



## CANCER SUPPORT

GRAMINEX Flower Pollen Extract

# Flower Pollen Extract and its Effect on Cancer

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## Clear cell adenocarcinoma of the female urethra showing strong immunostaining for prostate-specific antigen

K. KAWANO, M. YANO, S. KITAHARA and K. YASUDA

*Department of Urology, Dokkyo University School of Medicine, Koshigaya Hospital, Saitama, Japan*

### Case report

A 49-year-old woman presented with the chief complaint of voiding difficulty. On digital vaginal examination, a walnut-sized mass was confirmed around the urethra. Abdominal ultrasonography showed a mass at the bladder neck (Fig. 1), and cystoscopy revealed the tumour protruding from the posterior urethral wall at the bladder neck. Subsequent bone scintigraphy, systematic CT and serum chemistry showed no metastasis. The patient underwent transurethral biopsy of the tumor, and the histological diagnosis was carcinoma composed of high-grade cancer cells. Under the diagnosis of an invasive but localized urethral tumor, total cystourethrectomy was performed, including anterior vaginal wall and pelvic lymph node dissection. An ileal conduit was chosen for urinary diversion. The anterior vaginal wall was intact, but the tumour invaded the mucosa and muscle layer of the bladder trigone. No lymph node metastasis was detected. The patient was free of disease a year after surgery. Microscopy of the tumour showed clear-cell type adenocarcinoma, most of which contained clearly vacuolated cytoplasm and pleomorphic nuclei (Fig. 2a). The histological diagnosis was mesonephric adenocarcinoma. Immunohistochemical staining, using the two-step indirect immunoperoxidase technique with antibodies to PSA (L-1838, Dako, Glostrup, Denmark), showed strong cytoplasmic reaction in the tumour cells (Fig. 2b). Serum PSA levels were not measured before surgery, but at the follow-up serum PSA levels were estimated several times (Tandem-R, Hybritech, San Diego, CA, USA); all were below the detectable limit (<0.2 ng/mL).

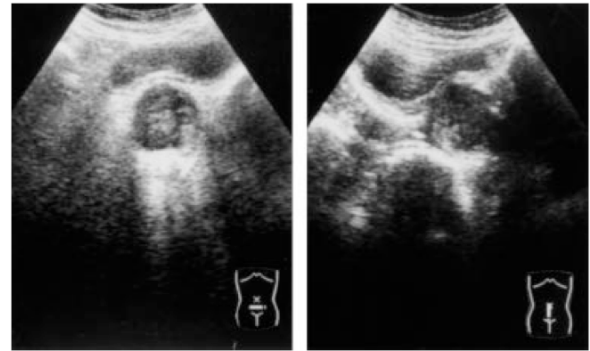
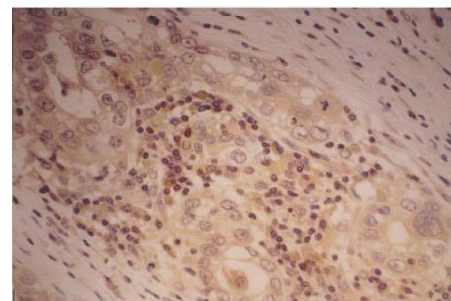
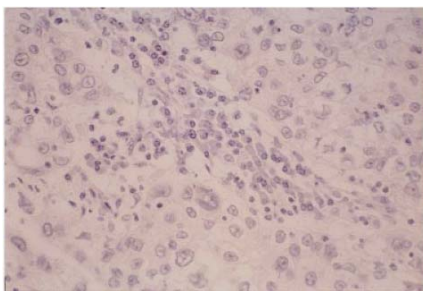


Fig. 1. Abdominal ultrasonograms showing the tumour at the bladder neck.

### Comment

The histogenetic derivation of clear cell adenocarcinoma in the female urethra remains controversial. Some authors suggested a mesonephric origin, from ultrastructural studies, and designated this tumour as a mesonephric adenocarcinoma, while Peven and Hidvegi [1] insisted on a Müllerian origin for this neoplasm, from an embryological perspective. From morphological and embryological findings, the female para-urethral ducts and glands have been considered to be homologous to the male prostate gland, and perhaps to the urogenital sinus. Furthermore, Pollen and Dreiling [2] strongly supported the homogeneity between the female paraurethral duct and male prostate gland on finding positive immunohistochemical staining using antibodies to PSA and PAP. They advocated that the tumour arises from the female para-urethral duct



**Fig. 2.** a, Sections of the tumour showing clear cell type adenocarcinoma (haematoxylin and eosin r400) and b, immunohistochemical staining for PSA showing a strong cytoplasmic reaction in the tumour (r600).

rather than embryonic remnants. Spencer et al. [3] and Zaviacic et al. [4] reported a neoplasm with a similar histological appearance and immunohistochemical characteristics as 'adenocarcinoma of Skene's para-urethral glands and ducts.' The present findings support the theory that the female clear cell adenocarcinoma arises from the para-urethral duct, as the present case was also positive immunohistochemically for PSA. However, there are variable terms for the neoplasm, e.g. mesonephroma, adenocarcinoma of Skene's para-urethral glands, and tumor of the female para-urethral duct. Therefore, the use of a more unified nomenclature is desirable. Clear cell adenocarcinoma of the female urethra is a comparatively rare entity; there are 42 cases of this neoplasm reported, including the present case, five of which had positive immunohistochemical staining for PSA. Only one was negative, but microscopy showed this tumour to be truly of para-urethral duct origin [5].

