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A selective small-molecule inhibitor of c-Jun *N*-terminal kinase 1

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ABSTRACT

Indiscriminately suppressing total c-Jun *N*-terminal kinase (JNK) activity is not an appropriate strategy because each JNK appears to have a distinct function in cancer, asthma, diabetes, or Parkinson's disease. Herein, we report that 7-(6-*N*-phenylaminohexyl)amino-2*H*-anthra[1,9-cd]pyrazol-6-one (AV-7) inhibited JNK1 activity, but not JNK2 or JNK3. We found that ultraviolet B (UVB) induced c-Jun phosphorylation and sub-G1 accumulation in JNK2$^{-/-}$ murine embryonic fibroblasts, which contain an abundance of JNK1, but not JNK2. These results demonstrate that AV-7 is an isoform selective small-molecule inhibitor of JNK1 activity, which might be developed as a therapeutic against diabetes.

Structured summary:
MINT-7148332: *JNK3* (uniprotkb:P53779) *phosphorylates* (MI:0217) *c-JUN* (uniprotkb:P05412) by *protein kinase assay* (MI:0424)
MINT-7148323: *JNK2* (uniprotkb:P45984) *phosphorylates* (MI:0217) *c-JUN* (uniprotkb:P05412) by *protein kinase assay* (MI:0424)
MINT-7148314: *JNK1* (uniprotkb:P45983) *phosphorylates* (MI:0217) *c-JUN* (uniprotkb:P05412) by *protein kinase assay* (MI:0424)

1. Introduction

The c-Jun *N*-terminal kinases (JNKs) are members of the mitogen-activated protein (MAP) kinase family [1]. The *jnk1* and *jnk2* genes are expressed ubiquitously, whereas *jnk3* expression is largely restricted to brain, heart, and testis [2]. The JNKs signal transduction pathway plays an important role in coordinating cellular responses including apoptosis [3], proliferation [4], and neoplastic transformation [5]. Ras-induced transformation requires c-Jun [6] and Ras induces phosphorylation of c-Jun by JNKs [7]. JNKs are constitutively activated in tumor cell lines [3] and antisense oligonucleotides targeted against JNKs inhibit tumor cell growth [8]. In contrast, double knockout of JNK1 and JNK2 caused marked increases in number and growth of Ras-induced tumor nodules in vivo [9]. We found that JNK2 deficient mice display significant suppression of skin papilloma development induced by 12-*O*-tetradecanoylphorbal-13-acetate (TPA) [10]. Importantly, the gene expression pattern induced by TPA in *jnk1* (*jnk1*$^{-/-}$) and *jnk2* deficient (*jnk2*$^{-/-}$) cells is different, suggesting distinct functions for each kinase [11]. The seemingly contradictory functions of JNKs were attributed to the idea that JNKs are activated differentially and probably activate different substrates based on stimuli or cell type. JNK1, but not JNK2, was reported to play a role in diabetes [12], and is believed to be a promising target for diabetes and insulin resistance [13]. Highly selective JNK1 or JNK2 inhibitors will aid greatly in understanding the precise functions of JNK1 and JNK2. However, until now, isoform selective inhibitors for JNK1 over JNK2 or JNK3 activities have not been developed. We report that 7-(6-*N*-phenylaminohexyl)amino-2*H*-anthra[1,9-cd]pyrazol-6-one (AV-7, Fig. 1), which was developed in-house using structure-based design, shows a selective inhibitory effect against JNK1, but not JNK2 or JNK3. Functionally, AV-7 suppressed JNK1-mediated c-Jun phosphorylation and reversed sub-G1 accumulation.

2. Materials and methods

2.1. Reagents and antibodies

AV-7 was synthesized as described in US Patent 61/102,089 [14]. Dulbecco's modified Eagle's medium (DMEM) and fetal

Abbreviations: JNK, c-Jun *N*-terminal kinase; MEF, mouse embryonic fibroblast; UVB, ultraviolet B

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AV-7

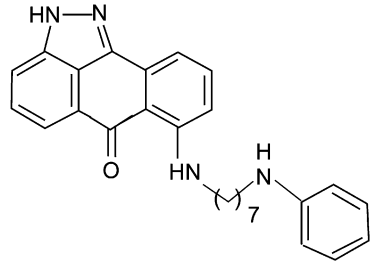

Fig. 1. Chemical structure of AV-7.

bovine serum (FBS) were purchased from Life Technologies Inc. (Grand Island, NY). Restriction and modifying enzymes were from New England BioLabs Inc. (Beverly, MA). The DNA ligation kit (version 2.0) was obtained from TAKATA Bio Inc. (Otsu, Shiga, Japan). The antibodies against JNK1 or JNK2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or from Cell Signaling Technology Inc. (Beverly, MA), respectively. Active JNK1, JNK2, and JNK3 were purchased from Upstate Biotechnology Inc. (Charlottesville, VA) and the 10X kinase buffer for the in vitro kinase assay was from cell signaling.

