals due to its role as a positive regulator of RNA polymerase I transcription,[24] SIRT7 is overexpressed in thyroid carcinoma.[25,26] The proto-oncogene *BCL6* is deacetylated and activated in an NAD^+-dependent manner, suggesting that a yet unidentified sirtuin acts as an activator of BCL6.[27] Constitutively active *BCL6* is found in most B-cell lymphomas, such as Burkitt's lymphoma, and downregulation of *BCL6* allows normal B-cells to exit the germinal center and differentiate.[28]

The first sirtuin inhibitor, sirtinol, was identified in a chemical genetic negative growth assay in yeast and has a reported IC_{50} of 68 μM for Sir2p.[29]

Another inhibitor of Sir2p, splitomicin,[30] was identified using a chemical genetic positive growth assay. Several relevant sirtuin crystal structures—those of SIR2-Af1, a sirtuin from *Archaeoglobus fulgidus*,[31] co-crystallized with NAD^+ and the human homologue SIRT2,[32] solved without NAD^+—show divergence in the substrate-binding pocket suggesting the possibility of developing selective inhibitors. The compound EX-527 has been identified as a selective inhibitor of SIRT1 with an IC_{50} of 98 nM.[33,34] Using a library of splitomicin analogues, the compound dehydrosplitomicin that inhibits the yeast sirtuin Hst1p[35] and the compound cambinol that inhibits both SIRT1 and SIRT2[2] were identified. Although cambinol has shown no activity against yeast, it has been shown to increase acetylation of p53, BCL6, and tubulin, in human cell lines.[2] Furthermore, it has shown selective toxicity against Daudi, a Burkitt's lymphoma cell line, and in mouse xenograft studies has shown efficacy in reducing tumor growth.[2] We report the development of cambinol analogues that have modest selectivity toward SIRT1 and SIRT2, leading to varied toxicity against Daudi cells. Our results suggest that SIRT2 may be a significant target for the treatment of Burkitt's lymphoma.

Materials and Methods

Reagents and antibodies

Cambinol, ADS010, ADS012, JP136, and ADS021 were synthesized as described below and shown in Figure 1. Compounds were purified to homogeneity as confirmed by thin layer chromatography and liquid chromatography-mass spectrometry. Unless otherwise noted, all reagents were purchased from Fisher Scientific. Trichostatin A and etoposide were purchased from Sigma. Antibodies to acetylated α-tubulin (clone 6-11B-1, Sigma, St. Louis, MO), α-tubulin (clone DM1A, Calbiochem, San Diego, CA), acetylated p53 (Cell Signaling, Danvers, MA), and p53 (Ab-6, Calbiochem) were obtained from commercial sources.

Synthesis of Cambinol

The synthesis of cambinol was performed using an approach described previously.[36] Briefly, ethyl benzoylacetate (2.9 mL, Fluka) and 2-hydroxy-1-naphthaldehyde (2.9 g, Fluka) were combined in equimolar amounts with catalytic pyrrolidine (~10 drops, Fluka) in ethanol (10 mL) in a flame-dried Erlenmeyer flask. After four hours, the reaction was quenched with excess glacial acetic acid (50 mL), and the resulting precipitate was isolated by vacuum filtration. The precipitate was recrystallized from glacial acetic acid and dried overnight under vacuum (3.5 g, 69% yield; ESI-MS (+) m/z = 301 $[M + H]^+$). The crystals (3 g) were dissolved in anhydrous pyridine (~30 mL, Fluka) under

argon and stirred on ice. One equivalent of sodium borohydride (380 mg, Fluka) was added to the solution and the reaction was allowed to proceed at room temperature. After 5 hours, the reaction was poured into excess 2 N HCl (~150 mL) and stirred vigorously. The precipitate (phenyl-β-keto lactone) was isolated by vacuum filtration, and washing with water neutralized the solid mass, which was dried under vacuum overnight. The β-keto lactone was recrystallized from saturated ethanol and dried under vacuum to remove all traces of ethanol (2.6 g, 87% yield; ESI-MS (–) $m/z = 301$ $[M - H]^-$).

Thiourea (972 mg, 1.4 equivalents, Fluka) was stirred in anhydrous dimethyl sulfoxide (60 mL, Aldrich) with sodium hydride (613 mg, 2.8 equivalents, Aldrich) for 15 minutes, the β-keto lactone (2.71 g, 1 equivalent) was added and was stirred for 4 hours at 90°C. The reaction was poured into excess 1 N HCl (~150 mL), and the precipitate was collected by vacuum filtration, neutralized by washing with water and dried overnight under vacuum. The precipitate (cambinol) was recrystallized from a mixture of ethyl acetate and *n*-heptane (605 mg, 19% yield; ESI-MS (–) $m/z = 359$ $[M - H]^-$).[Ψ]

Synthesis of ADS010

Methyl 3-trifluoromethylbenzoylacetate (1.231 g, Acros) and 2-hydroxy-1-naphthaldehyde (861 mg, Fluka) were combined in equimolar amounts with catalytic pyrrolidine (3 drops) in ethanol (3 mL) in a flame-dried vial. After four

[Ψ] An alternate synthesis for cambinol can be found in Appendix A.

hours, the reaction was quenched with excess glacial acetic acid (5 mL), and the resulting precipitate was isolated by vacuum filtration. The precipitate was recrystallized overnight from ethyl acetate and dried under vacuum (1.4 g, 77% yield). The rest of the synthesis proceeded as described above, except ADS010 was purified by silica gel chromatography (37 mg, 6% overall yield; ESI-MS (–) $m/z = 427$ $[M - H]^-$).

Synthesis of ADS012

Ethyl benzoylacetate (1.7 mL, Fluka) and salicylaldehyde (1.2 g, Aldrich) were combined in equimolar amounts with catalytic pyrrolidine (6 drops) in ethanol (6 mL) in a flame-dried Erlenmeyer flask. After four hours, the reaction was quenched with excess glacial acetic acid (~40 mL), and the resulting precipitate was isolated by vacuum filtration. The precipitate was recrystallized overnight from ethanol and dried under vacuum (2.1 g, 82% yield; ESI-MS (+) $m/z = 251$ $[M + H]^+$). The rest of the synthesis proceeded as described above for cambinol (137.5 mg, 24% overall yield; ESI-MS (–) $m/z = 309$ $[M - H]^-$).

Synthesis of JP136

The phenyl-β-keto lactone (66.7 mg) from the synthesis of cambinol was dissolved in dimethylformamide (~1 mL, Aldrich) under argon. Hydrazine monohydrate (13 μL, Fluka) was added, and the reaction was stirred for about 30 minutes. The volatile components were removed under vacuum and the solid was isolated by silica gel chromatography. The solid (JP136) was recrystallized from

a mixture of ethyl acetate and *n*-heptane (22.3 mg, 32% yield; ESI-MS (–) *m/z* = 315 $[M - H]^-$).

Synthesis of ADS021

The 3-trifluoromethyl-phenyl-β-keto lactone from the synthesis of ADS010 was subjected to the reaction described above for JP136 to yield ADS021 (33 mg, 42% yield; ESI-MS (–) *m/z* = 383 $[M - H]^-$).

HDAC Assay

Cambinol, ADS010, ADS012, JP136, and ADS021 were tested for their ability to inhibit the *in vitro* activity of bacterially expressed GST-SIRT1 and GST-SIRT2 [2]. The GST-SIRT2 expression plasmid was a gift of Dr. D. Moazed (Department of Cell Biology, Harvard Medical School, Boston, MA).[29] The SIRT1 cDNA was generously provided by Dr. E. Verdin (Gladstone Institute of Virology and Immunology, San Francisco, CA) [17] and cloned into the pGEX-4T-1 GST expression plasmid (GE Healthcare Life Sciences), expressed in, and purified from bacteria. The compounds were prepared as 30 mM solutions in dimethyl sulfoxide (Sigma) and dissolved in 50% glycerol for use in the assay as 3 mM stock solutions. For deacetylation assays, chemically acetylated $[^3H]$-acetyl-H4 peptide[37] was incubated with or without 500 μM NAD^+ and GST enzyme in a buffer containing 50 mM Tris-HCl (Ph 8.0), 150 mM NaCl, 1 mM DTT, and 5% glycerol. After 3 hours of incubation at 37°C, the reaction was

quenched by the addition of 5 μL of 1 N HCl containing 0.15 N acetic acid. Released [^{3}H]-acetic acid was extracted with 400 μL ethyl acetate and counted in 5 mL ScintiVerse II.

Cell culture

All cells were maintained at 37°C and 5% CO_2; the appropriate medium was supplemented with 1% L-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). Daudi cells were cultured in RPMI 1640 (Gibco) supplemented with 20% FCS (Gibco). NCI H460 cells were cultured in RPMI plus 10% FCS. Both cell lines were obtained from American Type Culture Collection (Manassas, VA).

Western blot analysis

NCI H460 cells were treated with the drugs for 16 hours and lysed in the presence of drugs in a buffer containing 20 mM Tris-HCl (Ph 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in the presence of Complete Mini protease inhibitors (Roche). Cell lysates were resolved by SDS-PAGE in 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Proteins were visualized using SuperSignal West Pico Chemilluminescent Substrate (Pierce, Rockford, IL).

