

## Cyclic Hydroxamic Acid Inhibitors of Prostate Cancer Cell Growth: Selectivity and Structure Activity relationships

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**BACKGROUND.** Clinical symptoms of prostatitis, prostatodynia, and benign prostatic hyperplasia are relieved by the pollen extract cernilton, and the water-soluble fraction of this extract selectively inhibits growth of some prostate cancer cells. A cyclic hydroxamic acid, DIBOA, has been isolated from this extract and mimics its cell growth-inhibitory properties, but the specificity of DIBOA for inhibition of prostate cell growth has not been reported.

**METHODS.** The in vitro growth inhibitory effects of DIBOA and nine structurally related compounds on DU-145 prostate cancer cells, MCF-7 breast cancer cells, and COS-7 monkey kidney cells were determined by treatment of the cells with various concentrations of the compounds for 2-6 days.

**RESULTS.** The compounds exhibited a wide range of potencies, but none of them exhibited selective inhibition of DU-145 cell growth. MCF-7 cells were more sensitive to DIBOA than either DU-145 cells or COS-7 cells. 3,4-dihydroquinoline-2 (1H)-one, compound (4), and 1-hydroxy-6-chloro-3,4-dihydroquinolin-2 (1H)-one, compound (7), selectively inhibited MCF-7 cell growth at a concentration of 10 µg/ml. 1-hydroxy-3,4-dihydroquinolin-2 (1H)-one, compound (3), and compound 7 were the most potent inhibitors of DU-145 cell growth. Treatment of DU-145 cells with 3 (100 µg/ml) substantially decreased the number of viable cells within 2 days, and no viable cells remained in the culture by day 4.

**CONCLUSIONS.** It is unlikely that DIBOA, compound (1), is responsible for the selective growth inhibition of prostate cancer cells by the water-soluble fraction of the pollen extract cernilton. Cell morphology results indicate that the growth-inhibitory effects of DIBOA and structurally related agents on DU-145 cells are due to their ability to cause cell death. *Prostate* 34:92-99, 1998. © 1998 Wiley-Liss, Inc.

**KEY WORDS:** hydroxamic acids; cancer; prostate; breast

### INTRODUCTION

The development of pharmacological agents for treatment of prostate cancer is a continuing challenge to biomedical research. A cyclic hydroxamic acid, 2,4-dihydroxy-2H, 1,4-benzoxazin-3 (4H)-one (DIBOA, 1), was reported recently to inhibit growth of the DU-145 human prostate cancer cell line [1,2].

DIBOA (1) was isolated from the water-soluble fraction of the pollen extract, Cernilton, which has been shown to cause symptomatic improvement in patients with benign prostatic hyperplasia, chronic prostatitis, and prostatodynia [3,4].

The water-soluble fraction (T60) of cernilton exhibited striking selectivity for inhibition of the growth of human prostate cancer cell lines when tested in vitro. Larynx, liver, bladder, testis, and breast cancer cell lines were not inhibited by T-60, but the hormone-independent prostate cancer cell line DU-145 was very sensitive to the extract. Two hormone-dependent prostate cancer cell lines were less sensitive than DU-145 to the growth inhibitory effects of T-60 [5]. The two reports of the inhibitory effects of DIBOA (1) on DU-145 did not include data obtained

with other cell lines [1,2]. We describe herein an investigation of the effects of DIBOA and several structurally related agents on the growth of DU-145 and two other cell lines. The results of this study demonstrate that these compounds are inhibitory not only to prostate cancer cells, but also to MCF-7 breast cancer cells and COS-7 cells. The structure of the agents are shown in Figure 1.

**Fig. 1.** Chemical structure of DIBOA (**1**) and nine structural evaluated for growth- inhibitory effects in this study. Chemical names; 2, 4-dihydroxy-2H-1, 4-benzoxazin-3 (4H)-one (**1**); 4-hydroxy-2H-1, 4-benzoxazine-3-one (**2**); 1-hydroxy-3, 4-dihydroquinolin-2 (1H)-one (**3**); 3, 4-dihydro-2 (1H)-quinolinone (**4**); 1-hydroxy-6-methoxy-3, 4-dihydroquinoline-2(1H)-one (**5**); 1, 6-dimethoxy-3, 4-dihydroquinolin-2(1H)-one (**6**); 1-hydroxy-6-chloro-3, 4-dihydroquinolin-2(1H)-one (**7**); 1, 3-dihydroindol-2-one (**8**); 1-hydroxy-1, 3-dihydroindol-2-one (**9**); 1, 3-dihydroxy-1, 3-dihydroindol-2-one (**10**).

