ADH1, an N-cadherin inhibitor, evaluated in preclinical models of angiogenesis and androgen-independent prostate cancer

Haiqing Li, Douglas K. Price and William D. Figg

The conversion from E-cadherin to N-cadherin has been observed in several human cancer types, including prostate cancer, with more homogenous expression of N-cadherin detected in high-grade prostate tumors. N-cadherin, *in vitro*, has been shown to promote cell mobility, migration and invasion of several cancer cell lines, indicating the possibility of N-cadherin as a molecular target of cancer therapy. Herein, we examined the potential of an N-cadherin inhibitor, ADH1, in reducing tumor angiogenesis *ex vivo* and delaying tumor progression *in vivo*. Our data demonstrate that ADH1, at the dosages evaluated, does not display either antiangiogenic activity in a rat aortic ring assay or antitumor potential in a PC3 subcutaneous xenograft tumor model. We detecteded cytotoxic activity in human umbilical vein endothelial cells, PC3, and Tsu-Pr1 cells, when ADH1 exposure was evaluated at 500 μmol/l or above.
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Molecular Pharmacology Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Correspondence to William D. Figg, PharmD, National Cancer Institute, Building 10/Room 5A01, 9000 Rockville Pike, Bethesda, MD 20892, USA
Tel: +1 301 402 3623; fax: +1 301 402 8606;
e-mail: wdfigg@helix.nih.gov

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Introduction

It is now well known that prostate cancer is the most common cancer among American males [1]. For patients with relapsing or progressive disease after hormonal therapy, most current treatments are palliative and few regiments alter survival [2]. Thus, the urgency to discover new molecular targets and treatments for these patients is clearly recognized.

N-cadherin, like E-cadherin, is a member of the classic cadherins, a family of transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion [3,4]. Classic cadherins are composed of three segments: an extracellular domain that mediates homophilic interactions between neighboring cells [5,6], a transmembrane domain and a cytoplasmic domain linked to the actin cytoskeleton via different catenins [7]. The cadherin molecule binds directly to β-catenin and γ-catenin (plakoglobin), which in turn interact with the actin-binding α-catenin [8,9].

In several human cancer types, including melanoma, prostate and breast, a cadherin switch – a conversion from E-cadherin into N-cadherin – has been observed [10–13]. A more homogenous expression of N-cadherin was detected in high-grade prostate tumors. Unlike E-cadherin, N-cadherin promotes cell mobility and migration. Its overexpression enhances the invasive capacity of bladder carcinoma cells [14], and induces cell migration, invasion and metastasis of breast cancer cells and melanoma cancer cells [15–17]. Furthermore, metastases derived from N-cadherin-expressing breast cancer cells continued to express both E-cadherin and N-cadherin at distant sites, indicating that N-cadherin can promote its metastatic effects even in the presence of E-cadherin [16]. In addition, N-cadherin was also detected in human endothelial cells and pericytes, and could play a role in angiogenesis [18]. Hence, N-cadherin may provide a new molecular target for therapeutic development, and N-cadherin inhibition may potentially inhibit tumor angiogenesis, decelerate cancer cell migration and delay metastasis.

ADH1, a short cyclic peptide, N-Ac-CHAVC-NH_2, has shown anti-N-cadherin activity *in vitro* by inhibiting N-cadherin-stimulated neurite outgrowth [19] and inducing apoptosis in cultured endothelial cells [20]. We examined the capability of ADH1 in inhibiting angiogenesis and tumor progression *in vivo*. The expression of N-cadherin in endothelial cells and neighboring smooth muscle cells in rat aorta [21] justified using a rat aortic ring assay to test the antiangiogenic function of ADH1. In addition, we tested the antitumor activity of ADH1 using a human prostate cancer xenograft model. The PC3 prostate cancer cell line used in this model is well established and expresses N-cadherin *in vitro* [22]. Our data demonstrate that ADH1 does not display either anti-angiogenic activity in a rat aortic ring assay *ex vivo* or

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antitumor potential in a PC3 xenograft tumor model *in vivo* at the dosages we used.