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## Authors

K. Kawano, MD, Urologist.  
M. Yano, MD, Urologist.  
S. Kitahara, MD, PhD, Associate Professor.  
K. Yasuda, MD, PhD, Professor.  
Correspondence: Department of Urology, Dokkyo University School of Medicine, Koshigaya Hospital, Saitama, Japan.

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

Kenneth P. Roberts,<sup>1</sup> Ramaswamy A. Iyer,<sup>2</sup> Girija Prasad,<sup>2</sup> Lee T. Liu,<sup>2</sup> Robert E. Lind,<sup>2</sup> and Patrick E. Hanna <sup>2,3\*</sup>

*1 Department of Urologic Surgery, University of Minnesota, Minneapolis, Minnesota*

*2 Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota*

*3 Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota*

**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 potency 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).

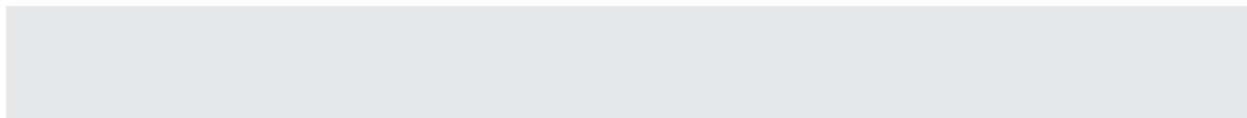
Compounds 1 and 4 also exhibited selectivity for inhibition of MCF-7 cells and are not close structural analogues of 7. Compounds 1, 4, and 7 may warrant further investigation of their inhibitory actions and breast cancer cells.

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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.



## The Secalosides, Novel Tumor Cell Growth Inhibitory Glycosides from a Pollen Extract

Jean-Claude Jaton,<sup>\*</sup>† Karen Roulin,† Keith Rose,† Francis M. Sirotnak,‡ Ari Lewenstein,§ Ge´rard Brunner,^ Catherine P. Fankhauser,^ and Ulrich Burger^

Department of Medical Biochemistry, University of Geneva, 1, rue Michel-Servet, CH-1211 Geneva-4, Switzerland, Laboratory of Molecular Therapeutics, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York 10021, Cerbios-Pharma S.A., 6917-Barbengo, Switzerland, and Department of Organic Chemistry, University of Geneva, 30, quai Ernest Ansermet, CH-1211 Geneva-4, Switzerland

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The pollen of rye (*Secale cereale*) was shown to contain a biologically highly active family of glycosides called the secalosides. Secalosides A and B (**1**), both of molecular formula C<sub>46</sub>H<sub>51</sub>NO<sub>24</sub>, were found to be epimeric esters of (2-oxo-3-indolyl)acetic acid (**4**). They are made up, in addition to this heterocyclic aglycon I (**4**), of three hexose building blocks and a carbocyclic aglycon II, which is an indan-derived dicarboxylic acid (**5**). In aqueous solution, secalosides A and B interchanged by epimerization at the chiral center of **4**. A further epimeric pair, secalosides C and D (**2**), contain one additional glucose building block. Secalosides A and B, the racemic aglycon I (**4**), and 2-oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid (**3**), which results from **4** by hydrolytic rearrangement, exhibited significant antitumor activity against S180 sarcoma *in vivo*. IC<sub>50</sub> values obtained were about 5 µg/mouse for the secalosides and 1 µg/mouse for **3** and **4**.

Cernilton is an extract from rye pollen that is produced commercially and used as a phytotherapeutic drug in Europe. This highly heterogeneous extract comprises two main components, namely, the hydrophilic Cernitin T-60 fraction and the hydrophobic Cernitin GBX1 fraction, both of which are devoid of allergens and high-molecular weight substances.

<sup>1</sup>Clinical trials have shown the efficacy of Cernilton in the treatment of benign prostatic hyperplasia<sup>1</sup> and chronic prostatitis<sup>2</sup> without reported side effects. Cernitin T-60 was found to be the active fraction *in vivo*, as it inhibited the growth of prostate cancer cells.<sup>3,4</sup> Recent reports have described the isolation and characterization of a prostate growth inhibitory substance in the Cernitin T-60 fraction, namely, the cyclic hydroxamic acid, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA).<sup>5,6</sup>

In another series of experiments carried out *in vivo*, the antitumor potential of Cernitin T-60 was

demonstrated on Lewis lung carcinoma implanted extraperitoneally in syngeneic mice.<sup>7</sup> Similar results were obtained with mice implanted with S180 sarcoma. The survival time of mice treated with Cernitin T-60 exceeded 100% with the percentage of cured mice being 20-40%. However, synthetic DIBOA, although highly cytotoxic against a large variety of human tumor cell lines in culture, was found not to be inhibitory in *in vivo* Lewis lung carcinoma or S180 sarcoma implanted in mice (Sirotnak, F. M. Unpublished data).

These encouraging data prompted us to identify the ingredients present in Cernitin T-60 that are active *in vivo*. We now report on the isolation and biological evaluation of a family of glycosides shown to be responsible for the *in vivo* biological activity of Cernitin T-60. We propose that these compounds be called secalosides A-D by virtue of their origin from rye pollen (*Secale cereale* L., Gramineae). The structures of the constituent aglycons as well as a partial gross structure for intact secalosides are presented herein. It is considered that one of these aglycons is responsible for most of the biological efficacy of the

