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12 Arylstibonic acids that inhibit the DNA binding of five B-ZIP dimers

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ABSTRACT

Previously, we identified an arylstibonic acid, NSC13778 that specifically binds to the basic region of the C/EBPα B-ZIP domain and disrupts DNA binding. We now examine a panel of 14 additional arylstibonic acid derivatives of NSC13778 for their ability to inhibit the DNA binding of five B-ZIP dimers (c-Fos|JunD, VBP, C/EBPα, C/EBPβ, and CREB). They show various specificities at inhibiting the DNA binding of five B-ZIP domains. NSC13746 inhibits the DNA binding of C/EBPβ and CREB at 100 nM and promiscuously inhibiting the DNA binding of all five proteins in the 1 μM range. Dialysis experiments indicate that NSC 13746 binding to the B-ZIP domain is reversible. Thermal denaturation studies indicate that NSC13746 binds the B-ZIP domain. Some compounds specifically inhibit DNA binding, with VBP and c-Fos|JunD being most easily disrupted. These compounds inhibit, with similar specificities to the pure B-ZIP domains, the DNA binding of nuclear extract to the AP1 DNA sequence but no inhibition is observed to SP1 containing oligonucleotide. Transient transfection assays indicate that NSC13746 can inhibit the TPA induced activation of two B-ZIP dependent reporters. These experiments suggest that arylstibonic acids are promising leads for inhibiting the DNA binding of a group of B-ZIP proteins in cells.

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1. Introduction

The B-ZIP domain dimerizes and binds to DNA in a sequence specific manner to regulate gene transcription. These transcription factors occur in approximately 55 genes in the human genome that have been grouped into 13 families with similar dimerization properties (Vinson et al., 2002; Newman and Keating, 2003). Many of these B-ZIP domain containing genes are immediate early genes (IEGs) that are induced in a variety of pathologies including cancer (Darnell, 2002). For example, c-Jun, a member of the AP1 family of B-ZIP transcription factors, was the first nuclear transcription factor described as an oncogene (Vogt et al., 1987; Vogt, 2001). However, no inhibitors of the DNA binding of these proteins are in clinical trials, and only one compound has been described that can bind to the B-ZIP domain to inhibit DNA binding (Rishi et al., 2005).

Targeting the DNA binding of the B-ZIP motif is a formidable task because of (a) the absence of any enzymatic pocket that may be targeted specifically and (b) the presence of a large and shallow interface (1000–3000 Å) that gets buried when B-ZIP proteins form homo- or heterodimers (Lo Conte et al., 1999; Jones and Thornton, 1996; Hu et al., 2000). Potentially, binding of a B-ZIP protein to DNA can be disrupted in two ways: (1) targeting molecules that bind to the major or minor grooves of DNA causing changes in its topology and steric hindrances such that the transcription factor cannot bind to DNA (Olenyuk et al., 2004; Dervan, 2001); or (2) targeting the B-ZIP domain itself. The B-ZIP class of transcription factors binds to the major groove of DNA as homo- and/or heterodimers via their basic DNA binding domain. The dimerization domains of the B-HLH-ZIP domains, which are structurally similar to the B-ZIP domain, have been successfully targeted. A synthetic peptidomimetic that inhibits the protein–protein interaction between Myc and Max heterodimer has been described (Berg et al., 2002). But due to poor cell permeability and high susceptibility to degradation, these peptides are poor pharmacological agents.

The potential clinical value of inhibiting the DNA binding of specific B-ZIP protein families has been demonstrated using transgenic mice (Oh et al., 2007; Gerdes et al., 2006). Previously, we designed potent dominant negatives for various B-ZIP and B-HLH-ZIP transcription factor families that dimerize with their wild type protein partners with high affinity and inhibit DNA binding (Krylov et al., 1995; Ahn et al., 1998; Moll et al., 2000; Rishi et al., 2004). These dominant negative proteins have been expressed in transgenic mice and shown to inhibit and/or regress papillomas in skin. For example, expression in the mouse epidermis of A-C/EBP, a dominant negative that inhibits the DNA binding of all C/EBP

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family members, prevents papilloma formation and can cause established papillomas to regress if expressed after papilloma formation (Oh et al., 2007). Expression in the mouse epidermis of A-Fos, a dominant negative that inhibits AP1 DNA binding, converts papillomas into benign sebaceous adenomas that are not able to convert into carcinomas (Gerdes et al., 2006). Additionally, inhibiting CREB by expressing A-CREB is able to prevent papilloma formation (Rozenberg et al., 2009). Together, these results suggest that inhibition of DNA-binding activity of B-ZIP transcription factors offers a promising molecular target to develop an effective anti-cancer therapy.

In a previous study, we used a high-throughput fluorescence anisotropy screen and three secondary screens to identify small-molecule inhibitors that disrupt the DNA binding of the B-ZIP domains (Rishi et al., 2005). From a diverse set of 1990 molecules, an arylstibonic acid, NSC13778, containing an antimony element, was identified that preferentially binds the C/EBPα basic region and inhibits DNA binding. Here we report the effect of 14 arylstibonic acid derivatives of the lead compound NSC13778 on the DNA-binding activity of five B-ZIP dimers: c-Fos|JunD (AP1), VBP, C/EBPα, C/EBPβ, and CREB. In the present study, two *in vitro* and an *in vivo* assay were used to characterize these compounds. Electrophoretic Mobility Shift Assay (EMSA) was used to screen 14 derivatives of NSC13778 for their ability to inhibit B-ZIP–DNA binding in a dose-dependent manner. The two water-soluble compounds that were positive by EMSA were further characterized using isothermal circular dichroism (CD) spectroscopy and CD thermal denaturation studies. NSC13746 was also evaluated *in vivo* by a transient transfections luciferase reporter assay and shown to inhibit B-ZIP mediated transcriptional activity.