Immunofluorescence

NCI H460 cells grown on coverslips for 24 hours were treated with 30 μM cambinol, ADS010, ADS012, JP136, or ADS021 and 2.5 nM trichostatin A

(Sigma) for 16 hours. Cells were permeabilized with 0.5% Triton X-100 in warm microtubule stabilizing buffer [80 mM PIPES (Ph 6.8), 1 mM $MgCl_2$, and 5 mM EGTA] and fixed with 0.5% gluaraldehyde for 10 minutes. Primary antibodies were targeted against α-tubulin (clone DM1A, Calbiochem) and acetylated α-tubulin (clone 6-11B-1, Sigma). Oregon green-conjugated anti-mouse IgG (Invitrogen) was used as the secondary antibody. Slides were visualized on a Nikon E800 microscope system equipped with a SPOT2 digital camera.

Toxicity assays

Daudi toxicity was measured by three different methods to fully elucidate the effects of the drugs. To compare with our previous work, cambinol and ADS010 were tested in a modified [^{3}H]-thymidine incorporation assay as described previously.[2,38] All compounds were also tested using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), in which cells are lysed, and the concentration of ATP is measured in each well. Cells plated in a 96-well microplate were treated with drugs for 72 hours (3 days) after which the CellTiter-Glo® reagent was added, lysing the cells and binding the released ATP with a luminescent substrate. The luminescence was quantified in a luminometer. Each drug was tested in triplicate. All compounds were also grown in 6-well plates and counted by trypan blue staining. Cells plated in a 6-well plate were treated with drugs for 72 hours (3 days) after which time an aliquot of cells was

removed and mixed with trypan blue stain and counted in triplicate with a haemocytometer.

Results and Discussion

To explore the structural determinants of the histone deacetylase inhibitory activity of cambinol,[2] we synthesized and screened simple analogues for their activity against SIRT1 and SIRT2 *in vitro*. Most of the twenty-six compounds were inactive against the sirtuins or did not behave differently than cambinol. Thus, we selected four compounds for further exploration: ADS010, ADS012, JP136, and ADS021 (Figure 1).

As mentioned previously,[2] ADS012 is inactive *in vitro* against both SIRT1 and SIRT2 (Figure 2), making it a useful negative control. ADS010 behaves similarly to cambinol *in vitro* (Figure 2). JP136 showed apparent selectivity for SIRT1 over SIRT2 (Table 1), with a selectivity index of 9.6. ADS021, which combines structural elements of ADS010 and JP136 (Figure 1), behaves like a combination of the two, with comparable *in vitro* IC_{50}s against SIRT1 with JP136 and against SIRT2 with ADS010 (Figure 2 and Table 1).

ADS010 Does Not Inhibit Cellular SIRT1, But ADS012 Does

Inhibition of cellular SIRT1 is measured by monitoring the acetylation state of p53, since it is the enzyme primarily responsible for p53 deacetylation.[2] To measure inhibition of cellular SIRT1, the cambinol analogues were incubated

overnight with etoposide-treated NCI H460 cells and analyzed by immunoblotting using acetyl-p53- and p53-specific antibodies. Etoposide treatment induces DNA damage, increasing the overall p53 content in the cells (first and last lanes of Figure 3A). However, most of this p53 is non-acetylated. When cambinol or its analogues are added to the cells, there is an increase in p53-acetylation proportional to the compound's activity. It appears that in cell-based assays, the compounds are active below their *in vitro* IC_{50}s. The maximum enhancement of p53 acetylation occurs at concentrations below the *in vitro* IC_{50}s. Cambinol induces p53 acetylation at 30 and 60 μM, as does JP136 and ADS021.

Surprisingly, ADS010, which is active *in vitro*, fails to induce p53 acetylation at 30 and 60 μM, suggesting that it is not active against cellular SIRT1. It is unclear why it is not active in the cell-based assay, however, lack of activity against SIRT1 is not due to poor cellular uptake (see below). ADS012, on the other hand, has moderate activity at both concentrations, suggesting that it has some activity against cellular SIRT1. This activity is not totally unexpected. We have previously shown that when the naphthyl group on splitomicin is changed to phenyl, *in vivo* yeast Sir2p inhibition is retained.[36] Furthermore, dihydrocoumarin was shown to inhibit both SIRT1 (IC_{50} = 208 μM) and SIRT2 (IC_{50} = 295 μM), and it increases heterochromatin silencing at concentrations similarly to splitomicin in yeast.[39] The large discrepancy between *in vitro* and *in vivo* activity is paralleled by splitomicin.[30] Since yeast media has an acidic pH,

mammalian tissue culture medium is neutral, and the *in vitro* histone deacetylase assay is performed at basic pH, the activity may simply be pH-dependent.

Cambinol and ADS010 Inhibit SIRT2, But the Other Analogues Do Not

Inhibition of cellular SIRT2 is best measured by monitoring the acetylation state of tubulin in the presence of low concentrations of trichostatin A.[2,17] The most straightforward and convincing method to ascertain the acetylation state of tubulin is through indirect immunofluorescence with specific antibodies against tubulin and acetyl-tubulin. An increase in acetyl-tubulin fluorescence, without a corresponding increase in overall tubulin fluorescence, reflects inhibition of SIRT2. As shown in Figure 3B, only cambinol and ADS010 appear to demonstrate any substantial increase in acetyl-tubulin acetylation, suggesting that they are the only compounds that inhibit cellular SIRT2. ADS012 does not have activity against SIRT2, as expected from the *in vitro* assay. It does not appear to be generally active in the cell-based assays, only gaining activity against SIRT1. Both JP136 and ADS021 are selective for SIRT1 in the cell-based assays, aligning with the *in vitro* data. Therefore, at the concentrations used in these experiments, JP136 and ADS021 are selective *in vivo* for SIRT1 and ADS010 is selective *in vivo* for SIRT2.

Cambinol and ADS010 Are Selectively Toxic for Burkitt's Lymphoma Cells in Multiple Assays

Cambinol has been shown to be toxic to several Burkitt's lymphoma cell lines, such as Daudi and Namalwa.[2] In testing the new analogues, only ADS010 was consistently toxic to Daudi cells (Table 2[φ]). The three compounds that did not inhibit cellular SIRT2 (ADS012, JP136, and ADS021) showed marginal activity against the lymphoma line, as measured by trypan blue exclusion, ATP level measurements, and a modified [^{3}H]-thymidine incorporation assay. On the other hand, although IC_{50}s and inhibition levels varied between the three assays, cambinol and ADS010 both consistently killed the Daudi cells. Although other targets cannot be ruled out, it seems likely that SIRT2 is the relevant target in Daudi cells. Certainly, based on the results for JP136 and ADS021, SIRT1 does not appear to be a valid drug target in Burkitt's lymphoma cells. As with cambinol, ADS010 is only toxic to Burkitt's lymphoma cells,[Ω] suggesting that a more potent selective inhibitor of SIRT2 could be a useful and selective anti-cancer therapeutic.

[φ] The graphs of the [^{3}H]-thymidine incorporation assays and ATP content measurements used to generate the data in Table 2 can be found in Appendices B and C.

[Ω] [^{3}H]-Thymidine incorporation assays for ADS010 against non-Burkitt's lymphoma cell lines are shown in Appendix B.

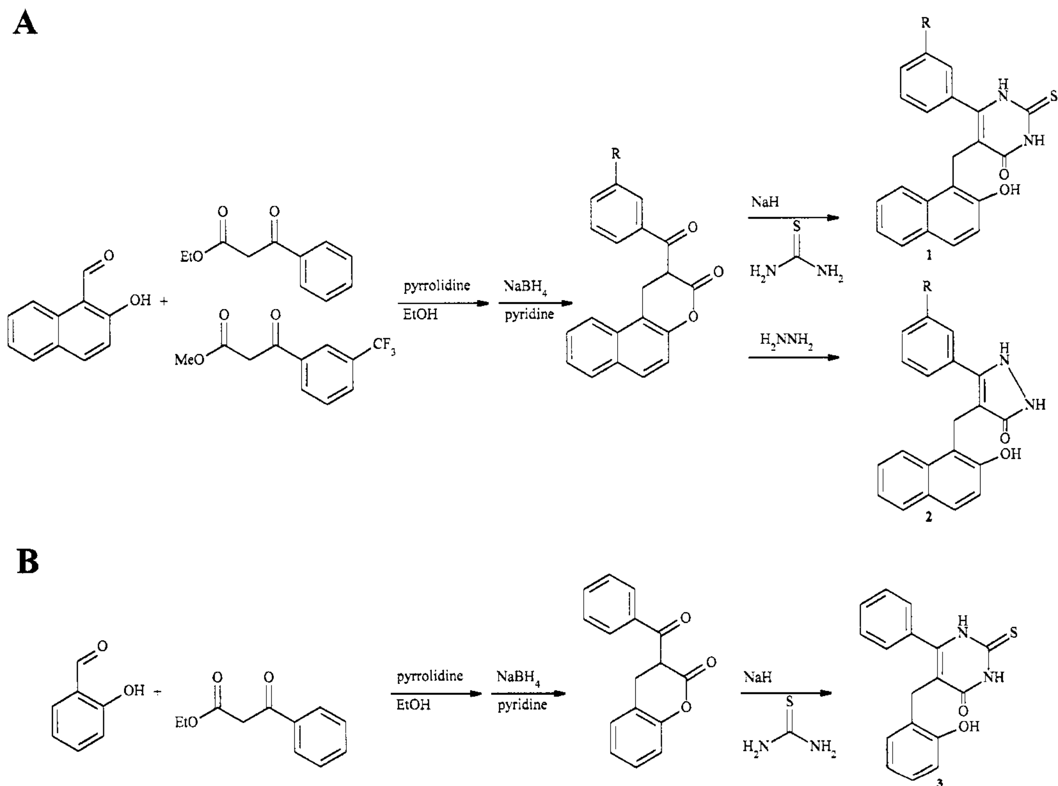
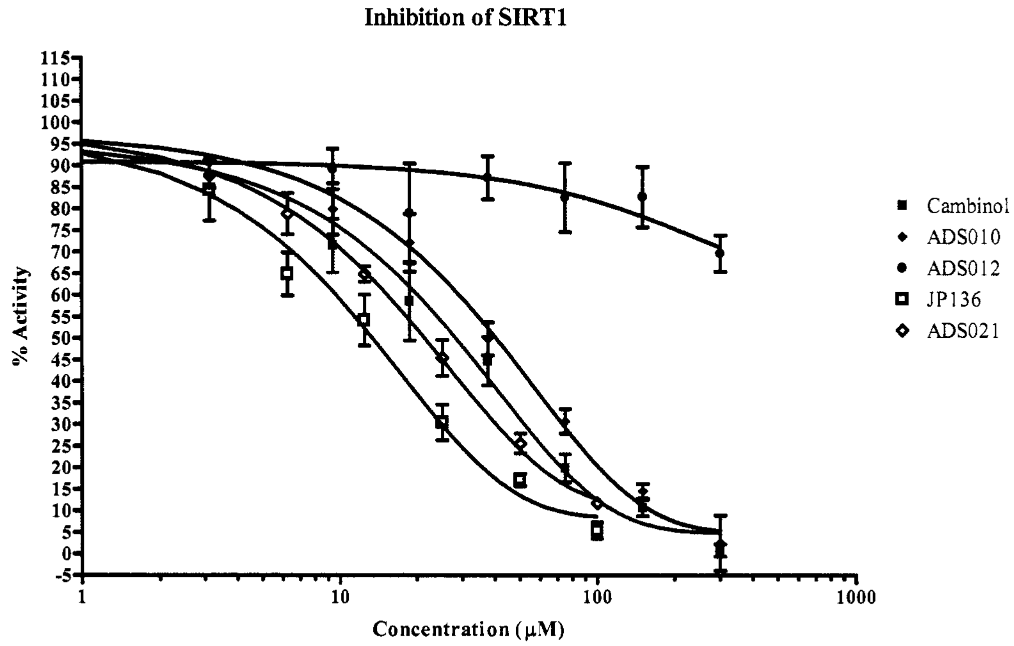


