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KN-62 Analogues as Potent Differentiating Agents of HL-60 Cells

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Abstract

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 leukemias.

Keywords

Promyelocytic leukemia; Calmodulin-dependent protein kinases; KN-62; HL-60 cells; Differentiating agents

1. Introduction

The retinoic acid receptors (RARs) are transcription factors that are involved in regulating myeloid differentiation [1]. 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-RAR α fusion protein. This aberrant RAR α fusion protein likely acts as a dominant negative to inhibit the normal role of the RARs in enhancing myeloid cell differentiation [2,3]. Treatment of APL patients with relatively high, pharmacological doses of retinoic acid (RA) likely overcomes this differentiation block and has had a remarkably

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Author Contributions

Aaron D. Schuler synthesized and screened all of the compounds in this paper and performed the initial data analysis. LeMoyne Mueller assisted with the screening of compounds. Jutong Si made the initial experimental observations that led to this drug screen and also performed the CaM kinase phosphorylation studies. Julian A. Simon designed the drug synthesis strategy, and Steven J. Collins supervised the overall experimental plan.

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beneficial therapeutic effect [4,5]. However, some 20–30% of patients with APL still relapse despite optimal RA therapy [5,6]. 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 [7]. 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 CaMKI α and CaMKII γ are the CaMKs that are predominantly expressed in myeloid cells, and these enzymes may have a regulatory role in blood cell differentiation [8].

We have recently observed that KN-62, a small molecule inhibitor of the CaMKs, enhances RAR α transcriptional activity and induces the differentiation of the RA-sensitive HL-60 human myeloid leukemia cell line [8]. 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 [7,9]. 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 RAR α transcriptional activity. Indeed CaMKI-mediated phosphorylation of RAR α enhances RAR transcriptional activity while CaMKII-mediated phosphorylation of RAR α 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.

2. Materials and Methods

2.1. Synthesis of KN-62 analogues

All reactions were carried out with dry solvents under an inert atmosphere, following synthetic procedures described previously [10,11]. The basic synthesis procedures are summarized in Supplemental Figure 1. Briefly, Boc-N-Me-L-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/hydrochloric acid (3:1) or dichloromethane/trifluoroacetic acid (1:1) [11], 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 μ L 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.

2.2. 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.

2.3. 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 μ M. 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 μ M 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 μ M in triplicate. KN-62 was examined in parallel with all experiments as an internal control.

2.4. 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.

2.5. 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.

2.6. Western blots

Western blots on HL-60 total cell lysates were performed as previously detailed [12]. Antibodies utilized in these Western blots included anti-CaMKII γ and anti-phosphospecific CaMKII (T286), both from Santa Cruz Biotechnology.

3. Results

3.1. Synthesis of KN62 related analogues

KN-62 (Figure 1A) consists of a tyrosine core (Figure 1B) to which three different chemical groups have been attached at distinct sites (designated R1, R2, and R3) (Figure 1C). As detailed in **Materials and Methods** and Supplemental Figure 1, we used a combinatorial approach to synthesize structural analogues of KN-62, incorporating different chemical groups at the R1, R2, and R3 positions (Figures 1D 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.

3.2. 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 (Supplemental Table) 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 μ M or less, and eight compounds exhibited an IC_{50} that was lower than KN-62 (Table 1 and Supplemental Figure 2A). The most potent of these compounds, AS-004, (Figure 2) has an IC_{50} approximately 5 times lower than KN-62 (0.38 vs. 1.6 μ M) (Table 1).

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 1 and Supplemental Figure 2B). Again AS-004, which proved most potent in inhibiting HL-60 proliferation (Table 1), 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 3). 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 4).

3.3. Inhibition of CaMKII autophosphorylation 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 [13]. 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 KN62-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 5). In contrast, AS-038, an analogue without differentiation inducing activity (Supplemental Table), did not exhibit activity in inhibiting CaMKII autophosphorylation (Figure 5).

3.4. 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 might also induce 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-RAR α fusion gene that characterizes APL [14], AS-004 enhanced CD11b surface antigen expression in a dose dependent manner (Figure 6, columns 1–4). Moreover, the addition of small, physiological concentrations of all-trans retinoic acid (ATRA) (1 nM) markedly enhanced the differentiation response of the NB4 cells to AS-004 (Figure 6, columns 5–8). These *in vitro* observations suggest that AS-004 might be of therapeutic benefit in the treatment of the ATRA-sensitive subsets of human myeloid leukemia.

4. Discussion

Differentiation therapy plays an important role in treating certain human myeloid leukemias, 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 1C) 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 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 1D) to yield compounds AS-044, AS-045, and AS-046 (Table 1 and Supplemental Table) greatly abrogated their differentiation-inducing activity compared with KN-62 with only AS-044 showing even minimal activity (Table 1). 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 2), 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 1). 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 1). 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 1) 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 improved compounds.

We have previously observed that CaMKI α and CaMKI γ are the CaMKs preferentially expressed in myeloid cells and that CaMKI enhances while CaMKII suppresses RAR activity [8]. 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 leukemias. It is a large molecule (MW=728), which might potentially be cleared rapidly from serum. However, if more drug-like CaM kinase II γ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

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-benzoxadiazole, Tosyl = p-toluenesulphonyl.

Figure 2.

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

Figure 3. 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.

Figure 4. 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 μ M KN-62; (B) 2 μ M KN-62; (C) 5 μ M KN-62; (D) 1 μ M AS-004; (E) 2 μ M AS-004; (F) 5 μ M AS-004.

Figure 5. 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 6.
CD11b expression of NB4 cells treated for five days with the indicated concentrations of AS-004 and/or ATRA.

Table 1

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

Analogue	R1 ¹	R2 ¹	R3 ¹	IC ₅₀ (μM) ²	30% CD11b (μM) ³
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

¹ R1, R2, and R3 groups are defined in Figure 1C, D, and E.

² IC₅₀ values were determined by the cell proliferation assay described in the text.

³ 30% CD11b is the concentration at which 30% of HL-60 cells are expressing CD11b on their surface as described in the text.