## MATERIALS AND METHODS

### Chemicals

DIBOA (**1**) was synthesized according to the published method [6]. Compounds 2-10 were prepared by standard synthetic procedures. The structure and purity of each compound were verified by nuclear magnetic resonance spectrometry, thin layer chromatography, infrared spectroscopy, and elemental analysis.

### Cell Lines

Du-145 cells are an androgen-insensitive human prostate cell line derived from a brain metastasis of prostate cancer and were used in this study to model the response of prostate carcinoma cells to hydroxamic acids [7]. MCF-7 cells are a human breast cancer cell line derived from a patient with metastatic mammary carcinoma [8]. COS-7 cells are an SV40 transformed cell line derived from simian CV1 cells [9]. MCF-7 and COS-7 cells were included to determine the degree of prostate specificity in the action of the hydroxamic acids. DU-145 cells and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and the MCF-7 cells were a gift from Dr. Norman Sladek, University of Minnesota.

### Growth Assays

Cells were plated at a density of 25,000 cells/ well in 24-well plates with RPMI media, supplemented with 4% FBS. Stock solutions of all compounds were prepared in DMSO (10mg/ml) and dilutions were made so that all cell media received the same DMSO exposure (1%). In control experiments, exposure to 1% DMSO in the culture media was shown to have no effect on cell growth for any of the cell types used in

these studies. The compound containing media were changed every 2 days. Treatments were carried out in triplicate, and each experiment was carried out at least twice. For cell enumeration, cells were trypsinized into a single-cell suspension and counted in an automated Coulter counter (Coulter Electronics, Hialeah, FL).

## RESULTS

**Time Course and Dose-Response Studies With DIBOA 1** The data shown in Figure 2 represents the effect of a range of concentrations of **1** on three types of cells during a 6-day treatment period. DU-145 cells were sensitive to compound **1** only at the highest concentration of 100µg/ml (Fig. 2A,A'). Growth inhibition was apparent on the second day and cell growth was only 10% of the control after 6 days. By comparison, the MCF-7 breast cancer cells were much more sensitive to the growth-inhibitory effects of **1** than were the DU-145 human prostate cancer cells (Fig. 2B,B'). MCF-7 cell growth was inhibited moderately by 1µg/ml of **1**, but was markedly inhibited by the higher concentrations of 10µg/ml and 100µg/ml. After 6 days, no MCF-7 cells were visible in the wells that had been treated in Figure 3C,C' indicate that the COS-7 cells were more sensitive to **1** than the DU-145 cells, but were less sensitive MCF-7 cells. At both 10µg/ml and 100µg/ml, growth of the COS-7 cells was inhibited after 2 days. Similar to the results obtained with the MCF-7 cells, no COS-7 cells were present after 6 days of treatment with 100µg/ml of compound **1**.

### Effect of **1** and Structurally Related Analogues on DU-145, MCF-7, and COS-7 Cells

To compare the selectivity and potence of **1** with compounds 2-10 (Fig.1), the three types of cells were treated with two concentrations (10µg/ml and 100µg/ml) for a 4-day period as described in Materials and Methods. The results are shown in Figure 3. Treatment of the DU-145 cells with 10µg/ml of each of the 10 compounds resulted in relatively modest growth inhibition of 10-30% of control values (Fig.3A). Similarly, the inhibition of COS-7 cells was 40% or less under these treatment conditions. Striking differences, however, were observed in the growth-inhibitory potencies of the compounds when MCF-7 cells were treated for 4 days with 10µg/ml of each agent. DIBOA (**1**) itself was approximately twice as effective as an inhibitor of MCF-7 cell growth than as an inhibitor of either DU-145 or COS-7 cells (Fig. 3A). Compound **4**, which is quite structurally dissimilar to **1**, caused the same degree of growth inhibition (40%) of MCF-7 cells as **1**, and exhibited a similar degree of selectivity for inhibition of MCF-7 growth in comparison to its effects on the other two cell lines (Fig. 3). Compound **7** caused 80% inhibition of the growth of MCF-7 cells at 10µg/ml, but only 17% and 40% inhibition of DU-145 and COS-7 cells,

respectively. Thus, compounds 1, 4, and 7 exhibited a selective growth inhibitory effect on the MCF-7 breast cancer cells. The difference in the effectiveness of 1, 4, and 7 for inhibition of MCF-7 cells in comparison to the other two cell lines was statistically significant ( $p < 0.05$ ).

