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Mixed Lineage Kinase Activity of Indolocarbazole Analogues

Chikara Murakata,[d] Masami Kaneko,[d] George Gessner,[b] Thelma S. Angeles,[b] Mark A. Ator,[b] Teresa M. O'Kane,[c] Beth Ann W. McKenna,[c] Beth Ann Thomas,[c] Joanne R. Mathiasen,[c] Michael S. Saporito,[c] Donna Bozyczko-Coyne[c] and Robert L. Hudkins[a,*]

[a]*Department of Medicinal Chemistry, Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA*
[b]*Department of Biochemistry, Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA*
[c]*Department of Neurobiology, Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA*
[d]*Kyowa-Hakko Kogyo Co., Ltd., Tokyo, Japan*

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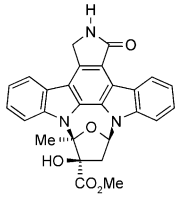
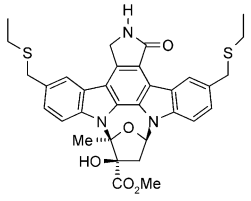
Abstract—The MLK1–3 activity for a series of analogues of the indolocarbazole K-252a is reported. Addition of 3,9-bis-alkylthiomethyl groups to K-252a results in potent and selective MLK inhibitors. The in vitro and in vivo survival promoting activity of bis-isopropylthiomethyl-K-252a (**16**, CEP-11004/KT-8138) is reported.

c-Jun NH_2-terminal kinases (JNKs) are stress-activated protein kinases (SAPK) that belong to the mitogen activated protein kinase (MAPK) family and respond to a variety of stimuli including oxidative stress, cytokines, and initiators of cell death.[1] The JNK/SAPK pathway, leading to activation of the transcription factor c-Jun, has been implicated in neuronal apoptosis,[2] and agents that block the JNK signaling cascade have been proposed as a therapeutic approach for preventing neuronal cell death observed in a variety of neurodegenerative diseases including Parkinson's, Huntington's, and Alzheimer's disease.[3] JNKs are the only kinases known to activate c-Jun and their activity is regulated through a sequential signaling cascade by a series of upstream kinases. The JNKs are phosphorylated and activated by the dual specificity MAPK kinases (MAPKK) MKK4 and MKK7, which are activated by multiple upstream MAPKK kinases including the mixed lineage kinases (MLKs).[1,4] The MLKs function as serine/threonine kinases although their catalytic domains have features of both tyrosine and serine/threonine kinases.[5] There are currently five known MLK family members: MLK1, MLK2, MLK3, DLK (dual leucine zipper kinase), and LZK (leucine zipper-bearing kinase). MLKs1–3 signal to JNKs via both MKK4 and MKK7 while DLK activates the JNK/SAPK pathway through MKK7.[4,6] Overexpression of the MLKs has been shown to induce activation of the JNK/SAPK pathway and lead to neuronal cell death.[4]



1 (+)K-252a



2 CEP-1347

Our research has focused on developing inhibitors of MLKs for the treatment of neurodegenerative diseases. Previously we reported that CEP-1347/KT-7515 (**2**), a derivative of the indolocarbazole natural product (+)K-252a (**1**) in clinical trials for Parkinson's disease, is an inhibitor of the JNK signaling cascade via MLK inhibition and displays a broad neuroprotective profile.[4,7] In culture, CEP-1347 protected cells after trophic factor withdrawal, DNA damage, oxidative stress,[4,8] Aβ toxicity,[9] and MPP^+ toxicity.[10] CEP-1347 rescued motor neurons in vitro and in vivo in developmental programmed cell death models.[11] In preclinical models of Parkinson's disease, CEP-1347 attenuated the MPTP-mediated loss of nigrostriatal dopaminergic neurons in mice[12] and primates[13] and effectively blocked the MPTP mediated-activation of JNK and MKK4 in