<sup>1</sup> \* To whom correspondence should be addressed. Tel.: 0041-22-7025495. Fax: 0041-22-3473334. E-mail: [Jaton@cmu.unige.ch](mailto:Jaton@cmu.unige.ch). † Department of Medical Biochemistry, University of Geneva. ‡ Memorial Sloan Kettering Cancer Center. § Cerbios-Pharma S.A. † Department of Organic Chemistry, University of Geneva. x Abstract published in *Advance ACS Abstracts*, April 1, 1997. **356 J. Nat. Prod.** **1997**, *60*, 356-360 S0163-3864(96)00655-6 CCC: \$14.00 © 1997 American Chemical Society and American Society of Pharmacognogy

intact glycosides. The complete structure elucidation of secalosides A and B is the subject of the following paper in this issue by Jaun *et al.*<sup>8</sup>

## Results and Discussion

The strategy for the isolation of active compounds present in the Cernitin T-60 extract is summarized in Figure 1. A functional *in vivo* assay was used to monitor the biological activity through the various fractionation steps (see the Experimental Section). The compounds from peaks 4.51 (secaloside A) and 4.52 (secaloside B) were found to be equally active *in vivo*. NMR spectroscopy in water and HPLC unveiled a slow interconversion of the two compounds suggesting an equilibrium between isomers. Similarly, another pair of isomers (peaks 3.51 and 3.52), which were called secalosides C and D, was isolated from the initial fraction 3 of the gel filtration. This biologically active pair was found to be structurally related to the secalosides A and B (*vide infra*).

FAB-MS gave an identical molecular weight of 1001 Da for secalosides A and B, while that of both the secalosides C and D was 1163 Da. The UV spectra of both compound pairs, recorded in water, were virtually indistinguishable with maxima at 280 nm and minima at 265 nm. Chemical microdeterminations<sup>9,10</sup> suggested that none of the secalosides contained an R-amino acid, a hydroxamic acid, or a fatty acid as a constituent. They all stained positively for phenolic compounds and for carbohydrates.<sup>9,10</sup> Acid hydrolysis in 1 N HCl for 6 h at 100 °C qualitatively unveiled the presence of D glucose in both pairs of secalosides, as determined by a specific enzymatic assay. In the enzymatic tests, none of the various R- and  $\alpha$ -glycosidases (see the Experimental Section) hydrolyzed hexoses from secalosides A or B. In contrast, when the mixture of secalosides C and D was digested for 16 h at 37 °C in the presence of R-glucosidase in phosphate buffer at pH 6.8, D-glucose was released. Quantitative measurements indicated that 0.85 and 1.05 mol of glucose were released per mole of secaloside C and D, respectively. The digest was further analyzed by RP-HPLC, which indicated that the R-glucosidase treatment yielded a new pair of compounds eluting with retention times indistinguishable from those of the intact secalosides A and B. This result is consistent with the difference of 162 mass units between the molecular weight of secalosides A and B and secalosides C and D.

The products of the acid hydrolysis mentioned above (1 N HCl, 6 h at 100 °C) were analyzed by RP-HPLC and monitored at 250 nm. Hydrolysates of both pairs of secalosides (A and B and C and D) gave rise to the same elution profile, demonstrating the presence of two aglycons. Aglycon I (**4**) with a UV absorption maximum in water at 250 nm exhibited from its HREIMS the molecular formula C<sub>10</sub>H<sub>9</sub>NO<sub>3</sub>. We consider that the genuine aglycon I underwent, under the hydrolysis conditions, an isomerization into

aglycon I $\phi$  (**3**). For this reason, the HPLC profile showed two peaks for this aglycon in a time-dependent ratio (*vide infra*). Aglycon II (**5**) had a UV absorption maximum in water at 282 nm and a minimum at 256 nm. Its HREIMS led to the apparent molecular formula C<sub>18</sub>H<sub>14</sub>O<sub>7</sub>. Later on it was recognized that aglycon II lost one molecule of water in the spectrometer inlet prior to ionization, so that its definitive formula therefore is C<sub>18</sub>H<sub>16</sub>O<sub>8</sub>.

These findings suggest that the isomeric secalosides A and B are made of five building blocks, namely one molecule each of aglycon I and aglycon II and three hexose subunits. This is consistent with a molecular formula of C<sub>46</sub>H<sub>51</sub>NO<sub>24</sub>, which is in full agreement with the experimental molecular mass of 1001 Da. Secalosides C and D, in addition, contain one extra glucose subunit; they have the molecular formula C<sub>52</sub>H<sub>61</sub>NO<sub>29</sub>, in agreement with the experimentally determined molecular mass of 1163 Da.

An analytical sample (1.2 mg) of the nitrogen-containing aglycon I $\phi$  was isolated by preparative HPLC from the products obtained by acid hydrolysis of secalosides A and B. The compound was identified by standard spectroscopic means as racemic 2-oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid (**3**). The assignment was confirmed by an independent synthesis following a literature procedure.<sup>11,12</sup> Nevertheless, various observations suggested that the structure of **3** did not properly reflect the constitution of the corresponding building block within the native glycosides. The HPLC profile of the hydrolysis products had shown next to **3** the peak of a transient compound with a similar retention time. Moreover, the NMR spectra of the glycosides (*vide infra*) are at variance with what one might expect, were the skeleton of **3** to be present. We therefore considered compound **3** to be an artifact resulting from a rearrangement during the hydrolysis procedure. Its most likely precursor is (2-oxo-3-indolyl)-acetic acid (OXIAA) (**4**). This compound, which is a catabolite of the plant growth hormone (3-indolyl)acetic acid (IAA),<sup>13,14</sup> has indeed been reported to undergo acidcatalyzed ring enlargement to give **3** *via* an opening/reclosure mechanism.<sup>11</sup> We have prepared racemic compound **4**,<sup>15</sup> which has the same retention time on the HPLC column as the transient species of the hydrolysate mentioned above, and have confirmed that **4** is the genuine aglycon I.