Treatment of the three types of cells for 4 days with 100  $\mu\text{g/ml}$  of compounds 1-10 resulted in a complete loss of selectivity for inhibition of MCF-7 cell growth by 1,4, and 7 (Fig.3B). At the 100  $\mu\text{g/ml}$  concentration, all 10 compounds inhibited growth of the three cell types, and apparent selectivity was exhibited only by compound 4, which caused 45% inhibition of the growth of DU-145 cells, but 83% inhibition of both MCF-7 and COS-7 cells (Fig. 3B). The weakest inhibitors were compounds 8 and 9.

### Growth Inhibition of DU-145 Cells: Dose-Response Comparison

For the purpose of comparing the DU-145 growth inhibition potency of 1 with that of several of its structural analogues, compounds 1-4 and 6-8 were studied at concentrations of 10, 25, 50, and 100  $\mu\text{g/ml}$  over a 4-day treatment period (Fig.4). As shown in Figure 3, compounds 1,3, and 7 appeared to exhibit similar effectiveness against DU-145 cells at concentrations of 10 and 100  $\mu\text{g/ml}$ . Figure 4, however, illustrates the significantly greater potency of 3 and 7 in comparison to 1,2,4,6, and 8, all of which exhibited similar dose-response curves. Compounds 3 and 7 inhibited cell growth by 70% and 80%, respectively, at 25  $\mu\text{g/ml}$ , whereas 1 caused less than 10% inhibition at this concentration. The differences in potency were maintained at the higher concentrations of 50 and 100  $\mu\text{g/ml}$ . Thus, both 3 and 7 were inhibitory to the growth of DU-145 human prostate cells than DIBOA (1). Neither 3 or 7, however, was selective for DU-145 cells, a characteristics they have in common with compound 1. Indeed, compound 7 appears to be a selective inhibitor of the growth of MCF-7 human breast cancer cells (Fig 3.)

**Fig. 2.** Effect of compound 1 on the growth of DU-145 cells (A,A'), MCF-7 cells (B,B'), and COS-7 cells (C,C'). Each cell type was treated with 0 ( $\blacklozenge$ ), 1 ( $\square$ ), 10 ( $\circ$ ) or 100 (O)  $\mu\text{g/ml}$  of compound 1. Cells were counted 2, 4, and 6 days after treatment was initiated. Data are expressed as total cells per well (A-C) and as percent of control (untreated) cells at each time point (A-C').

**Fig. 3.** The growth-inhibitory effect of compounds 1-10 on DU-145, MCF-7, and COS-7 cells. Cells were treated with the compounds at 10  $\mu\text{g/ml}$  (A) and 100  $\mu\text{g/ml}$  (B) and counted on the fourth day after initiation of treatment. Cell number is expressed as percent of control (untreated) cells. The degree of cell-growth inhibition was assessed for each compound compared to control with Student's t-test. \*Columns in A that represent a significant growth inhibition compared to control ( $P < 0.05$ ). \*\*Only column in B that

does not represent a significant decline in cell growth relative to control

**Fig. 4.** Growth-inhibitory dose response of representative compounds on DU-145 cells. Cells were treated with 0, 1, 10, 25, 50, or 100  $\mu\text{g/ml}$  of each compound. Cells were counted on day 4

### Effect of Compounds 1, 3, and 8 on DU-145 Cell Morphology

To characterize the effect of compounds with varying degrees of growth-inhibitory activity on DU-145 cells, morphology was analyzed 2 and 4 days after initiation of treatment with 100  $\mu\text{g/ml}$  of compounds 1,3, or 8 (Fig. 5). At 2 days of culture the control cells were approaching confluency, and reached confluency by 4 days of culture (Fig.5A,B). The control DU-145 cells were relatively small, polygonal-shaped cells that often exhibited cytoplasmic processes extending to make contact with neighboring cells. Numerous lysosomes and lipid droplets populated the cytoplasm, and the nuclei exhibited prominent nucleoli. The majority of the cells in the sample treated with compound 1 appeared identical to the control cells (Fig. 5C,D). However, at both 2 and 4 days of treatment some cells exhibited degenerative characteristics such as loss of attachment to the substratum and loss of distinct nuclear morphology. At day 4 the cells treated with compound 1 were not yet confluent, consistent with inhibition of cell growth compared to control. Compound 3 had dramatic effects of the DU-145 cells (Fig. 5E,F). On day 2 the majority of cells exhibited degenerative changes, but there were still viable cells present (Fig. 5E). The effect of compound 8 on cell morphology was essentially the same as that of compound 1 except that there were fewer degenerating cells, consistent with the less severe effect on cell growth. There was no morphologic evidence of cellular differentiation with treatment of any of these compounds.