*Corresponding author. Fax: +1-610-344-0065; e-mail: rhudkins@cephalon.com

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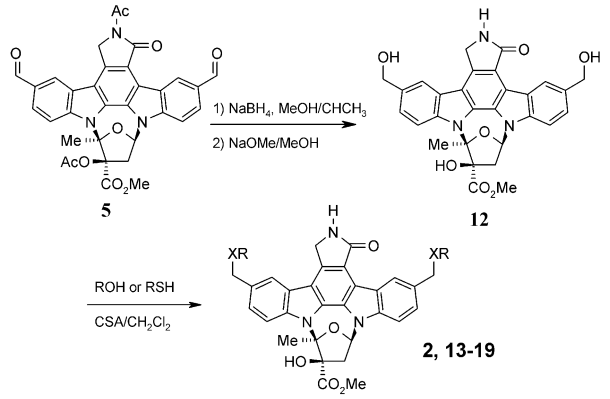
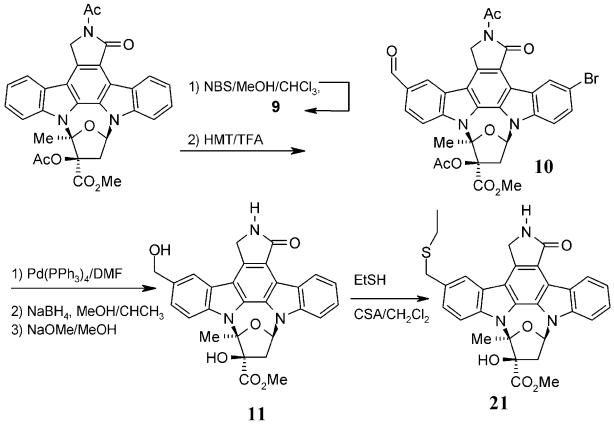
vivo.[14] This paper reports the MLK1–3 SAR for a set of indolocarbazole analogues and characterizes the survival promoting activity of CEP-11004 (**16**) in vitro and in vivo in MPTP models of Parkinson's disease.

The 3,9-[bis(alkoxymethyl)] (**13**–**14**) and 3,9-[bis(alkylthiomethyl)]-K-252a (**2** and **15**–**19**) derivatives were produced in good yield from 3,9-[bis(hydroxymethyl)]-K-252a (**12**) as shown in Scheme 1.[15] Diol **12** was prepared by converting K-252a (protected as a diacetyl) to 3,9-dialdehyde **5** ($TiCl_4$ and α,α-dichloromethyl methyl ether) followed by reduction to diacetyl-diol **6** (65%, $NaBH_4$, $CHCl_3$–methanol), and deprotection using catalytic NaOMe in methanol. The 3-ethylthiomethyl derivative (**20**) was prepared in a similar manner to the bis-derivatives from 3-formyl diacetyl-K-252a **7**. The 9-ethylthiomethyl derivative (**21**) was prepared as outlined in Scheme 2. Protection of the 3-position using 1 equivalent of NBS gave the 3-bromo compound (**9**), and then formylation gave the bromo-aldehyde **10**. Deprotection was successfully accomplished using $Pd(PPh_3)_4/H_2$. $NaBH_4$ reduction to alcohol **11**, removal of the acetate groups and thioether formation afforded 9-ethylthiomethyl K-252a (**21**).

The kinase inhibitory data for the derivatives is shown in Table 1. The objective was to identify an MLK inhibitor with >100-fold separation between the MLKs and the screen control kinases PKC (protein kinase C) and TrkA (NGF high affinity receptor tyrosine kinase). K-252a (**1**) is a potent, nonselective inhibitor of a number of serine/threonine and protein tyrosine kinases[15,16] that limits its utility as a therapeutic agent, or as a pharmacological tool. K-252a inhibited TrkA with an IC_{50} of 13 nM and PKC with an IC_{50} of 28 nM.[15] The most potent MLK inhibitors in the bis-thioether series were the ethylthiomethyl (**2**) and the isopropylthiomethyl (**16**) derivatives. Compound **2** displayed IC_{50} values of 38 nM, 51 nM and 23 nM for MLK1, MLK2, and MLK3, respectively, and good selectivity against PKC and TrkA (IC_{50} >10 μM). The IC_{50} values of **16** for the MLKs were 45 nM, 89 nM and 31 nM, respectively. Increasing the size of the alkyl group in the thioether side chain from ethyl (**2**) to *n*-butyl (**18**) resulted in a decrease in potency from 38 to 303 nM for MLK1, 51 to 627 nM for MLK2 and 23 to 143 nM for MLK3. Incorporating a dimethylamino group as in **19** or unsaturation (**17**) in the side chain was not favorable.



Scheme 1.



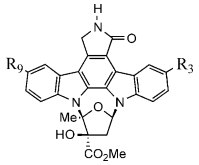
Scheme 2.