Figure 1: Synthesis of cambinol and analogues. **(A)** Synthetic scheme for the preparation of cambinol (**1**, R = H), ADS010 (**1**, R = CF_3), JP136 (**2**, R = H), ADS021 (**2**, R = CF_3). **(B)** Synthetic scheme for the preparation of ADS012 (**3**).

A



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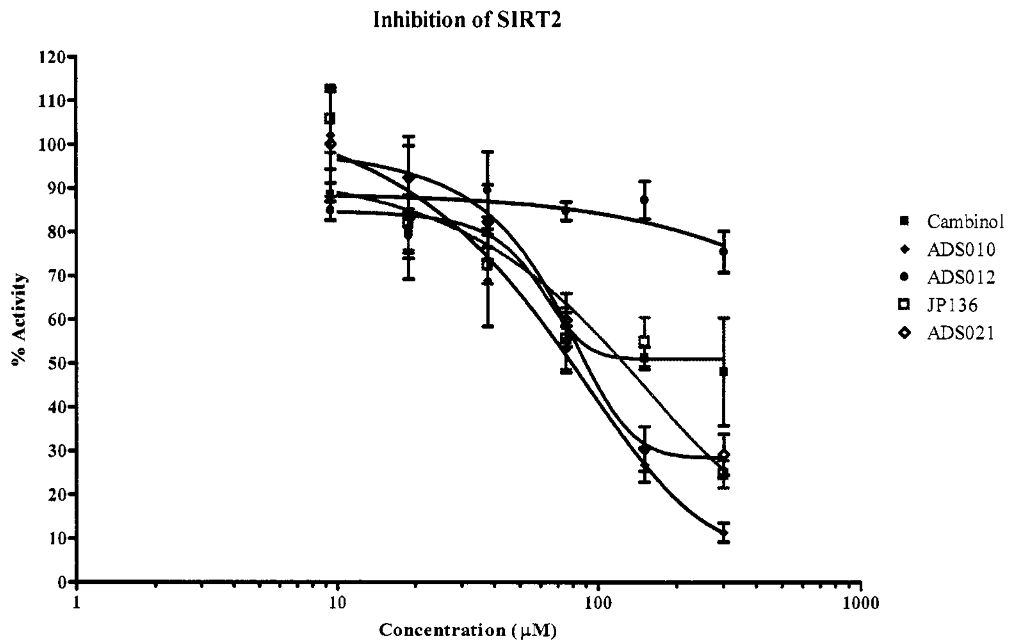
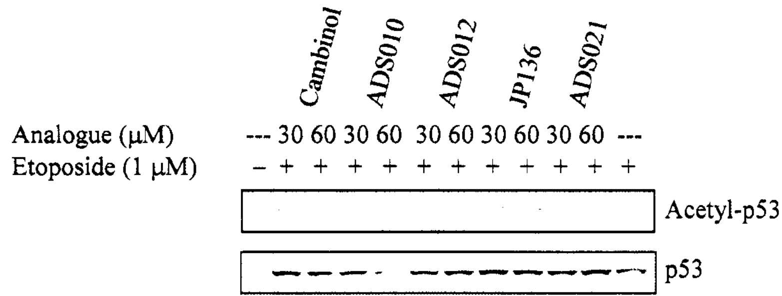


Figure 2: Inhibition of *in vitro* deacetylation by SIRT1 (**A**) and SIRT2 (**B**).

A



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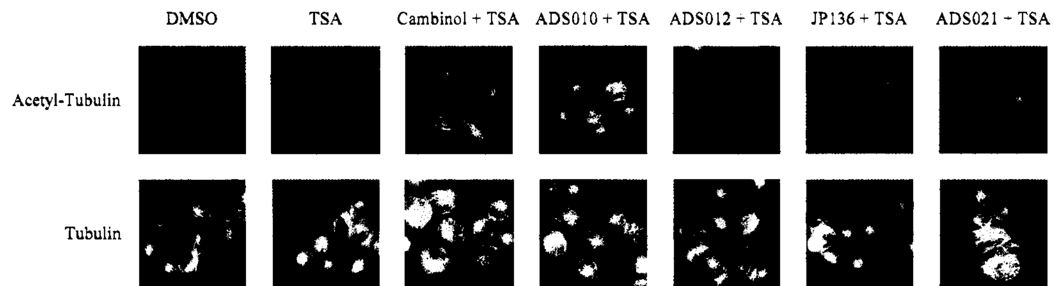

Figure 3: Activity of cambinol and analogues in cell-based assays. **(A)** SIRT1 inhibition is measured by monitoring the acetylation state of p53. Darker bands in the upper blot represent increased inhibition of SIRT1. **(B)** SIRT2 inhibition is measured by monitoring the acetylation state of tubulin in intact NCI H460 cells. Cells are treated with 2.5 nM TSA and 30 μM of the appropriate cambinol analogue for 16 hours, fixed, stained with antibodies against acetyl-tubulin and tubulin, and visualized with Oregon Green fluorescent antibody. The cells stained with acetyl-tubulin were exposed for 1.2 seconds, and cells stained with tubulin were exposed for 500 ms. A strong signal in the acetyl-tubulin stained cells indicates inhibition of SIRT2.

Table 1: *In vitro* IC_{50}s of cambinol and analogues against SIRT1 and SIRT2

Analogue	SIRT1 (μM)	SIRT2 (μM)	Ratio*
Cambinol	56 ± 2‖	59 ± 4‖	1.1
ADS010	40 ± 5	79 ± 11	2
ADS012	N/A	N/A	N/A
JP136	13 ± 3	125 ± 25	9.6
ADS021	21 ± 2	90 ± 11	4.3

* Selectivity for SIRT1 over SIRT2, as determined dividing the SIRT2 IC_{50} by the SIRT1 IC_{50}. ‖ From Heltweg *et al.*, ref. 2.

Table 2: Viability of Daudi cells treated with cambinol and analogues

Analogue	Haemocytometer*	ATP Level**	ATP Level‖ (μM)	Thymidine‖ (μM)
Cambinol	58 ± 6 %	65 ± 3 %	41 ± 3	8 ± 3
ADS010	44 ± 3 %	55 ± 4 %	36 ± 5	5 ± 4
ADS012	78 ± 10 %	92 ± 4 %	N/A	N/A
JP136	78 ± 7 %	101 ± 11 %	55 ± 1	ND
ADS021	82 ± 10 %	112 ± 4 %	N/A	ND

* % of DMSO control at 15 μM. ** % of DMSO control at 30 μM. ‖ IC_{50} values.

Chapter Two: KN-62 Analogues As Potent Differentiating Agents of HL-60 Cells[40]

Introduction

The retinoic acid receptors (RARs) are transcription factors involved in regulating myeloid differentiation.[41] Most cases of human acute promyelocytic leukemia (APL) are associated with the presence of the t(15;17) chromosome translocation, which results in the generation of the leukemogenic PML-RARα fusion protein. This aberrant RARα fusion protein likely acts as a dominant negative to inhibit the normal role of the RARs in enhancing myeloid cell differentiation.[42,43] Treatment of APL patients with relatively high, pharmacological doses of retinoic acid (RA) appears to overcome this differentiation block and has had a remarkably beneficial therapeutic effect.[44,45] However, some 20-30% of patients with APL still relapse despite optimal RA therapy.[45,46] Agents that enhance the activity of retinoic acid and the RARs may be of therapeutic benefit in enhancing the cure rate for this type of human leukemia.