Methods

Drug and cell lines

ADH1 was obtained from Adherex Technologies (Durham, North Carolina, USA). Human prostate cancer cell line PC3 was purchased from the American Type Culture Collection (Manassas, Virginia, USA). TSU-Pr1 was kindly provided by Dr Seong-Jin Kim (National Cancer Institute, Bethesda, Maryland, USA). Human umbilical vein endothelial cells (HUVECs) was purchased from Cambrex (Rockland, Massachusetts, USA).

Rat aortic ring assay

A rat aortic ring assay was performed as described in [23]. In summary, 12-well tissue culture plates were coated with 250 µl of Matrigel (Becton-Dickinson, Bedford, Massachusetts, USA), and allowed to gel for 30 min at 37°C and 5% CO_2. Thoracic aortas were excised from 8 to 10-week-old male Sprague-Dawley rats. After careful removal of fibroadipose tissues, the aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells and covered with an additional 250 µl of Matrigel. After the second layer of Matrigel had set, the rings were incubated with EGM-II (Clonetics, San Diego, California, USA) and endothelial cell growth factors provided as the EGM-II Bulletkit (Clonetics) overnight. The culture medium was subsequently changed to EGM-II, supplemented with 2% fetal bovine serum, 0.25 µg/ml amphotericin B and 10 µg/ml gentamicin. Aortic rings were treated daily with either the vehicle (phosphate-buffered saline), carboxyamido-triazole (CAI) (12 µg/ml) or ADH1 (10–200 µmol/l, duplicate) for 4 days and photographed on the 5th day using a $\times 2.5$ objective. CAI, a known antiangiogenic agent, was used as positive control. Experiments were repeated five times using aortas from five different rats. The area of angiogenic sprouting was quantified using Adobe PhotoShop [23].

Human prostate cancer xenograft model

All animal experiments were carried out in accordance with institutional guidelines for animal welfare. PC3 (1×10^6) cells were injected subcutaneously into 5- to 6-week-old male severely combined immunodeficient mice. When tumor volume reached around 150–200 mm^3, animals were randomized into two groups ($n = 5$ each). Each group was treated with intraperitoneal bolus injections of either the drug vehicle (saline) or ADH1 (200 mg/kg) 5 days a week for 4 weeks. Tumors were measured with a caliper three times a week and their volumes were calculated using the formula $\pi/6ab^2$, where a is the longest dimension of the tumor and b is the width [24]. Before euthanasia, the mice were anesthetized with 2% isofluorane. Tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Additional tumor tissues were harvested, snap-frozen in liquid nitrogen, and subsequently processed for protein isolation and Western blot analysis.

Antibodies and Western blot

The following antibodies were used: N-cadherin, clone 32, 1 : 2500; α-catenin, clone 5, 1 : 250; actin, clone A4, 1 : 5000; all from BD Biosciences (San Diego, California, USA). Tumors were homogenized and isolated using modified radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl, pH7.4, 150 mmol/l NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 5 mmol/l ethylenediaminetetraacetic acid and 1% deoxycholate) with Complete protein inhibitor cocktail (Roche, Indianapolis, Indiana USA). Lysates were then tested for protein concentration using the bovine serum albumin method (Pierce, Rockford, Illinois, USA). For each sample, 30 µg protein was boiled for 3 min, loaded in a lane in a 4–12% gradient NuPAGE Novex Bis–Tris gel (Invitrogen, Carlsbad, California, USA), electrophoresed under reducing conditions and transferred onto nitrocellulose membrane. Membranes were blocked in 5% milk in TBS/T (Tris buffered saline with 0.1% Tween) and incubated with primary antibody overnight at 4°C. Blots were then washed, incubated with horseradish peroxidase-linked secondary antibody, washed again and then developed with an enhanced chemiluminescence kit (Amersham, Arlington Heights, Illinois, USA).

3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay

5000 PC3, TSU-Pr1 (a N-cadherin-positive bladder carcinoma cell line) and HUVECs were plated in each well of 96 well plates. After overnight culture, cells were treated with different concentrations of ADH1 (from 100 nmol to 1 mmol/l) for 48 h. Subsequently, a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay kit (American Type Culture Collection, Manassas Virgina, USA) was used to test ADH1 cytotoxicity *in vitro*.