Of significance, the bis-thioether series (**2**, and **15**–**19**) showed good selectivity with IC_{50} values >1 μM for PKC and TrkA. The diol **12** displayed an improvement in potency for MLK1 (15 nM) and MLK3 (11 nM); however, the alcohol proved to be a 3 nM PKC inhibitor. The bis-ether analogues were also prepared and evaluated. The bis-methyl ether **13** and bis-ethyl ether **14** were potent MLK1–3 inhibitors, however, the selectivity was poor compared to the thioether analogues. To evaluate the contribution of the individual ethylthiomethyl side chains toward kinase activity the 3-ethylthiomethyl (**20**) and 9-ethylthiomethyl (**21**) analogues were prepared. Each mono-substituted derivative showed comparable MLK1–3 activity with the 9-ethylthiomethyl **21** being slightly more potent than **20**. However, **20** and **21** showed inferior selectivity (5- to 20-fold) compared to bis-**2** or -**16**. The 9-substituted derivative **21** was a potent (61 nM) inhibitor of TrkA, while both **20** (220 nM) and **21** (400 nM) were more potent inhibitors of PKC than **2** and **16**.

Information from this SAR reveals that MLK1, MLK2, and MLK3 tolerate substitution in the 3- and the 9-positions of K-252a with alkylthiomethyl groups that are not allowable at TrkA kinase and PKC.

Compound **16** (CEP-11004/KT-8138) was evaluated for survival promoting activity in three in vitro neuronal death models. Although each model system differed in the stimulus used to induce death, the mode of death was characterized as apoptotic, as previously demonstrated,[8–10] Compound **16** demonstrated dose dependent rescue of neuronally differentiated rat pheochromocytoma PC12 cells following NGF withdrawal (Fig. 1A) as well as of primary embryonic rat cortical neurons following exposure to the amyloidogenic peptide, Aβ1-42 (Fig. 1B). In addition, **16** demonstrated dose dependent rescue of retinoic acid differentiated human SH-SY5Y cells following treatment with MPP^+, the toxic metabolite of MPTP (Fig. 1C). In each of these in vitro model systems, the efficacy of **16** was observed at concentrations between 30 and 1000 nM. Maximal efficacy in these models typically ranged between 40 and

Table 1. Kinase activity



Entry	R^3	R^9	IC_{50} (nM)				
			MLK1[a]	MLK2[a]	MLK3[a]	PKC[b]	TrkA[c]
2	CH_2SEt	CH_2SEt	38±17	51±9	23.1±0.1	>10,000	>10,000
12	CH_2OH	CH_2OH	15±3	nd	11±1	3	nd
13	CH_2OMe	CH_2OMe	16±2	16±2	11±2	450	270
14	CH_2OEt	CH_2OEt	19±2	20±1	11±1	2600	210
15	CH_2S^nPr	CH_2S^nPr	90±10	nd	56±2	>10,000	>10,000
16	CH_2S^iPr	CH_2S^iPr	45±8	89±14	31±17	980	>10,000
17	$CH_2SAllyl$	$CH_2SAllyl$	162±43	nd	94±16	>10,000	>1000
18	CH_2S^nBu	CH_2S^nBu	303±53	627±152	143±21	>7600	>1000
19	$CH_2S(CH_2)_2NMe_2$	$CH_2S(CH_2)_2NMe_2$	360	nd	123[d]	1600	>1000
20	CH_2SEt	H	43±7[d]	140[d]	44±12	222	>1000
21	H	CH_2SEt	28±4	28±3	21±3	400	61

nd, not determined.
[a]MLK assays were conducted as described in ref 4.
[b]PKC (rat brain homogenate) was conducted as described in ref 15.
[c]TrkA activity was measured as described in refs 15 and 16b.
[d]Average of two determinations.

100% rescue relative to the untreated basal condition. Consistent with blockade at the level of the MLKs, **16** dose dependently inhibited MPP^+ induced increases in the phosphorylation of both the p54 and p46 isoforms of JNKs (Fig. 1D) in SH-SY5Y cells. Inhibition of phosphorylation of JNKs was complete at 300–1000 nM concentrations.