**Figure 1.** Fractionation steps employed to isolate the secalosides A-D from Cernitin T-60. "IC<sub>50</sub>" values represent the inhibitory concentration of various fractions leading to a 50% reduction of S180 tumor cell growth as compared to untreated mice. *Secalosides Journal of Natural Products, 1997, Vol. 60, No. 4 357*

The aglycon II, a markedly air-sensitive compound, was found to be water soluble and to have the

molecular composition C<sub>18</sub>H<sub>16</sub>O<sub>8</sub>. Its UV spectrum was similar to that of the secalosides, and it exhibited a complex bathochromic shift when recorded in 0.1 N NaOH. The <sup>1</sup>H-NMR spectrum showed the presence of two benzene rings with a 1,2,4- and a 1,2,4,5-substitution pattern, respectively. Allylic coupling revealed that an aliphatic -CHCHCH- fragment was connected at both ends to the tetrasubstituted aromatic ring. This suggested an indan skeleton, with the additional trisubstituted aromatic ring being attached to a benzylic position. The structure of 5,6-dihydroxy-3-(4-hydroxy-3-methoxyphenyl) indan-1,2-dicarboxylic acid (**5**) accounts best for these observations and for the observed <sup>13</sup>C-NMR spectra. The methoxy group was localized on the basis of long-range couplings. The ease by which compound **5** lost water in the mass spectrometer prior to ionization suggested that the two carboxyl functions were *cis* oriented and, hence, predestined for anhydride formation.

However, none of the spectral parameters provided reliable information as to the relative configuration of the adjacent aromatic side chain.

According to their one- and two-dimensional <sup>1</sup>H-NMR spectra including COSY-DQF16,17 recorded in D<sub>2</sub>O at 400 MHz, secalosides A and B share many structural features. Both were shown to contain the spin systems of aglycon I (**4**) and aglycon II (**5**) in a 1:1 ratio. In addition, each contained one R-glucopyranose and one α-glucopyranose moiety, readily identified by their respective anomeric protons. The third hexose subunit of the glycosides did not show an anomeric proton, and its constitution was not immediately obvious. <sup>1</sup>H-NMR spectroscopy revealed, besides these constitutional features, that secalosides A and B interchange in solution. In D<sub>2</sub>O the equilibrium was reached within less than 24 h with an A/B ratio of roughly 1.3:1. Initially it was thought that this interchange, which seriously impeded the spectral assignment, might be catalyzed by traces of acid. When the spectra were recorded in a 3:2 mixture of MeOH-*d*<sub>4</sub>/C<sub>5</sub>D<sub>5</sub>N it was found, however, that the interchange was greatly accelerated by the base, and instead of being slowed down, it reached equilibrium in less than 1 h.

Between the two glycosides, the largest chemical shift differences were observed for the AB proton resonances of an ABM-spin pattern appearing at high field. Due to an H/D exchange, this pattern simplified in D<sub>2</sub>O slowly to an AB-appearance at δ 2.22 and 2.68 for secaloside A and at δ 2.42 and 2.48 for secaloside B, respectively. The isotopic exchange was faster than the isomerization of the glycosides. We assigned these resonances to the methylene groupings of the (2-oxo-3-indolyl)acetyl fragment (i.e., aglycon I). The <sup>1</sup>H-NMR spectra of the glycosides, recorded in DMSO-*d*<sub>6</sub>, showed a free NH resonance at δ 10.4. Consequently, aglycon I must be

bound *via* its carboxylic function to the remainder of the molecule. It was concluded, therefore, that secalosides A and B are esters of (2-ox-3-indolyl)-acetic acid (**1**). They are epimers with respect to the chiral center H-C(3)\* of the nitrogen-containing heterocycle, and their mutual interconversion resulted from epimerization at this site. This is probably also true for secalosides C and D (**2**). The large number of partially overlapping signals observed in both the <sup>1</sup>H and <sup>13</sup>C-NMR spectra of secalosides A and B, being in equilibrium, greatly hampered the assignment. It was not possible to determine the total molecular connectivity of secalosides A and B on the basis of the work done in water or pyridine/methanol. A solution to this structural problem is presented in the following paper in this issue.<sup>8</sup>

Biological experiments carried out so far with the pollen extract Cernitin T-60 have suggested that the latter can inhibit the growth of various tumor cell lines *in vivo* (S180 sarcoma, Lewis lung cancer, and mammary adenocarcinoma EO771) (Sirotnak, F. M. Unpublished data). Biological evaluation of a mixture of secalosides A and B, using the packed cell volume (PCV) assay, as described in the Experimental Section, has shown that these glycosides are highly active *in vivo*. A 10-day treatment of mice with a dose of about 5-10 μg/ animal sufficed to kill 50% of implanted S180 tumor cells in the peritoneal cavity of the animals (Table 1). The racemic aglycon I (**4**) and the artifactual isomeric aglycon I<sub>c</sub> (**3**) were included in the evaluation. Both are found to exhibit very strong antitumor activity against S180 sarcoma. Whereas the cell growth inhibitory concentration at 50% (IC<sub>50</sub>) is about 5 μg/mouse for secalosides A and B, it is about 1 μg/mouse or less for the aglycons I and I<sub>c</sub> (Table 1). Because of the lack of available material, the activity of aglycon II could not be evaluated.