## DISCUSSION

Cernilton, a pollen extract, exhibits clinical effectiveness in the treatment of benign prostatic hyperplasia and chronic prostatitis [3,4]. In vitro studies demonstrate that the relevant biological activity of cernilton resides in the water-soluble cernitin T-60 fraction rather than in the hydrophobic fraction and that the water-soluble fraction selectively inhibits the growth of DU-145 human prostate cancer cells, but does not inhibit MCF-7 human breast cancer cells [5]. DIBOA (1, Fig.1) was isolated from the water-soluble fraction of cernilton and exhibited growth-inhibitory action on DU-145 cells [1,2]. In contrast to the results reported from studies with the water-soluble fraction, the data shown in Figure 2 indicate that DIBOA (1) does not selectively inhibit the growth of DU-145 cells, but is rather a more potent inhibitor of the growth of MCF-7 human breast cancer cells. Thus, the reported selectivity of the water-

soluble cernitin T-60 fraction for inhibition of DU-145 cells is unlikely to be attributable to the action of DIBOA (1). Further, compound 1 effected inhibition of the growth of MCF-7 and COS-7 cells at 10 $\mu$ g/ml, a concentration which did not slow the growth of DU-145 cells. Thus, in the present studies, 1 was found to be a more effective growth inhibitor of both MCF-7 and COS-7 cells than of DU-145 cells and, at a concentration of 10 $\mu$ g/ml, exhibited selectivity for MCF-7 cells (Fig. 3). The selectivity was lost when a concentration of 100 $\mu$ g/ml of 1 was used (Fig.3). Although, this study was not designed to determine the mechanism whereby these hydroxamic acids inhibit cell growth, it is evident from the cell morphology data that DU-145 cells are killed by these compounds, and the extent of cell death seems to correlate with the degree of cell-growth inhibition. Whether or not the compounds also have an effect on the kinetics of cell division cannot be determined from these experiments.

One objective of this study was to obtain information about the structural requirements for inhibition of the growth of DU-145 cells by 1. The hydroxyl group in the 2 position of compound 1 renders the compound capable of undergoing ring opening under aqueous conditions to generate, successively, an  $\alpha$ -keto aldehyde and an isocyanate [10]. Both of the latter are reactive chemical species that can form covalent adducts with cellular constituents and contribute to inhibition of cell growth. Compound 2, however, which does not contain a 2-hydroxyl group and cannot undergo ring opening (Fig.1), was approximately equal in potency to 1 as an inhibitor of DU-145 growth (Fig.4). Further, compounds 3 and 7, which contain neither the 2-hydroxyl group nor the 1-oxygen atom of 1, were both more potent inhibitors of the growth of DU-145 cells than was 1 (Fig.4). Thus, if compounds 1-3 and 7 inhibit cell growth by a common molecular mechanism, the mechanism does not involve generation of reactive  $\alpha$ -keto aldehydes or isocyanates.

Cyclic hydroxamic acid analogues of 4 have been reported to exhibit antimicrobial activity, and compound 1, but not 4, was mutagenic to *Salmonella typhimurium* TA98 and TA100 [10-12]. Thus, the growth inhibitory effects of such agents are not unexpected. An unanticipated result of the present study, however, was the relatively potent and selective growth inhibitory effect of compound 7 on MCF-7 human breast cancer cells (Fig. 3).

Compounds 1 and 4 also exhibited selectivity for inhibition of MCF-7 cells and are not close structural analogues of 7. Compounds 1, 4, and 7 may warrant further investigation of their inhibitory actions and breast cancer cells.

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**Fig.5.** Effect of compounds 1, 3, and 8 on Du-145 cells were plated on 8-well microscope slides at the same density used in the cell growth assays. Cells were allowed to attach to the microscope slide for 24h before treatment with a compound. At 2 and 4 days after initiation of treatment, cell morphology was analyzed by Normarski differential-interference-contrast microscopy. Cells were viewed in culture media, without fixation, under coverslips. **A,C,E,G:** Cell morphology recorded on day 2, **B,D,F,H:** Cell morphology on day 4. Cells in A and B were not treated with any compound (control); cells in C and D were treated with 100 $\mu$ g/ml of compound 8. Magnification in general is 600x, while magnification of insets in B, D, F, and H is 1,000x. Cells treated with compound 1 begin to exhibit degenerative morphology (irregular shape and detachment from the substratum) and a noticeable decrease in cell number by day 4(D). Note the dramatic effect of compound 3 in cell

morphology, as well as cell number, at both 2 and 4 days (E and F0, respectively). The effect of compound **8** on cell morphology is intermediate between that of compound **1** and control.