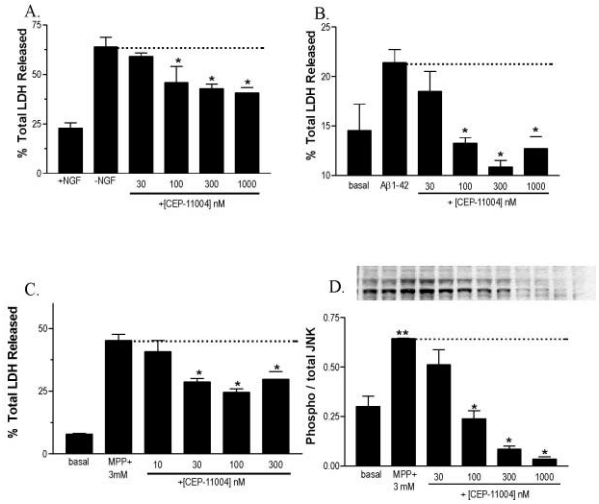


Figure 1. CEP-11004 protection in models of apoptotic neuronal cell death: (A) NGF withdrawal induced death of differentiated PC12 cells, (B) $A\beta_{1\text{-}42}$ induced death of primary cortical neurons and (C) MPP+ induced death of retinoic acid differentiated SH-SY5Y cells. (D) CEP-11004 inhibits MPP+-induced JNK activation at concentrations that provide neuroprotection. (D insert) Immunoblot analysis of phospho JNK (p54 and p46 bands) from duplicate analyses (left to right) in untreated basal control cultures and in 3 mM MPP^+ treated cultures in the absence and presence of increasing concentrations of **16**. Data was replicated in a minimum of two independent experiments. *=significance at $p \leq 0.05$ by Student and Dunnett *t*-tests.

Systemic administration of **16** inhibits the MPTP-mediated increase in phospho-MKK4 in the substantia nigra and attenuates the MPTP mediated loss of striatal dopaminergic terminals. The magnitude of activity and the doses of **16** needed for inhibition of these parameters in MPTP-treated mice is essentially equal to that reported for **2**.[12,14] The biochemical efficacy was assessed by treating mice with **16** at 1 and 10 mg/kg/sc, 2 h prior and 2 h post-MPTP (40 mg/kg/sc) (Fig. 2A). The substantia nigra was removed four hours after MPTP and levels of phospho-MKK4 and total MKK4 levels measured by immunoblot.[12] MPTP elevated levels of phospho-MKK4 in the substantia nigra by 4-fold. Administration of **16** (CEP-11004) at 10 mg/kg/sc inhibited this activation by 50%. For assessment of neuroprotective activity, mice were treated with **16** at doses of 0.1, 1 and 10 mg/kg/sc, 18 h prior to administration of MPTP (20 mg/kg/sc) and then daily for 4 days. [^{3}H]GBR-12935 binding was utilized as an index of striatal dopaminergic terminal density.[12] Administration of **16** attenuated the MPTP-mediated decrease in striatal dopaminergic terminals at a dose of 1 mg/kg, while at the higher 10 mg/kg dose, the protective activity was reduced. These data are shown in Figure 2B.

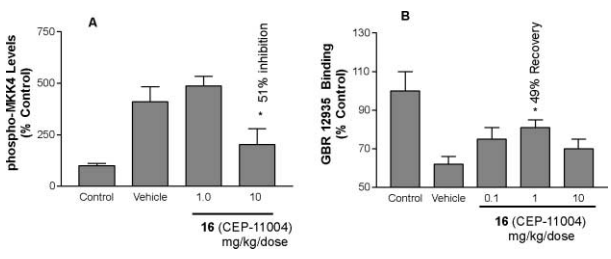


Figure 2.

The SAR is reported for a series of 3,9-bis-alkylthiomethyl and 3,9-bis-alkoxymethyl K-252a analogues resulting in potent and selective MLK inhibitors. CEP-11004/KT-8138 (**16**) demonstrates inhibition of the JNK pathway following treatment of cells with MPP^+ and demonstrates in vivo protection of dopaminergic terminals within the striatum projecting from neurons within the substantia nigra of mice following administration of MPTP. Taken together, the data demonstrating **16** (CEP-11004) protection against multiple insults in a variety of cells underscores the role of the MLK family as a convergence point within signal cascades culminating in apoptotic neuron death. Furthermore, the in vivo demonstration of protection of terminal loss following MPTP administration to mice underscores that targeting inhibition of the JNK pathway at the level of the MLKs may be an effective strategy for blocking neurodegeneration associated with Parkinson's disease.

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