**Table 1.** *In vivo* Biological Activities of T-60 Pollen Extract and Purified Fractions Derived Thereof

extract/compd	dose (μg/mouse)	PCV <sub>a</sub> (%L)	T/C cell growth <sub>b</sub> (fraction of control)
Control	0	470	1
T60 batch TMO57	1000	0	0
	500	0	0
	250	43	0.09
	125	270	0.57
<b>1</b>	60	0	0
	40	10	0.02
	20	82	0.17
	10	115	0.24
	5	210	0.45
<b>3</b>	2.5	330	0.7
	20	0	0
	10	0	0
	5	0	0
	2.5	0	0
<b>4</b>	1.25	140	0.30
	0.625	290	0.62
	20	0	0
	10	0	0
	5	0	0
	2.5	0	0

1.25	75	0.16
0.625	170	0.36

<sup>a</sup> PCV, packed cell volume. <sup>b</sup> T/C, treated vs control mice cell growth. **358** *Journal of Natural Products*, 1997, Vol. 60, No. 4 Jaton et al.

In *in vitro* cytotoxicity assays (L1210 leukemia, S180 sarcoma, KB cells), neither Cernitin T60 pollen extract nor mixtures of secalosides A and B and C and D showed any activity, in spite of the high activities demonstrated *in vivo*. The data are particularly significant with regard to S180 sarcoma, as secalosides are active when given ip to S180-bearing mice. Putative mechanism(s) of action may involve activation of the animal's immune system for the following reasons: (1) when mice were treated with cyclophosphamide (an inhibitor of cell proliferation) 24 h prior to the implantation of S180 tumor cells, the effect of secalosides was abolished; (2) when secalosides were administered ip together with 2-chloroadenosine (an inhibitor of macrophage activation), no effect was detectable; (3) tumor-transplanted mice strains, immunodeficient in T/B cells (nude, SCID, or beige mice) did not respond to treatment with the secalosides (to be published elsewhere). Perhaps the important observation reported here is that most, if not all, of the biological activity of the secalosides appears to reside in a small moiety of the intact glycosides, i.e., the (2-oxo-3-indolyl)acetyl fragment (4) or its tetrahydroquinoline counterpart (3). Synthetic samples of the racemic compounds 4 and 3 appear to be as active as the whole intact secaloside pair on a molar basis. A further potentialization of action may be obtained upon attachment of one hexose unit, as predicted from the general gross structure of the glycoside.

## Experimental Section

**General Experimental Procedures.** The <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of the secalosides and of the aglycons were recorded on a Bruker AMX-400 spectrometer (9.4 T) operating at 400 and 100 MHz, respectively. Mass spectra of glycosides were measured by direct insertion on a MS 50 S instrument (Kratos Ltd, Manchester, England) under conditions of electron impact ionization (70 eV) with a probe temperature raised first to 150 °C for about 1 min and then to 350 °C. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV spectra were recorded in water on a Varian DMS-80 spectrophotometer within the wavelength range of 400-200 nm. Methods for the detection of biochemical compounds on paper and thin layer chromatograms were carried out as described in ref 9.

**Test Material.** Cernitin T-60 was obtained as a commercial preparation from AB Cernelle, S-4320 Vegeholm, Sweden. The water-soluble T-60 fraction (pH 4.0-4.4) contained about 60% of the total rye pollen.

**Extraction and Isolation** (cf. Figure 1). A portion (400 mL) of the 25% w/v water-soluble T-60 fraction was extensively dialyzed with distilled water for 48 h at 4 °C using Spectra/Por dialysis membrane tubing with a cutoff size of 1000 Da. The diffusate (outside fluid) was changed after 24 h. The "inside bag" content was evaporated to a small volume (100 mL) prior to lyophilization. The weight of this fraction, designated "A1", was 26 g. The diffusate was discarded. A second dialysis step was carried out with a Spectra/Por membrane tubing (cutoff 2000 Da). Fraction A1 (26 g) was redissolved in 100 mL of H<sub>2</sub>O and dialyzed for 1 week at 4 °C. Diffusates (2 L) were collected every 2 days, evaporated, and lyophilized. The yield of recovered yellowish powder, a fraction designated A1.2, was 6 g. This fraction (6 g) was then loaded on three Sephadex G25 (superfine grade, 4.6 cm × 120 cm) columns equilibrated and developed in distilled water at a flow rate of 20 mL/h at 4 °C; 10 mL fractions, distributed into five distinct zones, were collected and monitored at 280 nm. Fractions under each peak were lyophilized and tested for biological activity (*vide infra*). Material exhibiting *in vivo* activity in mice was contained in peaks 3 and 4. Each peak was rerun on G-25 SF columns (2.6 × 100 cm) under the same elution conditions and yielded four to five well-separated subfractions. Major peak subfractions thus obtained were subjected to the biological activity test. HPLC was performed on a Varian 5000 apparatus using columns packed with a Merck reversed-phase Lichro-Cart C18. The analytical column (100 mm × 4.0 mm i.d.) was operated at 1 mL/min, monitoring at 280 nm. Solvent A was 0.1% aqueous TFA, and solvent B was 0.1% TFA in acetonitrile. A gradient of 5-20% over 20 min followed by a plateau for 5 min at 60% acetonitrile was used. Major peaks were collected in Eppendorf tubes and dried down in a vacuum centrifuge (Savant SVC 200). For preparative runs, purification was achieved with a Macherey-Nagel column (250 mm × 21 mm i.d.) Nucleosil 300 Å 5- $\mu$ m C8 particles. A linear gradient (10-48% acetonitrile in 0.1% aqueous TFA) was applied for 45 min at a flow rate of 5 mL/min.