2. Materials and methods

2.1. Compounds

NSC13778 and 14 arylstibonic acid derivatives were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD.

2.2. Protein expression and purification

Amino acid sequences of B-ZIP domain proteins used in this study are given elsewhere: c-Fos (Olive et al., 1997), JunD (Rishi et al., 2005), VBP (Moll et al., 2000), C/EBPα and C/EBPβ (Krylov et al., 1995), CREB and its dominant negative A-CREB (Ahn et al., 1998). All recombinant proteins were expressed in *Escherichia coli* BL21(LysE) strain and purified as described previously (Rishi et al., 2004). Final protein purification step involved HPLC using Vydac C14 reverse phase column. A linear gradient from 0% to 100% acetonitrile containing 0.01% trifluoroacetic acid over 45 min with a flow rate of 1 ml/min was used to elute the proteins. All proteins have a 13 amino acid N-terminal φ10 epitope that was used for immuno detection of proteins in Western blots.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

28 mer oligonucleotides were purchased from Sigma–Genosys and were HPLC purified. Single strand oligonucleotide was end labeled with [γ^{32}P]ATP using T4 phage polynucleotide kinase. Labeled oligo was purified using a G-50 column (GE Healthcare, UK) according to manufacturer instructions and annealed to complementary unlabeled oligo resulting in radiolabeled double stranded DNA. Sequences of oligonucleotides used for EMSA experiments were: AP1: 5′-GTCAGTCAGAATGACTCATATCGGTCAG-3′; VBP: 5′-GTCAGTCAGATTACGTAATATCGGTCAG-3′; C/EBP: 5′-GTCAGTCAGATTGCGCAATATCGGTCAG-3′; CREB: 5′-GTCAGTCAGATGACGTCATATCGGTCAG-3′. Underlined nucleotides are the binding sequences for B-ZIP proteins. Protein–DNA interaction in presence and absence of a small-molecule was studied using EMSA. Six HPLC purified proteins, four homodimers (VBP, C/EBPα, C/EBPβ, CREB) and one heterodimer (c-Fos|JunD form AP1) were used in band shift experiments. Before EMSA protein stock solutions (10 μM) in CD buffer were heated to 50 °C for 5 min and cooled to room temperature. The heterodimer between c-Fos and JunD domains was generated by heating equimolar concentrations of c-Fos and JunD proteins at 65 °C in presence of 1 mM DTT for 15 min and cooling the mixture to room temperature. For EMSA, samples were prepared by mixing 10 nM dimer protein with the required concentration of small-molecule (0.1, 1.0 and 10 μM) in 15 μl gel shift reaction buffer (25 mM Tris; pH 8.0, 50 mM KCl, 0.5 mM EDTA, 2.5 mM DTT, 1% bovine serum albumin and 10% glycerol) and incubated for 5 min at 37 °C. 7 pM ^{32}P-radiolabeled double stranded oligonucleotide was added and final volume was made up to 20 μl with gel shift reaction buffer and equilibrated at 37 °C for 15 min. Finally, the mixture was incubated for additional 5 min at room temperature and DNA|protein complexes were resolved on a 7.5% native PAGE using 25 mM Tris–borate running buffer (pH 8.0) with 0.3 mM EDTA at 150 V potential (11 mA current) for 90 min. Gels were dried and autoradiographed after 18 h exposure using a Kodak MR X-ray film. The shifted bands represent DNA|protein complexes.

2.4. Dialysis experiments

2 μM CREB B-ZIP dimer protein in absence and presence of 100 μM NSC13746 was dialyzed against CD buffer for 24 h at 4 °C using a Slide-a-Layer dialysis tubing (Pierce) with a molecular weight cut off of 8000. Dialyzed samples without heating and after heating to 50 °C for 5 min were mixed with radioactive probe and ran on 7.5% native PAGE as described above. Gel was dried and exposed to X-ray film for 5 h and developed subsequently.

2.5. Nuclear extracts

Nuclear extracts were made from mouse liver (Gorski et al., 1986) with some modifications. Mouse liver (10–15 g) was homogenized in 30 ml of homogenization buffer (HB) (10 mM Hepes (pH 7.6), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% glycerol, and 2 M sucrose) using a Teflon-glass homogenizer. Fifteen strokes of homogenizer were enough to get 90% of cells lysed. 50 ml of HB buffer was added to make the final volume to 85 ml. 28 ml of homogenate was overlaid on 10 ml HB in three centrifuge tubes and spun at 24,000 rpm for 30 min at −2 °C. Individual pellets containing nuclei from three tubes were pooled and re-suspended in 50 ml of 9:1 v/v HB and glycerol, divided into three parts and layered on 10 ml of HB buffer in three tubes and centrifugation step was repeated. Pellets were pooled again and suspended in nuclei lysis buffer (10 mM Hepes (pH 7.9), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol). Nuclei lysis was enhanced by 10 strokes in a glass Dounce homogenizer kept on ice and incubated for 40 min. Final centrifugation was performed for 30 min at 35,000 rpm and 4 °C. The pellet was discarded and supernatant was aliquoted and stored at −70 °C. Protein concentration of nuclear extract was measured by Bradford method (Bio-Rad, CA). For EMSA 5 μg of nuclear extracts were incubated with or without compounds (37 °C for 15 min) in gel shift buffer that contained 10 mM Hepes (pH 8.0), 6% glycerol, 80 mM KCl, 0.05 mM EDTA, 1 mM $MgCl_2$ and 1 mM DTT. In addition, 1 μg of polydI–dC was added to each reaction mixture to inhibit non-specific binding. 70 pM of radiolabeled DNA was added, mixture was incubated at room temperature