Results

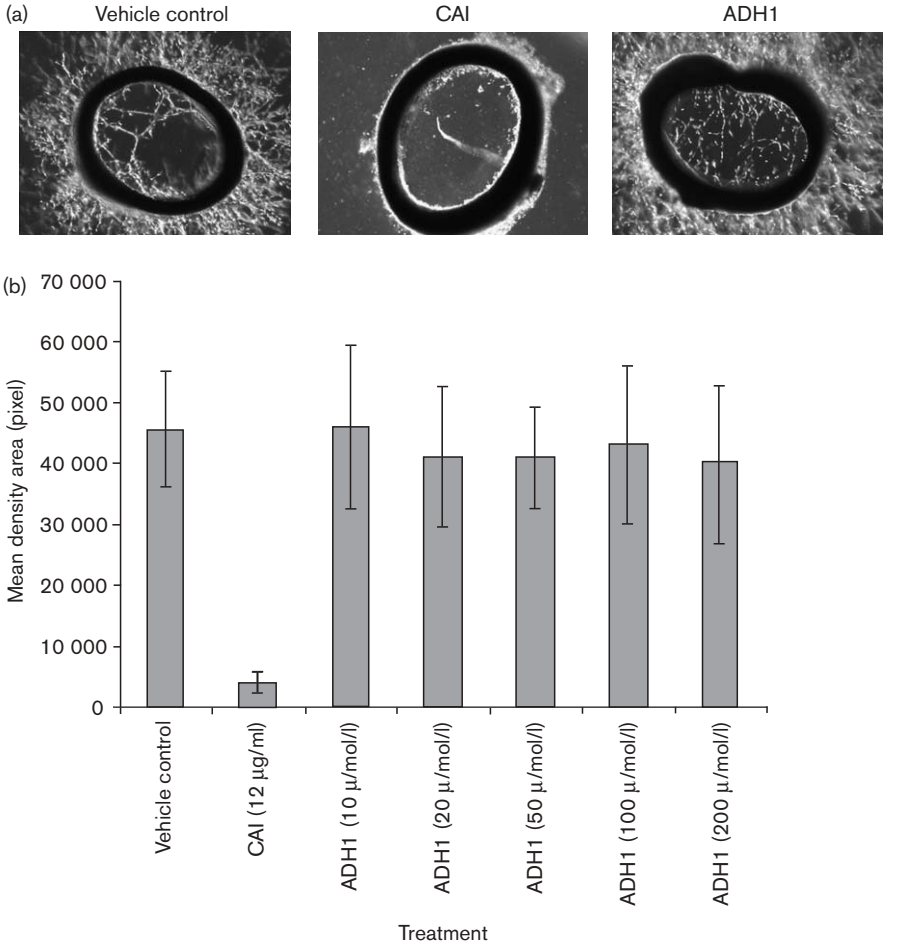
ADH1 treatment on rat aortic angiogenesis *ex vivo*

As shown in Fig. 1a, CAI, a known antiangiogenic agent, dramatically suppressed microvessel outgrowth from rat aortic rings. Angiogenesis was not blocked, however, by ADH1 at any concentration from 10 to 200 µmol/l ($n = 10$). The area of angiogenic sprouting, reported in pixels, was quantified using Adobe PhotoShop and the result is shown in Fig. 1b.