Calmodulin-dependent protein kinases (CaM kinases or CaMKs) are a family of ubiquitous proteins that are expressed in a variety of tissues and are involved in regulating tissue growth, development, and function.[47] These enzymes are regulated by changes in intracellular Ca^{++} concentration, which in

turn regulate levels of the Ca^{++}/calmodulin (CaM) complex. Ca^{++}/CaM binds to the CaMKs and enhances their enzymatic activity. The most widely studied CaMKs include CaMKs I, II, and IV and their different isoforms, and these distinct CaMKs regulate the development and activity of multiple different cell types. We have previously observed that CaMKIα and CaMKIIγ are the CaMKs that are predominantly expressed in myeloid cells, and these enzymes may have a regulatory role in blood cell differentiation.[48]

We have recently observed that KN-62, a small molecule inhibitor of the CaMKs, enhances RARα transcriptional activity and induces the differentiation of the RA-sensitive HL-60 human myeloid leukemia cell line [48]. In addition, this compound markedly enhances the effect of low dose RA to trigger the differentiation of the NB4 promyelocytic leukemia cell line. KN-62 blocks the binding of Ca^{++}/CaM to the CaMKs and thus acts as a general CaMK inhibitor exhibiting IC_{50}s of 0.8 μM, 0.8 μM, and 3 μM, against CaM kinases I, II, and IV respectively.[47,49] In exploring the molecular basis for the activity of this CaM kinase inhibitor to regulate leukemia cell differentiation, we observed that the CaMKs have opposing activities in regulating RARα transcriptional activity. Indeed CaMKI-mediated phosphorylation of RARα enhances RAR transcriptional activity while CaMKII-mediated phosphorylation of RARα inhibits its activity. Thus, although KN-62 can inhibit both CaMKI and II, its role

in enhancing RAR activity and triggering myeloid cell differentiation likely results from inhibiting the activity of CaMKII.

In the present study, we synthesized a variety of structural analogues of KN-62 and investigated whether any of them have increased differentiation-inducing activity against HL-60 cells compared with the parental KN-62 compound. The analogues displayed a wide range of activity in inducing HL-60 differentiation, and one (AS-004) was five times more potent than KN-62 in inhibiting proliferation and inducing differentiation of HL-60.

Materials and Methods

Synthesis of KN-62 analogues

All reactions were carried out with dry solvents under an inert atmosphere, following synthetic procedures described previously.[50,51] The basic synthesis procedures are summarized in Figure 4. Briefly, Boc-N-Me-tyrosine (**1**; most analogues) or Boc-N-Me-D-tyrosine (**1**; AS-044, AS-045, AS-046) (1.0 eq) was stirred at room temperature with EDC (1.1 eq), HOBt (1.1 eq), and secondary amine (1.1 eq) in dimethylformamide. After 24 hours, volatile materials were removed under vacuum, and the resulting residue was resuspended in ethyl acetate/water (2:1), and the layers were separated. The organic layer was washed with brine, dried over sodium sulfate, and passed through a plug of silica. The flow-through was collected and evaporated to dryness under vacuum. The

product (**2**) was dissolved in dichloromethane/methanol (3:1) and divided equally into glass vials, one for each sulfonic ester. After evaporating the solvent from each vial, each aliquot was used for a different subsequent synthesis. In these syntheses, the starting material (**2**) was dissolved in tetrahydrofuran/dichloromethane (1:1), treated with NaH (1.2 eq), and coupled with the appropriate sulfonyl chloride (2 eq). After 18 hours, excess amounts of two scavenger resins, PS-Trisamine and MP-Carbonate (Argonaut), were added with additional dichloromethane. After 3 hours, resins were filtered off and washed with dichloromethane and tetrahydrofuran. The organic washes were evaporated to dryness and dissolved in ethyl acetate/saturated sodium bicarbonate (2:1). The organic layer was removed, dried over sodium sulfate, filtered, and evaporated to dryness. The sulfonic esters (**3**) were treated with either ethyl acetate/concentrated hydrochloric acid (3:1) or dichloromethane/trifluoroacetic acid (1:1),[51] depending on the reactivity of the sulfonic ester, to remove the Boc protecting group. The deprotected compounds were divided up as described above, one for each sulfonamide. In the final reaction to make the sulfonamides (**4**), the free amine precursors were stirred in dichloromethane and triethylamine (1 eq), followed by addition of the appropriate sulfonyl chloride (2 eq). After 18 hours, excess amounts of two scavenger resins, PS-Trisamine and MP-Isocyanate (Argonaut), were added with additional dichloromethane. After 90 minutes, the resins were filtered off and washed with dichloromethane and

dimethylformamide. The organic washings were evaporated to dryness and dissolved in 200 μL of dimethylsulfoxide. The crude products (**4**) were purified by preparative HPLC (Agilent) and confirmed by ESI-MS (Agilent). Final products were prepared as 5 mM stock solutions in DMSO for biological assays.

Cell Culture

HL-60 and other myeloid leukemia cell lines were cultured in RPMI 1640 with 5% heat-inactivated fetal calf serum (Hyclone) at 37°C in an atmosphere with 5% carbon dioxide. The newly synthesized analogues were dissolved in dimethylsulfoxide (DMSO) and added to the culture media to give a constant DMSO concentration of no higher than 0.2%, a concentration that does not alter HL-60 proliferation/differentiation.

Cell Proliferation Assay

HL-60 cells were seeded at a concentration of 5×10^4 cells/mL in 12 well plates and incubated for 5 days with each compound. Compounds were initially screened at concentrations of 0.5, 1, 2, 5, and 10 μM. After 5 days, cells were counted by trypan blue staining and the proliferation/viability of the treated cells was compared with cells treated with 0.2% DMSO alone. Any compound that appeared to have an IC_{50} of 10 μM or lower was again screened in triplicate at each concentration. The triplicate data was plotted in Excel (Microsoft) to calculate IC_{50} values for each active compound. A particularly potent compound, AS-004, was tested at concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5,

and 10 μM in triplicate. KN-62 was examined in parallel with all experiments as an internal control.

Morphological assessment

10^5 cells were deposited onto a glass microscope slide, using a Shandon 3 Cytospin (Thermo Electron), air dried, and stained with Wright-Giemsa stain.

Measurement of CD11b Expression

Compound-treated cells (10^6/mL) were incubated with PE labeled anti-CD11b antibody (Becton Dickinson Biosciences) and propidium iodide (Sigma) in PBS that contained 2% human antibody serum (Gemini Biotech). After a brief incubation on ice, the cells were rinsed in buffer and subjected to fluorescent-activated cell sorter (FACS) analysis on a Becton Dickinson FACS SCAN. The percent of cells expressing CD11b was plotted in Excel (Microsoft) to determine the concentration at which the cells have at least 30% CD11b expression.

Western blots

Western blots on HL-60 total cell lysates were performed as previously detailed.[52] Antibodies utilized in these Western blots included anti-CaMKIIγ and anti-phosphospecific CaMKII (T286), both from Santa Cruz Biotechnology.

Results

Synthesis of KN62 related analogues

KN-62 (Figure 5A) consists of a tyrosine core (Figure 5B) to which three different chemical groups have been attached at distinct sites (designated R1, R2, and R3) (Figure 5C). As detailed in **Materials and Methods** and Figure 4, we used a combinatorial approach to synthesize structural analogues of KN-62, incorporating different chemical groups at the R1, R2, and R3 positions (Figures 5D and E). We synthesized and purified 45 different KN-62 analogues and compared their activity with KN-62 in inhibiting proliferation and inducing differentiation of HL-60 myeloid leukemia cells.

Effect of KN-62 analogues on HL-60 proliferation and differentiation

Since the terminal differentiation of HL-60 cells is associated with their reduced proliferation, we first screened the KN-62 analogues for their ability to inhibit HL-60 proliferation. In contrast to KN-62, 26 of the 45 compounds did not exhibit growth inhibitory activity against HL-60 cells (Table 3) indicating that the biological activity of this compound class is dependent upon the specific combination of R1, R2 and R3 structural elements. However, the other 19 analogues inhibited HL-60 cell proliferation with an IC_{50} of 10 μM or less, and eight compounds exhibited an IC_{50} that was lower than KN-62 (Table 4[Π]). The

[Π] The graph used to generate the proliferation IC_{50} values can be found in Appendix D.

most potent of these compounds, AS-004, (Figure 6) has an IC_{50} approximately 5 times lower than KN-62 (0.38 vs. 1.6 μM) (Table 4).

The surface antigen CD11b is a convenient marker to quantify and compare HL-60 cell differentiation since CD11b is minimally expressed on immature promyelocytes, but its expression is enhanced as the cells terminally differentiate to neutrophils. Therefore, to ascertain whether the KN-62 analogues were stimulating HL-60 differentiation in addition to inhibiting their proliferation, we measured the CD11b levels in the analogue-treated HL-60 cells. For each compound, we identified the concentration at which treated cells exhibited 30% CD11b expression or higher. Several of the compounds appeared to be particularly potent and induced CD11b expression at significantly lower concentrations than KN-62 (Table 4[Φ]). Again AS-004, which proved most potent in inhibiting HL-60 proliferation (Table 4), also exhibited the greatest potency in inducing CD11b expression. Most of the compounds that reduced proliferation of HL-60 cells also increased CD11b expression on these same cells at comparable concentrations (Figure 7). The enhanced CD11b expression induced by AS-004 was consistent with the marked morphological changes induced by this compound. Indeed AS-004 induced banded and segmented neutrophils in HL-60 cell cultures at significantly lower concentrations than KN-62 (Figure 8).