2.2. Construction of His-c-Jun bacterial expression vector

Human c-Jun cDNA was used for PCR as a template. A fragment of c-Jun (aa 1-201) was amplified using primers, hc-Jun-sense: 5′-GAC GAC GAC AAG ATG ACT GCA AAG ATG GAA ACG AC-3′ and hc-Jun antisense: 5′-GAG GAG AAG CCC GGT TTA GGG TTG CTC GGG AAA GGC CAG-3′. The amplified DNA fragment was purified from agarose using the QIAEX II DNA extraction kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The eluted DNA was introduced into the pET-46 Ek/LIC His-fusion bacteria expression vector using the Ek/LIC cloning kit according to the manufacturer's instructions (Novagen, Darmstadt, Germany). The fusion expression vector (pHis-c-Jun-1-201) was confirmed by restriction enzyme mapping and sequencing.

2.3. Purification of His-c-Jun-1-201

Briefly, pHis-c-Jun was transformed into BL21 *Escherichia coli* and a single colony was cultured overnight in a 37 °C shaking incubator. This culture (0.5 ml) was inoculated into 50 ml of bacterial culture media and incubated until OD_{600} = 0.6. Protein expression was induced with IPTG ([final] = 0.5 mM) and incubated for 4 h in a 25 °C shaking incubator. The bacteria were harvested by centrifugation, washed twice with ice-cold 1X PBS and His-c-Jun proteins were purified using Ni–NTA agarose bead chromatography (QIAGEN Inc.). Purified protein was confirmed by Western blotting with a c-Jun antibody and Coomassie blue R-250 staining.

2.4. In vitro kinase assay

Wildtype His-c-Jun-1-201 protein was used in an in vitro kinase assay with active JNK1, JNK2, or JNK3 (Upstate). Reactions were performed at 30 °C for 30 min with 20 ng active kinase, 2 μg His-c-Jun protein, 50 μM unlabeled ATP and 10 μCi [γ-^{32}P] ATP. Reactions were stopped with 6X SDS sample buffer. Samples were boiled and separated by 12% SDS–PAGE and visualized by autoradiography.

2.5. Cell culture

JNK^{WT}, $JNK1^{-/-}$, or $JNK2^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with 10% heat-inactivated FBS in a 37 °C, 5% CO_2 incubator. The cells were maintained by splitting at 80–90% confluence and media changed every 3 days.

2.6. Western blotting

The proteins were extracted with NP-40 lysis buffer and freezing and thawing. Proteins were resolved by SDS–PAGE, transferred onto PVDF membranes, hybridized with appropriate antibodies and then visualized using the ECL detection kit (Amersham Biosciences, Piscataway, NJ).

2.7. MTS Assay

To estimate proliferation, JNK^{WT}, $JNK1^{-/-}$, or $JNK2^{-/-}$ MEFs were seeded (1×10^3) into 96-well plates in 100 μl of 10% FBS/DMEM and incubated at 37 °C, 5% CO_2. Proliferation was measured by adding 20 μl of the CellTiter 96® Aqueous One Solution (Promega, Madison, WI, USA) and then incubated for 1 h at 37°C, 5% CO_2. To stop the reaction, 25 μl of a 10% SDS solution were added and absorbance measured at 492 and 690 nm.

2.8. Sub-G1 analysis

JNK^{WT}, $JNK1^{-/-}$, or $JNK2^{-/-}$ MEFs (2×10^5) were seeded into 60-mm dishes and cultured for 16 h at 37 °C, 5% CO_2. Cells were

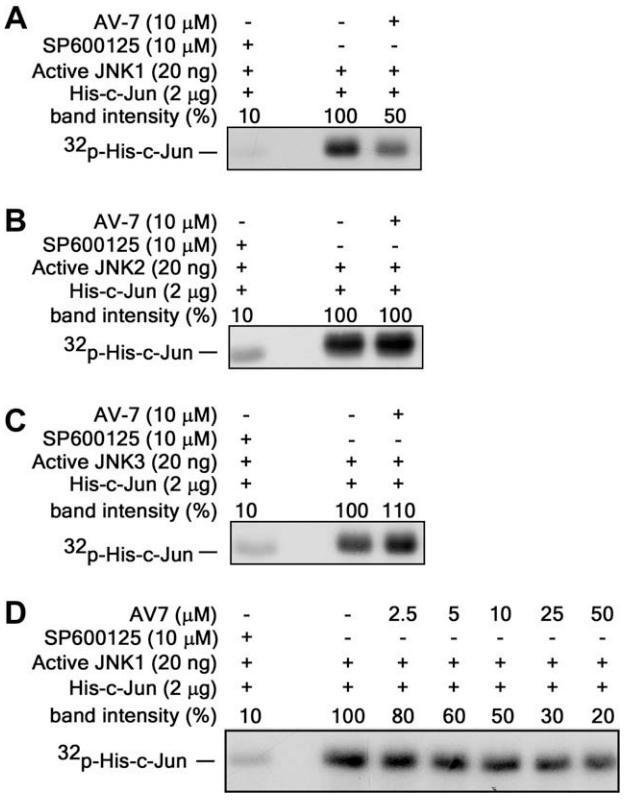

Fig. 2. AV-7 inhibits JNK1, but not JNK2 or JNK3 in vitro. (A–C) Respective in vitro kinase assays were conducted using 2 μg of a His-c-Jun bacterial-expressed protein, [γ-^{32}P]ATP, AV-7 compound (10 μM), and 20 ng of each commercially active JNK1, JNK2, or JNK3, respectively. The ^{32}P-labeled phosphorylated c-Jun was visualized by autoradiography. (D) An in vitro kinase assay was performed with different doses of AV-7 (2.5–50 μM) and the ^{32}P-labeled phosphorylated c-Jun was visualized by autoradiography.