The expression of N-cadherin and α-catenin in prostate tumors

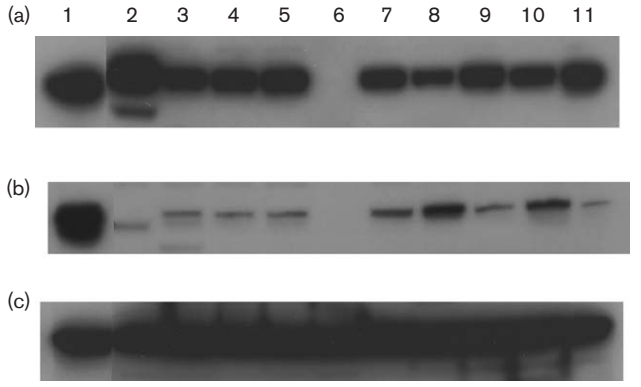
The expression of N-cadherin was confirmed in eight out of nine tested PC3 xenograft tumors (Fig. 2a), indicating the target for ADH1 was available. α-Catenin was detectable in most tumors (Fig. 2b), albeit at a low level, raising the question of the quantity of cadherin–catenin

Fig. 1



(a) Representative images of rat aortic rings treated with the vehicle control (phosphate-buffered saline), carboxyamido-triazole (CAI) or ADH1. (b) Bar graphs of the total microvessel outgrowth area measured as numbers of pixels in response to the vehicle control ($n=5$), CAI (12 μg/ml, $n=5$) or ADH1 (from 10 to 200 μmol/l, $n=10$ for each concentration).

Fig. 2



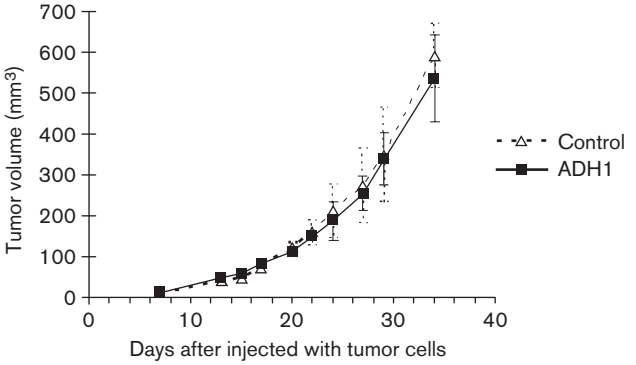
The expression of N-cadherin and α-catenin in cultured PC3 cells and PC3 tumors. Lane 1, mouse cerebellum (a positive control); lane 2, PC3 cells; lanes 3–11, PC3 xenograft tumors. (a) N-cadherin. (b) α-catenin. (c) Actin expression in cerebellum, PC3 cells and PC3 xenograft tumors.

complex *in vivo*. Actin staining was used to show the amount of protein loaded (Fig. 2c).

ADH1 treatment on N-cadherin-positive prostate tumor growth *in vivo*

When PC3 xenograft tumors reached around 150–200 mm^3 in volume, mice were randomized and treated with i.p. bolus injections of either the drug vehicle (saline) or ADH1 (200 mg/kg) 5 days a week for 4 weeks. No significant difference in tumor growth was observed between the N-cadherin-negative tumor and the N-cadherin-positive tumors (data not shown). Using data from N-cadherin-positive PC3 tumors, ADH1 did not inhibit PC3 tumor growth at a detectable level, compared with the vehicle control (Fig. 3). A similar result was observed when all the prostate tumors were included (data not shown). No phenotype difference was observed between tumors treated with ADH1 and those with control.

Fig. 3



ADH1 treatment did not delay the growth of N-cadherin-positive PC3 tumors, compared to vehicle control ($n=4$ for each treatment).

ADH1 treatment on PC3, TSU-Pr1 and human umbilical vein endothelial cells *in vitro*