[Φ] The graph used to generate the 30% CD11b expression values can be found in Appendix E.

Inhibition of CaMKII autophosphoryation by the different KN-62 analogues

The binding of the Ca^{++}/calmodulin complex to CaMKII triggers autophosphorylation of the CaMKII holoenzyme that markedly enhances its enzymatic activity.[53] Since KN-62 likely acts as a CaMK inhibitor by inhibiting Ca^{++}/calmodulin binding to the regulatory region of the CaMKs, we would predict that KN-62 as well as the KN-62-related analogues that trigger HL-60 differentiation would also inhibit this CaMKII autophosphorylation. We used anti-phosphospecific CaMKII antibodies to assess the autophosphorylation of CaMKII in HL-60 cells treated with different KN-62 analogues and observed a direct correlation between the activity of these analogues in inducing HL-60 differentiation and inhibiting CaMKII autophosphorylation. Both KN-62 and AS-004, which are active HL-60 differentiation inducers, markedly reduced CaMKII autophosphorylation (Figure 9). In contrast, AS-038, an analogue without differentiation inducing activity (Table 3), did not exhibit activity in inhibiting CaMKII autophosphorylation (Figure 9).

Effect of AS-004 on other myeloid leukemia cell lines

The above studies identified AS-004 as the most potent KN-62 analogue in inhibiting proliferation and inducing differentiation of HL-60 cells. We also determined whether this compound induces the differentiation of other myeloid leukemia cell lines. We did not observe any effect of this compound on the proliferation/differentiation of a number of different ATRA-unresponsive human

myeloid leukemia cell lines including K562, KCL-22, KG-1 and THP-1 (data not shown). In contrast, in the NB4 cell line, which was derived from a patient with M3 APL and harbors the PML-RARα fusion gene that characterizes APL,[54] AS-004 enhanced CD11b surface antigen expression in a dose dependent manner (Figure 10, columns 1-4). Moreover, the addition of small, physiological concentrations of all-trans retinoic acid (ATRA) (1 nM) markedly enhanced the differentiative response of the NB4 cells to AS-004 (Figure 10, columns 5-8). These *in vitro* observations suggest that AS-004 might be of therapeutic benefit in the treatment of ATRA-sensitive subsets of human myeloid leukemia.

Discussion

Differentiation therapy plays an important role in treating certain human myeloid leukemia, particularly APL. We recently observed that the general CaM kinase inhibitor KN-62 markedly enhances the differentiation of retinoic acid-sensitive human myeloid leukemia cell lines. We undertook the present study in an attempt to synthesize structurally similar analogues of KN-62 that exhibit more potent activity in inducing leukemia cell differentiation. Our combinatorial chemistry approach to the synthesis of these analogues involved substituting different chemical groups at three sites (designated R1, R2, and R3) of a core tyrosine-related scaffold (Figure 5C) and gave us insight into which structural motifs were important for maximum differentiation inducing ability. Our

observations suggest that KN-62 is not the optimal inducer of leukemia cell differentiation. Nevertheless, there are particular elements of the KN-62 structure that are important for mediating this activity.

Examination of the different KN-62 analogues and their activity in inducing HL-60 differentiation indicates that the basic modular structure of the molecule appears to be very important, as does the tyrosine core. For example, changing the chirality of the tyrosine (R1=D-PP instead of PP, Figure 5D) to yield compounds AS-044, AS-045, and AS-046 (Tables 3 and 4) greatly abrogated their differentiation-inducing activity compared with KN-62 with only AS-044 showing even minimal activity (Table 4). Groups present at the sulfonamide position of KN-62 (R3) also appear critical in mediating the differentiation-inducing potency of these compounds. AS-004, which differs from KN-62 by substituting 4-benzothiadiazole (BTD-4) for 5-isoquinoline (5-IsoQ) at the R3 position (Figure 6), was the most active compound tested, exhibiting a five fold greater differentiation-inducing potency than KN-62. BTD-4 at the R3 position led to activity in all of the analogues containing the L-tyrosine core (Table 4). On the other hand, the sulfonic ester position (R2) does not appear to be as relevant to the activity of the compounds as R3. For example, many of the active analogues harbored different chemical groups at the R2 position (Table 4). Furthermore, there is a relatively poor correlation between activity and the groups present at the R2 position. For example, AS-004 is the most potent compound in both of our

assays, and it has 5-isoquinoline in the sulfonic ester position. Replacing this group with BTD-4 (AS-029, Table 4) slightly reduces its activity. However, the second most active compound in our proliferation assay (AS-047) has BTD-4 in the sulfonic ester position, but changing the group to 5-isoquinoline (AS-048) reduces rather than increases its activity. Taken together these results suggest that the R2 position does not play as critical a role in compound activity. Finally, groups present at the secondary amine position (R1) appear to be important for the activity of the analogues. For example, compounds with phenylpiperazine (PP) in that position were generally more active than compounds containing benzylpiperazine (BP). Moreover, the two morpholine analogues (AS-047, AS-048) were quite potent in the proliferation assay, suggesting that further optimization of the secondary amine position could lead to even better compounds.

We have previously observed that CaMKIα and CaMKIIγ are the CaMKs preferentially expressed in myeloid cells and that CaMKI enhances while CaMKII suppresses RAR activity.[48] Thus a KN-62 analogue that preferentially inhibits CaMKII vs. CaMKI should exhibit enhanced activation of RAR and enhanced myeloid differentiation compared with an analogue that inhibits CaMKI and II equally well. However, our experimental efforts to determine whether AS-004 is a more potent inhibitor of CaMKI vs. CaMKII than KN-62 have not revealed significant differences in the activity of these compounds (data not shown).

While AS-004 is more potent than KN-62 in inducing HL-60 differentiation, there are potential problems with it being a viable drug candidate for the treatment of human leukemia. It is a large molecule (MW=728), which might potentially be cleared rapidly from serum. However, if more drug-like CaM kinase IIγ inhibitors can be developed, our data suggests that they could be potent anti-leukemic agents particularly in boosting activity of retinoic acid in treating promyelocytic leukemia.

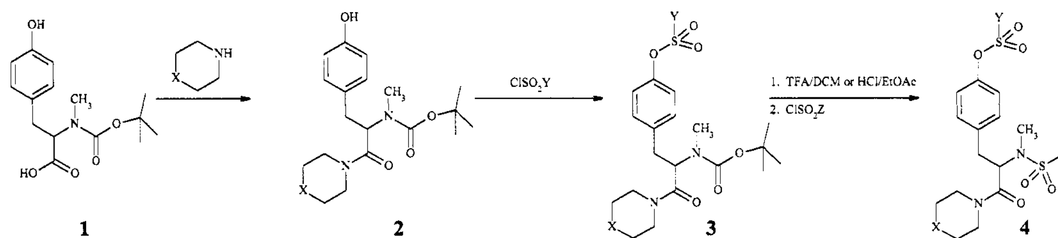

Figure 4: Synthetic preparation of KN-62 analogues. Steps 1-4 of this synthesis are described in detail in Materials and Methods. X = O, N-Ph, or N-CH_2-Ph. Y, Z = 5-isoquinoline, 8-quinoline, dansyl, 4-benzothiadiazole, 5-benzothiadiazole, 4-benzoxodiazole, or tosyl.