**Chemical Hydrolysis.** The major, and separately, the minor epimer of glycoside 1 (secalosides A and B) and the major, and separately, the minor epimer of glycoside 2 (secalosides C and D), ca. 1-2 mg each, were hydrolyzed in 1 N HCl (100-200  $\mu$ L) at 100 °C for periods ranging from 5 min to 6 h in small sealed glass tubes; 18 hydrolyzates were dried down, ashed three times with 300  $\mu$ L of H<sub>2</sub>O and finally redissolved in 0.1% aqueous TFA prior to RP-HPLC, MS, and/or NMR analyses. Each glycoside gave the compounds 3 and 5. Insoluble material was discarded.

**Enzymatic Hydrolysis.** Glycoside mixtures 1 and 2 were subjected to a series of enzymatic digestions using either exoglycosidases or endoglycosidases, such as R- and  $\alpha$ -glucosidases, R- and  $\alpha$ -amylases,

R-mannosidase, and R- and  $\beta$ -galactosidases, using procedures recommended by the manufacturer (Boehringer Mannheim). Digests were analyzed by RP-HPLC. The enzymatic determination of glucose was carried out by the Gluco-Quant test, according to the ad-hoc procedure (Boehringer Mannheim).

**Secaloside A (1)** (major epimer) was obtained as a grayish amorphous powder: mp 178 -186 °C dec; [R]<sub>D</sub> +4.6° (c 0.6, MeOH); UV (H<sub>2</sub>O)  $\lambda_{max}$  320 (sh), 280, strong end absorption; exhibited comparable 1H-NMR data in MeOH-d<sub>4</sub> (400 MHz) as described in ref 8; FAB-MS *m/z* [M + H]<sup>+</sup> 1002 (C<sub>46</sub>H<sub>51</sub>NO<sub>24</sub>).

**Secaloside B (1)** (minor epimer) was obtained as a grayish amorphous powder: mp 192-201 °C dec; [R]<sub>D</sub> +6.8° (c 0.4, MeOH); UV (H<sub>2</sub>O)  $\lambda_{max}$  320 (sh), 280, strong end absorption; exhibited comparable 1H-NMR data in MeOH-d<sub>4</sub> (400 MHz) as described in ref 8; FAB-MS *m/z* [M + H]<sup>+</sup> 1002 (C<sub>46</sub>H<sub>51</sub>NO<sub>24</sub>).

**2-Oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid (3)** (racemic) was obtained as a colorless powder: mp 218-219 °C (lit.12 mp 220 °C). The UV, IR, 1H-NMR, 13C-NMR, and MS data were identical with those of an authentic sample (refs 11 and 12). *Secalosides Journal of Natural Products, 1997, Vol. 60, No. 4 359*

**5,6-Dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-indan-1,2-dicarboxylic acid (5)** was obtained as a colorless, air-sensitive powder: UV (H<sub>2</sub>O)  $\lambda_{max}$  (log  $\epsilon$ ) 282.6 (0.17), 250 (sh), 201 (1.41) nm; 1H-NMR (D<sub>2</sub>O, 400 MHz)  $\delta$  3.76 (1 H, dd, J) 9.6, 8.8 Hz, H-2), 3.80 (3 H, s, OCH<sub>3</sub>), 4.42 (1 H, br d, J) 8.8 Hz, H-3), 4.76 (1 H, br d, J) 9.6 Hz, H-1), 6.41 (1 H, d, J e 0.8 Hz), 6.84 (1 H, dd, J) 8.2; 1.8 Hz, H-6 $\phi$ ), 6.90 (1 H, d, J) 8.2 Hz, H-5 $\phi$ ), 6.93 (1H, d, J) 1.8 Hz, H-2 $\phi$ ), 6.97 (1H, s); 13C-NMR (D<sub>2</sub>O, 100 MHz)  $\delta$  54.1 (d, C-3), 55.0 (d, C-1), 58.6 (q, OMe), 59.6 (d, C-2), 114.3 (d), 115.1 (d), 115.4 (d), 118.2 (d), 124.2 (d), 133.5 (s), 138.8 (s), 141.4 (s), 146.4 (s), 146.5 (s), 147.5 (s), 150.2 (s), 179.2 (-CO<sub>2</sub>), 180.1 (-CO<sub>2</sub>); EIMS (70 eV) *m/z* 342 (70) [M<sup>+</sup> - H<sub>2</sub>O], 314 (38), 270 (100), 169 (32); HREIMS *m/z* 342.0748 (calcd for [C<sub>18</sub>H<sub>16</sub>O<sub>8</sub> - H<sub>2</sub>O], 342.0739).

**Packed Cell Volume (PCV) Assay.** The measurement of the biological activity of various peak fractions was performed by one of us (F.M.S.) at the Sloan Kettering Cancer Center, New York, NY. Briefly, mice (ca. 20 g) were injected at day 1 with 2-3  $\times$  10<sup>5</sup> cells from S180 sarcoma into the peritoneal cavity. They were then treated at days 3, 5, 7, and 9 with different amounts of the samples to be analyzed. At day 10, mice were sacrificed and weighed. The

volume of cells remaining within the peritoneal cavity was determined after 10 000 rpm centrifugation of the collected ascitic fluid, which yielded the percentage of cellular volume (V<sub>c</sub>). After draining of ascitic fluid and drying of the peritoneal cavity, mice were reweighed and the mass difference reflected ascite volume (V<sub>a</sub>) of mice. PCV volume is defined as %V<sub>c</sub> - V<sub>a</sub>. The mean PCV value of treated mice (T) was compared to that of control mice (C). A ratio T/C of, e.g., 0.5, IC<sub>50</sub> (inhibitory concentration at 50%) reflects a cell growth of 50% as compared to control. Data are the average of two experiments using six to seven mice/group (see Table 1).

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## Antitumour potential of pollen extract on Lewis lung carcinoma implanted intraperitoneally in syngeneic mice.