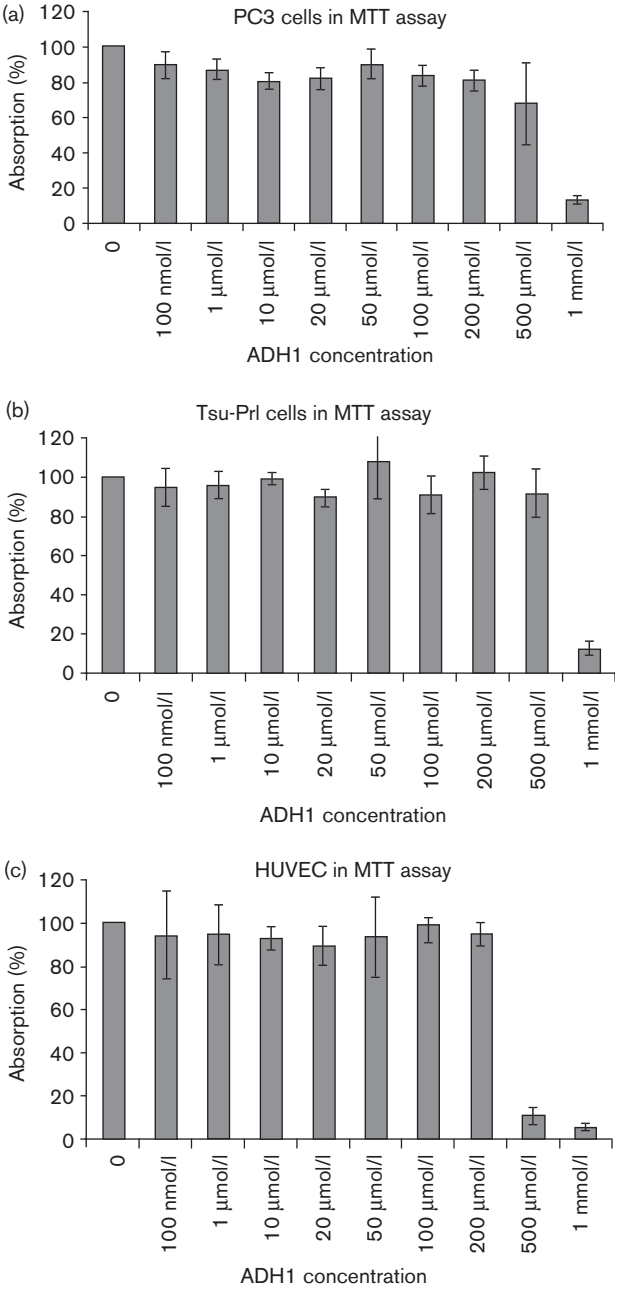
After 48 h treatment, only the highest tested concentration of ADH1 (1 mmol/l) inhibited cell growth in either PC3 (Fig. 4a) or TSU-Pr1 cells (Fig. 4b). HUVECs were more sensitive to ADH1 treatment, exhibiting cell death at 500 μmol/l (Fig. 4c).

Discussion

The expression pattern of N-cadherin suggests its possible roles in promoting both cancer cells and their environment [12,13]. We tested the potential of an N-cadherin inhibitor, ADH1, in inhibiting either tumor progression or tumor angiogenesis *in vivo*.

To test the antiangiogenic activity of ADH1 *ex vivo*, we used a rat aortic ring assay. A sustained concentration of 200 μmol/l ADH1 failed to reduce the microvessel outgrowth of rat aorta. ADH1 at a higher concentration (500 μmol/l) induced HUVEC death, indicating an anti-N-cadherin therapy may still be useful as an antiangiogenic tool. An obvious obstacle in developing ADH1 as an angiogenic inhibitor is whether this high concentration is clinically achievable. Recent reports from 2006 ASCO Annual Meeting showed that in one clinical trial, up to 148 μg/ml C_{max} was possible and ADH-1 was generally well tolerated in these patients [25]. More recently, concentration peaks as high as 400 μmol/l (273.6 μg/ml in salt form) have been safely achieved in patients (personal communication from Adherex Technologies Inc.). Nonetheless, we do not know the full exposure from these limited data.

Fig. 4



ADH1 treatment only affected cell growth at a high concentration (48 h treatment, $n=3$ for each concentration). (a) For PC3 cells, only 1 mmol/l ADH1 inhibited cell growth. (b) Same for TSU-Pr1 cells. (c) Human umbilical vein endothelial cells (HUVECs) had a higher sensitivity to ADH1 treatment. Cell death is detected at 500 μmol/l level. MTT, 3-(4, 5-dimethylthiazol-Zyl)-2, 5-diphenyl tetrazolium bromide.