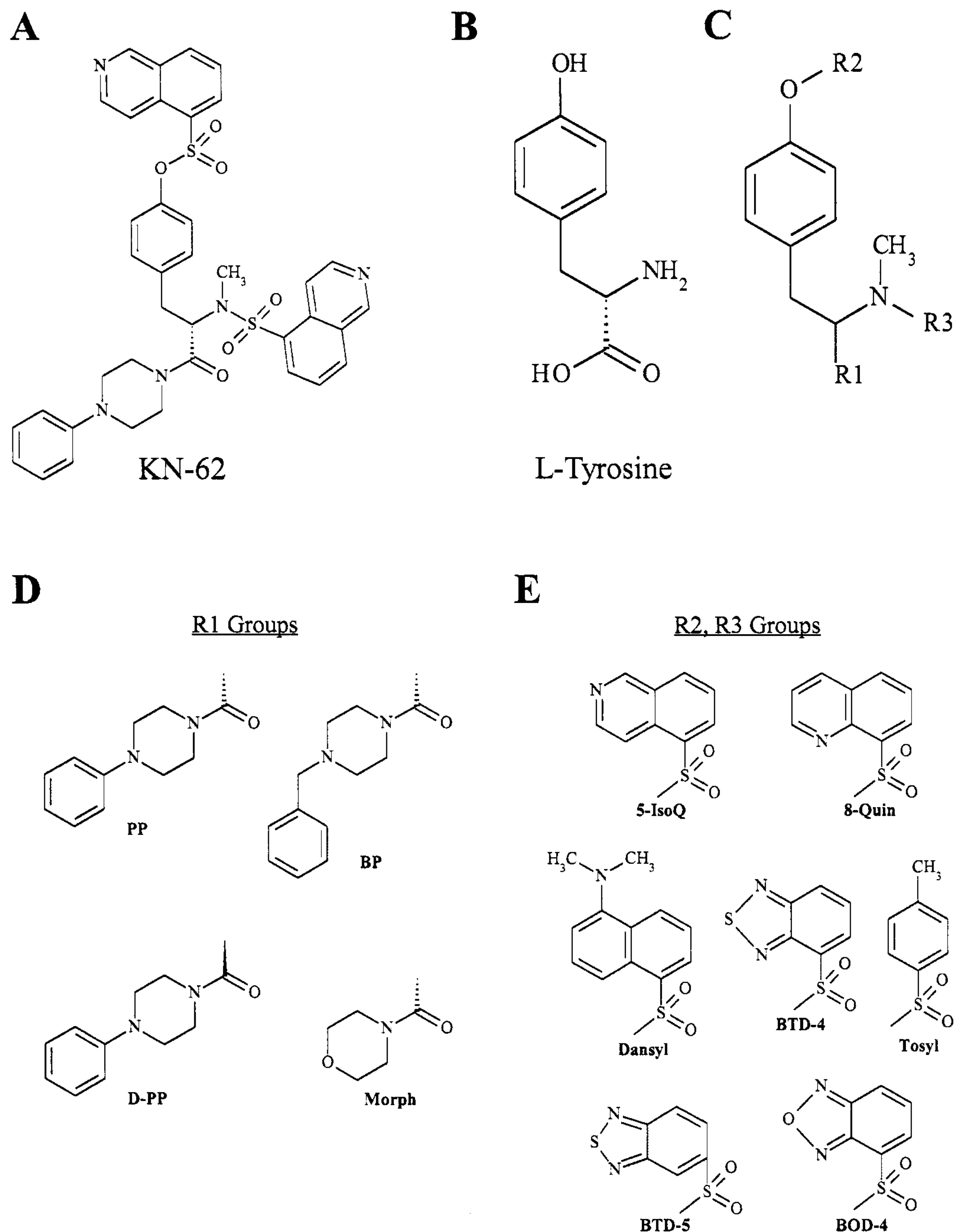

Figure 5: Structure of KN-62 and analogues. Chemical structures of **(A)** KN-62; **(B)** L-tyrosine and **(C)** N-Me-tyrosine analogues. **(D)** R1 groups: PP = phenylpiperazine, D-PP = phenylpiperazine attached to D-tyrosine, BP = benzylpiperazine, Morph = morpholine; **(E)** R2 and R3 groups: 5-IsoQ = 5-isoquinoline, 8-Quin = 8-quinoline, Dansyl = 5-dimethylaminonaphthalene-1-sulphonyl, BTD-4 = 4-benzothiadiazole, BTD-5 = 5-benzothiadiazole, BOD-4 = 4-benzoxodiazole, Tosyl = *p*-toluenesulfonyl.

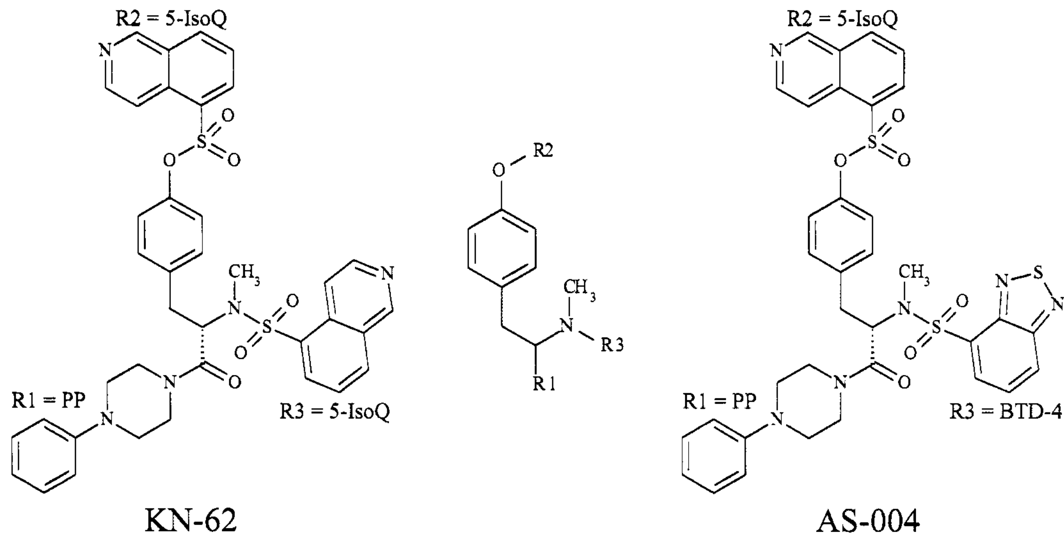

Figure 6: Comparison of the chemical structures of KN-62 vs. AS-004. AS-004 differs from KN-62 at the R3 position.

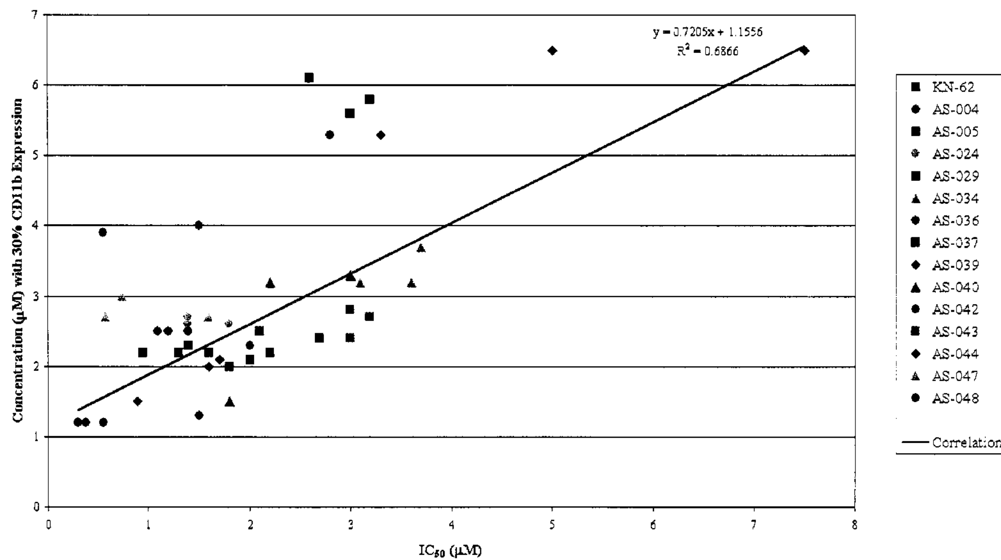

Figure 7: Correlation between inhibition of HL-60 cell proliferation and stimulation of differentiation displayed by the different KN-62 analogues. The legend on the right correlates each analogue to its representative symbol in the plot. The most significant outliers, AS-037 and AS-048, were excluded from the correlation calculation, since they appear to have significant off-target activity.

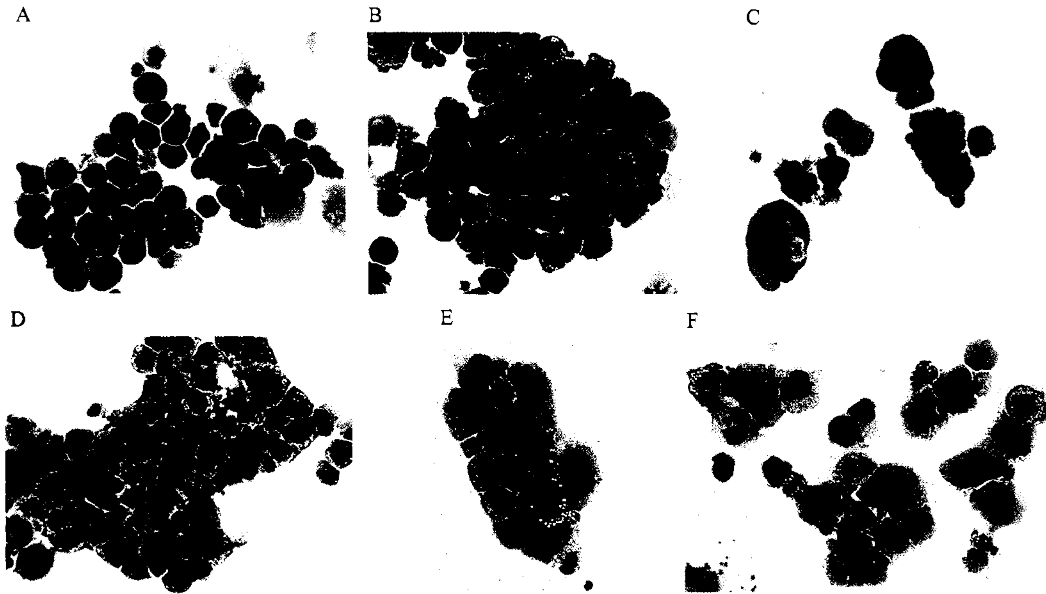

Figure 8: Morphological appearance of KN-62 vs. AS-004 treated HL-60 cells. Wright-Giemsa stained HL-60 cells treated for five days with (**A**) 1 μM KN-62; (**B**) 2 μM KN-62; (**C**) 5 μM KN-62; (**D**) 1 μM AS-004; (**E**) 2 μM AS-004; (**F**) 5 μM AS-004.