*Furusawa-E; Chou-SC; Hirazumi-A; Melera-A*

*Department of Pharmacology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii, USA.*

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A defined pollen extract of selected plants is used to treat chronic prostatitis or benign hyperplasia. The antineoplastic potential of the water-soluble fraction (Cernitin T60) of the pollen extract against Lewis lung carcinoma implanted i.p. in syngeneic mice was investigated. Cernitin T60 was not cytotoxic against KB cells at concentrations up to 2.5 mg/ml. Cernitin T60 (0.5 g/kg) significantly prolonged the lifespan of mice carrying the tumour without any apparent side effects. Cernitin T60 demonstrated beneficial therapeutic effects in an additive fashion on the life-span of mice when it was combined with standard cytotoxic antineoplastic drugs such as adriamycin [doxorubicin], cisplatin, vincristine, methotrexate, fluorouracil, or thioguanine. The antineoplastic potential of Cernitin T60 was completely abolished by treatment with inhibitors of macrophage functions (2-chloroadenosine or carrageenan); the antineoplastic potential of Cernitin T60 was not abolished following treatment with the T-cell inhibitor, cyclosporin A. Cernitin T60 appears to be a potent immunostimulator of macrophages.

One fraction, designated FV-7, in the water soluble ingredient of the pollen extract Cernilton was found to be inhibitory to the growth of a prostate cancer cell line. Characterization of FV-7 by high-resolution mass spectrometry and nuclear magnetic resonance identified the fraction as hydroxamic acid, 2,4-dihydroxy-2H-1, 4-benzoxazin-3 (4H)-one (DIBOA). To confirm this further, we synthesized an authentic sample of DIBOA and found subsequently that the synthetic DIBOA was structurally indistinguishable from FV-7. Furthermore, in a separate experiment we compared the in vitro effects of FV-7 and DIBOA on the growth of a prostate cancer cell line and found that in both cases the effect was inhibitory and that the inhibition curves obtained for both compounds were virtually identical.

## Isolation and Characterization of a Cyclic Hydroxamic Acid from a Pollen Extract, Which Inhibits Cancerous Cell Growth in Vitro

Xin Zhang, Fouad K. Habib, Margaret Ross, Ulrich Burger, Ari Lewenstein, Keith Rose, and Jean-Claude Jaton

Department of Medical Biochemistry and Organic chemistry, University of Geneva, Geneva, Switzerland, Department of Surgery/ Urology, Western general Hospital, Edinburgh, U.K., and Cernitin SA, Lugano, Switzerland

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### Introduction

Cernilton is a multipotent extract from rye pollen which is commercialized by Cernitin SA, Lugano, Switzerland; it is one of the most popular phytotherapeutic drugs in Europe. The extract contains two main components, namely the hydrophobic Cernitin GBX1 fraction, both of which are devoid of allergens and other high molecular weight substances.

Clinical trials reported the efficacy of this drug in the treatment of benign prostatic hyperplasia (BPH) and chronic prostatitis with no reported side effects. Subsequently, it was demonstrated in vitro that the water soluble Cernitin T-60 fraction was the active component in the "Cernilton" mixture as this inhibited the growth of prostate cancer cells and primaty culture from BPH specimen. In addition, in vivo studies on rats showed significant reductions in the weight of the rat prostate following an intake of Cernilton over a period of 21 days. Significantly there was no change in any of the other organs of the animals tested.

More recently we have identified a number of constituent components in Cernitin T-60 of which only one fraction, designated V-7 (FV-7), was found to be biologically active with respect to its strong inhibitory effect on the growth of prostate cell lines; this prompted us to identify and characterize this active ingredient. We now report on the isolation, characterization, and biological evaluation of one natural component present in the pollen extract and believed to be responsible for the biological activity of this drug.

### Results

**Purification of Biologically Active Fractions from a Pollen Extract (Cernitin T-60).** The aim of the present investigation was to single out one or more components present in a pollen extract (referred to as T-60 water soluble fraction) which could be responsible for the growth inhibition of the human prostate cell line DU-145. The first step of purification consisted of the dialysis of the spissum through a spectrapor dialysis membrane with a cutoff limit of 1 kDa. Using the in vitro DU-145 cell growth inhibition assay, the material inside the bag proved to be inactive (as tested in the range of 0.1-0.5 mg/mL) and was discarded. All of the inhibitory activity was recovered in the diffusate.

Diffusate (2g) was then loaded onto a Sephadex G-25 superfine column. Up to eight fractions could be resolved. They were separately lyophilized, and the total recovery was  $\approx$  90% of the starting material. Each fraction was assayed for the inhibitory activity: only one fraction (FV) was found to exhibit significant inhibitory activity toward DU-145 prostatic cancerous cell line at a concentration range of about 100 $\mu$ g/mL. Fraction V, which eluted at the same position as N-2-4-DNP-L-alanine (MW=255 Da) on the same column (i.e., 3.7- fold void volume), accounts for  $\approx$ 10% of the components present in the diffusate, i.e.,  $\approx$  3.6% (w/w) of the Cernitin T-60 fraction extract. Fractions IV and VI also exhibited some inhibitory activity, which

was likely to be due to the cross-contamination by fraction V.

**Purification of the Active Fraction V.** Pooled fraction V (~50mg from five to six preparative G-25 runs) was then loaded onto a Sephadex G-10 column, equilibrated, and developed in distilled water. Fraction V yielded eight subfractions. The inhibitory activity was found to be associated with the seventh subfraction only (elution volume = 9.2-fold void volume), at a concentration as low as 15-20µg/mL. FV-7 accounts for ≈ 13% of the whole FV, which in turn corresponds to .3% (w/w) of the whole Cernitin T-60.