A fundamental question is whether N-cadherin is required in tumor angiogenesis *in vivo*. Using an anti-N-cadherin antibody, N-cadherin has been shown to be important for smooth muscle cells to adhere to endothelial cells [21]. After arterial injury in a rat carotid artery model, N-cadherin was upregulated dramatically and transiently to enhance smooth muscle cell migration during wound repairing [26]. This emphasizes the importance of N-cadherin-mediated adhesion, a crucial step for blood vessel maturation. Tumor blood vessels,

however, are notoriously leaky immature vessels and loosely covered with pericytes. Although N-cadherin plays an important role in vessel maturation and pericyte adherence in physiological angiogenesis, its function may be lost on tumor neovasculature. Hence, the necessity of N-cadherin in tumor vasculature warrants further investigation.

In patients with prostate cancer, the reports of positive N-cadherin expression in cancer cells have been quite variable, from 5 to 60% of primary tumors [10,27]. The pattern of N-cadherin and E-cadherin expression is complementary, with more homogeneous N-cadherin detected in high-grade tumors [10]. Although the role of N-cadherin in enhancing migration, invasion and/or metastasis has been demonstrated in cultured cancer cells, there is little understanding as how N-cadherin functions *in vivo*, in the presence or absence of E-cadherin. *In vitro*, N-cadherin transfection in squamous carcinoma cells has resulted in downregulation of E-cadherin, while antisense N-cadherin induced E-cadherin expression [28]. Nevertheless, it is unknown to what extent, if any, that N-cadherin regulates E-cadherin expression *in vivo*. In recent years, with the discovery of β-catenin's role in the Wnt signal pathway [29,30], the importance of E-cadherin to regulate the β-catenin pool has been widely noted. It is not clear, however, whether the N-cadherin has a similar function. The downstream factors of the N-cadherin pathway are also poorly understood. Although α-, β- and γ-catenin have been shown to colocalize with N-cadherin, whether the α-catenin–actin cytoskeleton network is required for N-cadherin function has not been clearly defined.

The failure to impede PC3 xenograft tumor growth by ADH1 in our study emphasizes the complicated relationships within the cadherin–catenin family *in vivo*, and illustrates the urgency to understand the molecular mechanism of the N-cadherin pathway. The similar growth in N-cadherin-negative tumors and N-cadherin-positive tumors, as well as the ineffectiveness of ADH1 treatment, indicates that N-cadherin may not be a decisive factor in these PC3 xenograft tumors. It is possible that in PC3 prostate cancer cells, the function of N-cadherin may be limited by the low level of α-catenin protein. In other N-cadherin-positive prostate cancer cell lines, such as PPC-1 and ALVA-31, α-catenin protein is absent [22]. This differs from in N-cadherin-negative cell lines, DU-145 and LNCaP, in which α-catenin proteins are abundant [22]. However, even in a cell line with both N-cadherin and α-catenin positive, such as TSU-Pr1. Here, we need to emphasise that TSU-Pr1 is positive for both protein expressions. Even in an N-cadherin, however, α-catenin-positive cancer cell line such as TSU-Pr1 [22], only high concentrations of ADH1 (1 mmol/l) inhibited cell growth, indicating the real problem may still lie in the required high concentration for ADH1 activity, and the capability to achieve it *in vivo*. In previous studies in prostate cancer patients, 20–40% of tumors showed heterogeneous or negative α-catenin expression, and it was highly correlated with abnormal E-cadherin expression and a poor prognosis [31–34]. If an α-catenin–actin cytoskeleton link is essential for N-cadherin function, increased N-cadherin expression may not be important in patients with reduced α-catenins. Subsequently, anti-N-cadherin treatments may not be fruitful in these patients. Similarly, aberrant β-catenin or γ-catenin expression is also common, occurring in 10–40% of prostate cancer patients [27,33]. Hence, a thorough investigation is necessary to illuminate the role of N-cadherin and the usefulness of anti-N-cadherin therapy in cancer patients with abnormal catenin expression. Clearly, without a better understanding of the N-cadherin pathway and cadherin–catenin complex, it will be difficult to predict whether N-cadherin can serve as a legitimate target to develop new cancer therapeutics.

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