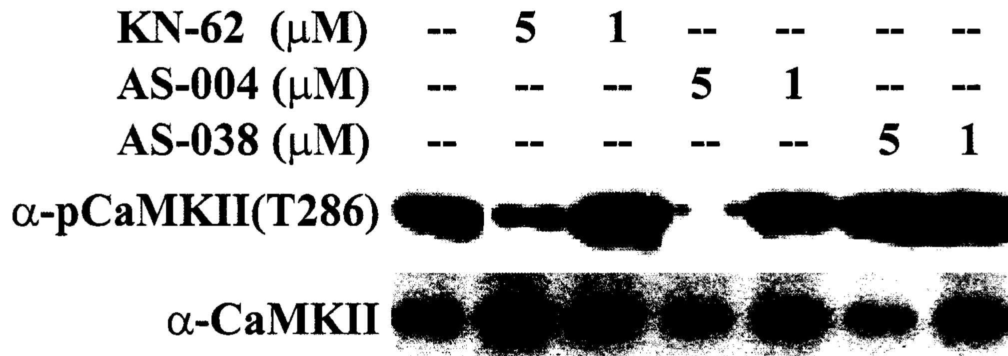

Figure 9: CaMKII phosphorylation in HL-60 cells treated with KN-62, AS-004, and AS-038. Western blots using the indicated CaMKII or phospho-specific CaMKII antibodies were performed on HL-60 total cell lysates treated for five days with the indicated compounds.

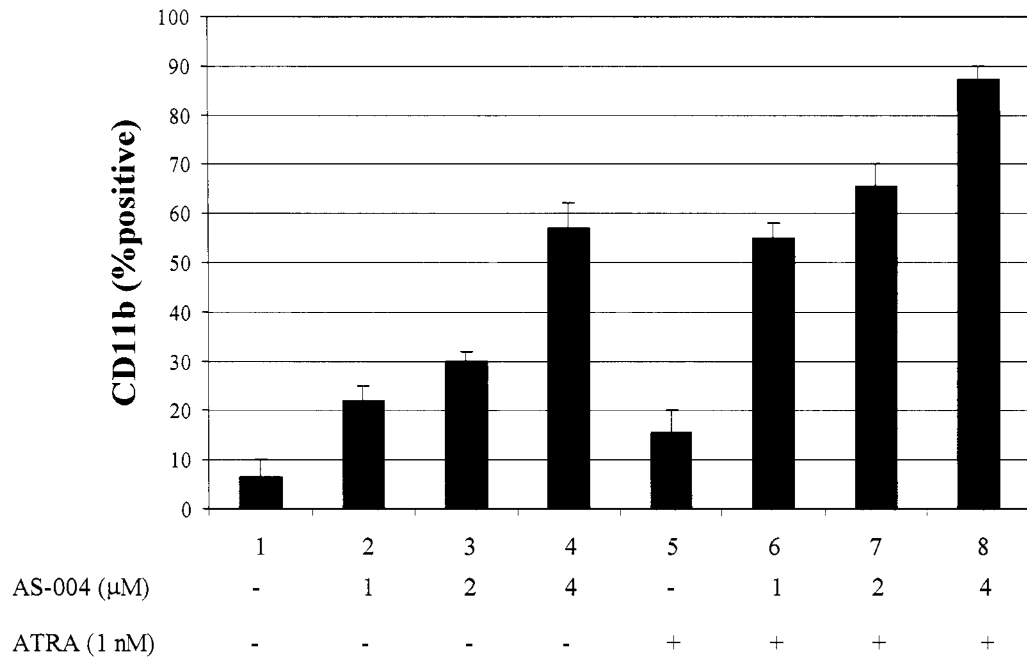

Figure 10: CD11b expression of NB4 cells treated for five days with the indicated concentrations of AS-004 and/or ATRA.

Table 3: KN-62 analogues that do not inhibit HL-60 cell proliferation

Analogue	R1*	R2*	R3*
AS-002	PP	5-IsoQ	8-Quin
AS-003	PP	5-IsoQ	Dansyl
AS-006	BP	BTD-4	5-IsoQ
AS-008	BP	BTD-4	Dansyl
AS-010	BP	BTD-4	BTD-5
AS-012	BP	BTD-5	8-Quin
AS-013	BP	BTD-5	Dansyl
AS-015	BP	BTD-5	BTD-5
AS-016	BP	5-IsoQ	5-IsoQ
AS-017	BP	5-IsoQ	8-Quin
AS-021	BP	8-Quin	5-IsoQ
AS-022	BP	8-Quin	8-Quin
AS-023	BP	8-Quin	Dansyl
AS-025	BP	8-Quin	BTD-5
AS-026	PP	BTD-4	5-IsoQ
AS-027	PP	BTD-4	8-Quin
AS-028	PP	BTD-4	Dansyl
AS-030	PP	BTD-4	BTD-5
AS-031	PP	BTD-5	5-IsoQ
AS-032	PP	BTD-5	8-Quin
AS-035	PP	BTD-5	BTD-5
AS-038	PP	Dansyl	Dansyl
AS-041	PP	Tosyl	Dansyl
AS-045	D-PP	8-Quin	BTD-4
AS-046	D-PP	BTD-4	BTD-4

* R1, R2, and R3 groups are defined in Figure 5.

Table 4: Effect of KN-62 analogues on HL-60 cell proliferation and differentiation

Analogue	R1[*]	R2[*]	R3[*]	IC_{50} (μM)[‖]	30% CD11b (μM)[#]
KN-62	PP	5-IsoQ	5-IsoQ	1.6	2.25
AS-004	PP	5-IsoQ	BTD-4	0.38	1.2
AS-005	PP	5-IsoQ	BTD-5	1.5	2.05
AS-007	BP	BTD-4	8-Quin	10	ND
AS-009	BP	BTD-4	BTD-4	6.5	3.95
AS-014	BP	BTD-5	BTD-4	8	ND
AS-019	BP	5-IsoQ	BTD-4	2.5	7
AS-024	BP	8-Quin	BTD-4	1.3	2.6
AS-029	PP	BTD-4	BTD-4	1.1	2.2
AS-033	PP	BTD-5	Dansyl	10	ND
AS-034	PP	BTD-5	BTD-4	4	3.3
AS-036	PP	8-Quin	BTD-4	1.1	2.5
AS-037	PP	8-Quin	BOD-4	3.1	5.9
AS-039	PP	Dansyl	BTD-4	1.4	1.8
AS-040	PP	Dansyl	BOD-4	2.5	2.6
AS-042	PP	Tosyl	BTD-4	1.5	1.7
AS-043	PP	Tosyl	BOD-4	3.2	2.6
AS-044	D-PP	5-IsoQ	BTD-4	5	6.05
AS-047	Morph	BTD-4	BTD-4	0.92	2.8
AS-048	Morph	5-IsoQ	BTD-4	2	4.5

[*] R1, R2, and R3 groups are defined in Figure 5. [‖] IC_{50} values were determined by the cell proliferation assay described in the text. [#] 30% CD11b is the concentration at which 30% of HL-60 cells are expressing CD11b on their surface as described in the text.

End Notes

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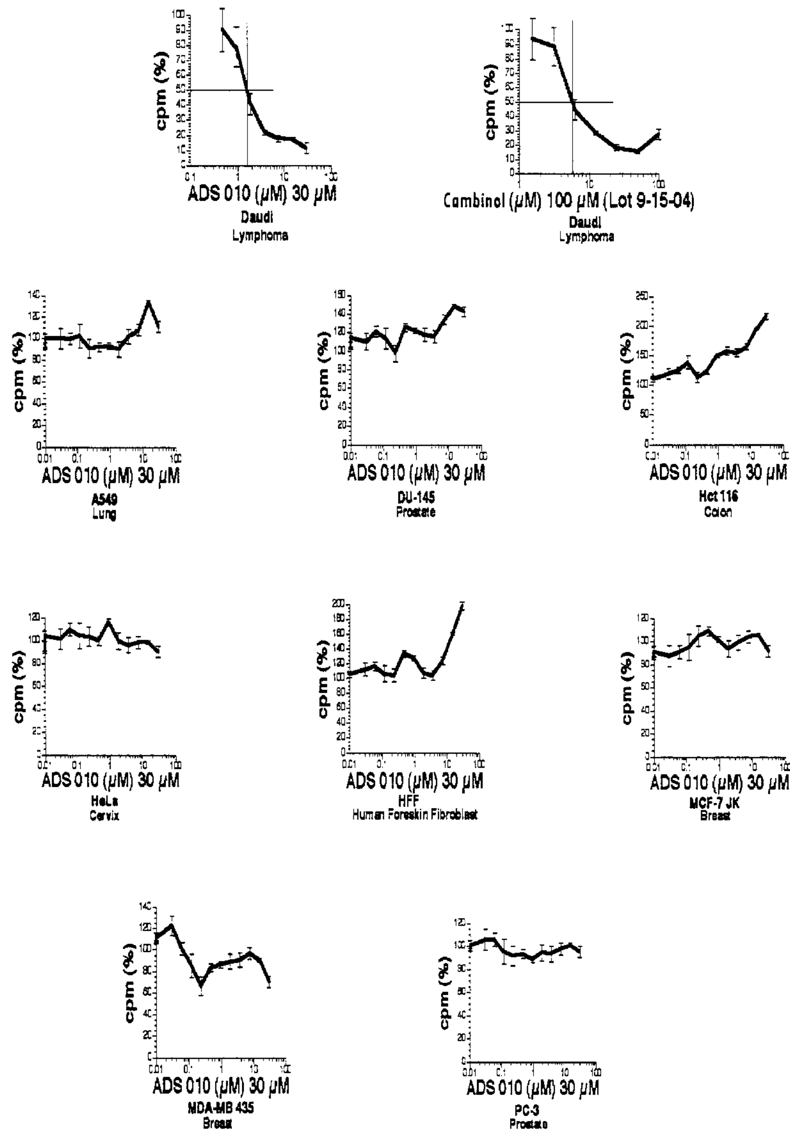
Appendix A:
Alternate Synthesis of Cambinol

Unlike the synthesis shown in the text, this synthesis can be scaled up for the production of tens of grams of cambinol. Put phenyl-β-keto-lactone (4.93 mmol) into vacuum-purged, argon-filled round bottom flask and dissolve in toluene (8 mL). Add piperidine (5.45 mmol), and heat solution to reflux with stirring, azeotropically removing water into a Dean-Stark trap. After one hour, remove reaction from heat and remove solvent under vacuum. Dissolve solids in toluene/diglyme (1:1), and add thiourea (9.95 mmol). Return solution to dehydrating reflux, and after about 2.5 hours, add DBU (1 mmol) to catalyze conversion to cambinol. After 18 hours of additional reflux under dehydrating conditions, cool reaction to room temperature and pour into 50 mL 1 M HCl. After 5 hours of stirring, add ethyl acetate to the acidic solution, and isolate the organic layer. Wash the organic layer twice with water and once with brine, then dry it over sodium sulfate. Transfer the solution to a dry flask through a cotton plug and remove solvent under vacuum.

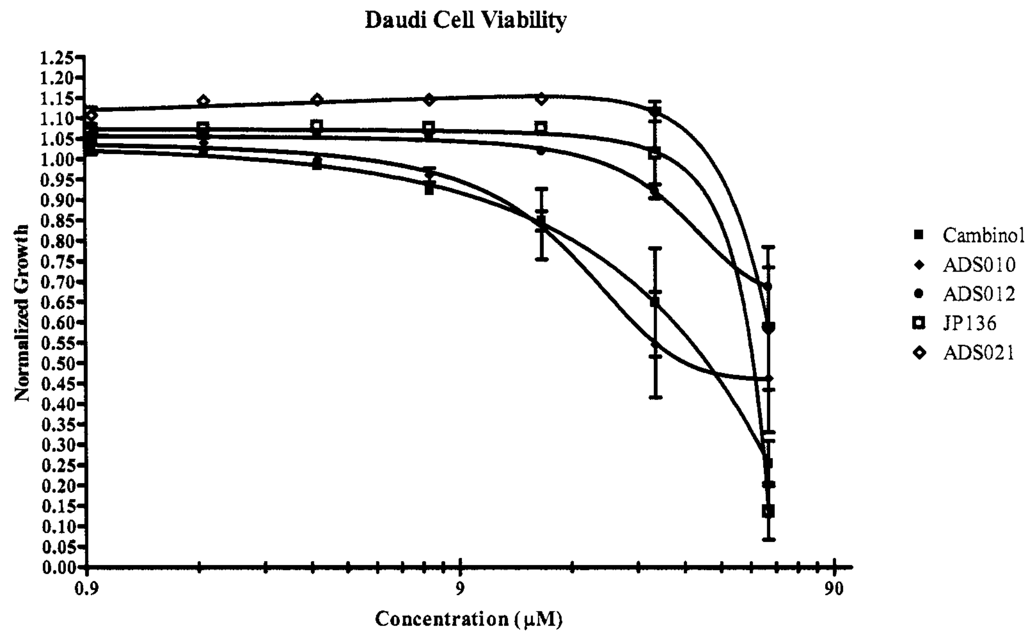
The crude cambinol is initially purified by silica gel chromatograpy. The fractions containing cambinol are pooled, and the solvent is removed under vacuum. The mostly pure cambinol is recystallized twice from ethyl acetate/*n*-heptane to yield pure (93% as confirmed by HPLC) white crystals.

Appendix B: [3H]-Thymidine Incorporation Assays

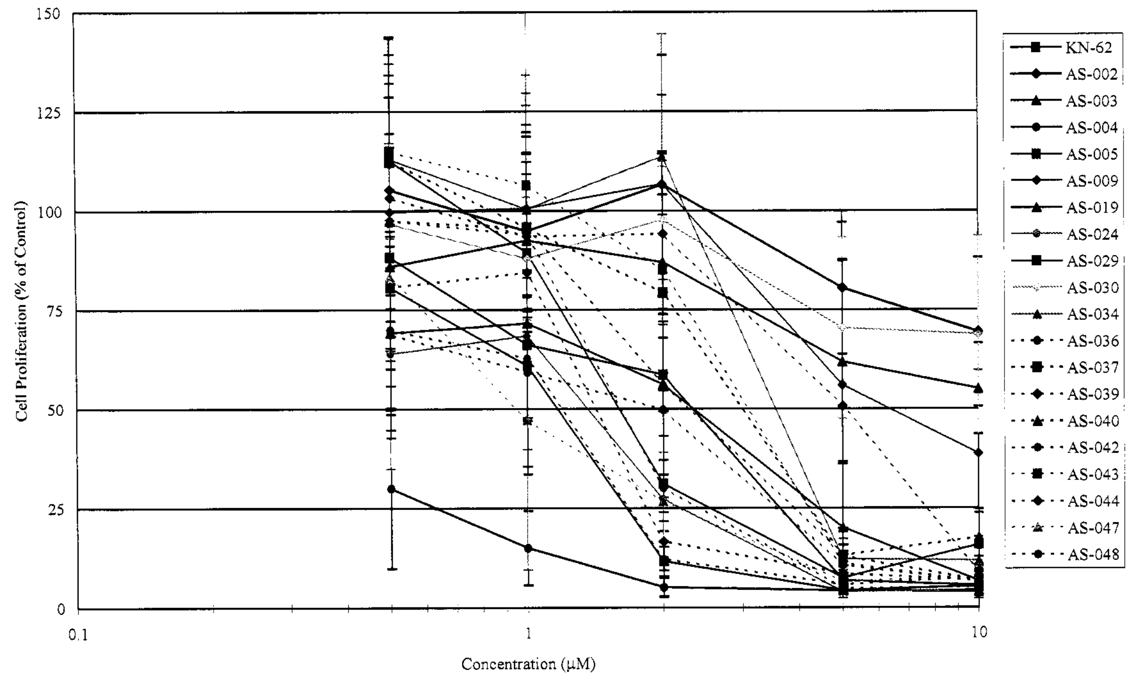
ADS010 (like Cambinol) kills Daudi cells as measured by [^{3}H]-thymidine incorporation, but it does not kill non-Burkitt's lymphoma cell lines. The cpm (%) is measured as described in Chapter One, Materials and Methods.



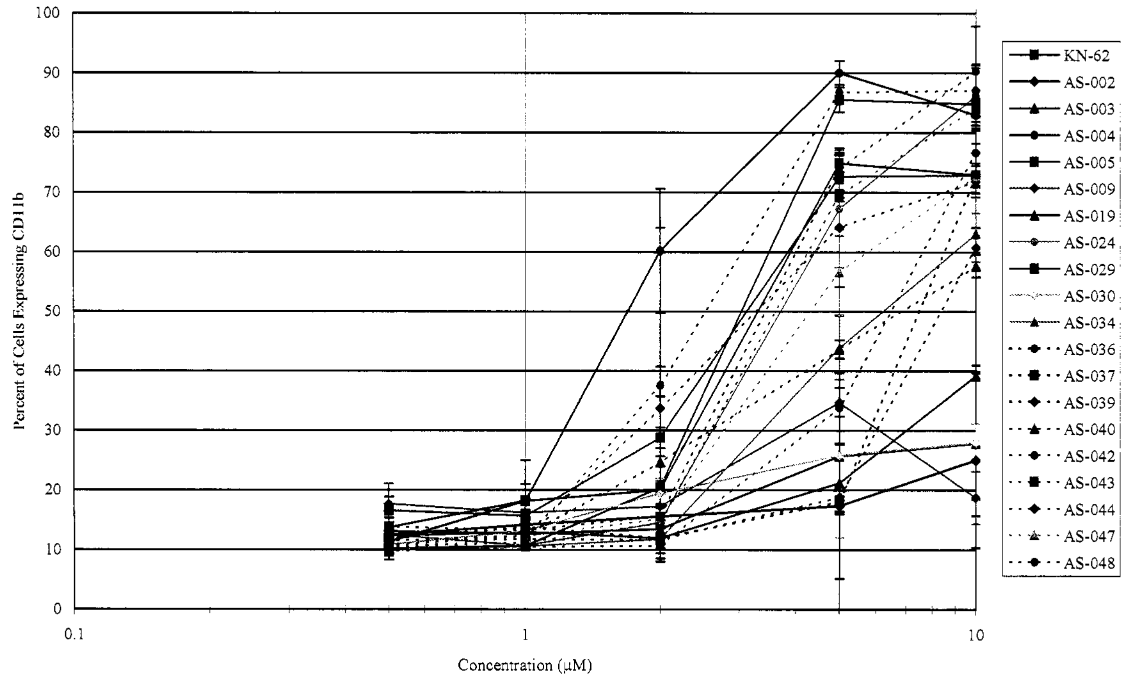
Appendix C:
Effect of Cambinol Analogues on Daudi ATP Content



Appendix D:
Effect of KN-62 Analogues on HL-60 Proliferation



Appendix E:
Effect of KN-62 Analogues on HL-60 Differentation



Vita

Aaron D. Schuler was born in Tacoma, Washington. He spent all of his childhood there, before attending Harvey Mudd College in Claremont, California, where he earned a Bachelor of Science degree in Chemistry in 2001. After completing his undergraduate degree, he moved to Seattle, enrolling in the Biomolecular Structure and Design Program at the University of Washington. In 2002, he joined the laboratory of Dr. Julian Simon at the Fred Hutchinson Cancer Research Center, completing his Doctor of Philosophy in Chemistry in 2006.