G-10 pooled fraction v-7 (500µg) was further loaded on a Lchro CART C18 reverse phase column (4.6 mm x 100 mm) on a Varian 5000 liquid chromatograph to isolate the active compound to homogeneity, i.e., in a form suitable for chemical characterization. The eluting system was .1% TFA, pH 2.0, with an acetonitrile, was separated from several minor contaminants which were estimated to account for no more than 10% of the total fraction V-7, as monitored at 254 nm. The in vitro biological test confirmed that the major HPLC peak had dramatic inhibitory effect on DU-145 cells at 10µg/mL.

**Chemical characterization of the Natural Product Contained in Fraction V-7. Ultraviolet Spectrum (UV).** The UV spectra of the HPLC-purified component V-7 at pH 7.0 exhibited a shoulder at 279 nm, an absorption maximum at 253 nm, and a minimum at 232 nm.

**Mass Spectrometry (MS).** The strongest signals were obtained at 200° C during evaporation of the bulk of the sample. The spectrum showed a strong signal at *m/e* 181, which was thought to be the most reasonable candidate molecular ion (data not shown). High-resolution, narrow scan data provided an assigned mass of 181.0375. The only composition found within the maximum estimated error (0.1 mmu) was C<sub>8</sub>H<sub>7</sub>NO<sub>4</sub>, 181.0375 calcd (error 0.1 mmu).

**Nuclear Magnetic resonance (NMR).** The HNMR spectrum of the natural product V-7 recorded in DMSO-d<sub>6</sub> showed seven H resonances. Four of these, appearing as a multiplet at 7.0-7.3 ppm, immediately suggested the presence at 10.9 ppm was indicative of an acidic hydrogen atom. Two further resonances at 8.1 and 5.7 ppm, respectively, were found to couple with 5.5 Hz (vicinal coupling). Two hydrogen atoms of the molecule (at 10.9 and 8.1 ppm) underwent isotopic exchange with D<sub>2</sub>O.

The C-NMR spectrum showed eight resonances at 92.0, 112.9, 117.1, 122.5, 123.8, 128.7, 140.6, and 157.6 ppm. Together with the results from high-resolution MS, these spectra definitely confirm the molecular formula C<sub>8</sub>H<sub>7</sub>NO<sub>4</sub> for the compound V-7.

Two-dimensional heteronuclear shift correlation spectroscopy (H/C-COSY) revealed that only five of the eight C atoms are bound to hydrogen, i.e., three of the C atoms of the compound bear no H (those at 157.6, 140.6, and 128.7 ppm). The compound has neither a CH<sub>2</sub> nor a CH<sub>3</sub> group. Consequently, and in agreement with the isotope exchange experiment mentioned above, the two remaining H atoms must be bound to a heteroatom. The most reasonable fit between the structure and the NMR spectra was found for 2, 4-hydroxy-2H-1, 4-benzoxazin-3 (4H)-one (DIBOA).

#### DIBOA

DIBOA is a cyclic hydroxamic acid, FV (G-25) and FV-7 (G-10) were the only two fractions of all the Sephadex fractions which exhibited a positive blue complex formation, characteristic for hydroxamic acids, with the ferric chloride spray method. The ultimate proof that fraction V-7 was indeed identical to the known compound DIBOA resides in the independent chemical synthesis. This was carried out and then naturally occurring product V-7 was found indistinguishable by NMR, MS, UV spectra, and HPLC profile from an authentic synthetic sample of DIBOA.

**Figure 1.** Growth of the androgen insensitive DU-145 human prostate cancer cell line following treatment with different concentrations of either fraction V-7 or DIBOA. Cells at a density of 1.5x 10<sup>3</sup> cells/ well were incubated for periods up to 6 days, and the results are expressed as percentage of thymidine incorporated relative to the untreated control. Each point is the mean of three separate experiments each run eight times. Bars represent coefficient of variation.

**Comparison of the Biological activity of V-7 and synthetic DIBOA.** At an initial cell density of 1.5x 10<sup>3</sup> DU-145 cells/ well, the inhibitory patterns for both the naturally occurring fraction V-7 and an authentic synthetic sample of DIBOA were tested at 1, 10, and 100µg/mL. The results outlined in Figure 1 demonstrate an identical inhibitory pattern for the two compounds, thus confirming that there is no difference between DIBOA and V-7 in terms of their biological activities. At a concentration of 1µg/mL of either V-7 or DIBOA, no growth inhibition can be observed from day 1-6. However at a concentration of 10µg/mL of, the inhibitory effect at day 1 was found to be in the region of 50%, but this increased to 80% at day 5. When concentrations of 100µg/mL inhibitory material were used, a complete shutdown of the proliferative effects was achieved from day 1 and this remained as such up to day 6. Similar patterns of inhibition were recorded when the initial cell density was increased to 2.5 x 10<sup>3</sup> cells/well (results not shown), suggesting

that the inhibitory effect is independent of cell concentration.

## Discussion

We described here the isolation of a cell growth inhibitor, designated fraction V-7 from a commercial pollen extract, Cernitin T-60. V-7 was shown to be inhibitory at a concentration as low as 5-10 $\mu$ g/mL when added to human prostatic cell line DU-145 in culture, and its content was evaluated to average 1% of the pollen extract. High-resolution mass spectrometry and nuclear magnetic resonance allowed us to characterize V-7 as the cyclic hydroxamic acid, 2,4-dihydroxy-1,4-benzoxazin-3(4H)-one (DIBOA). Furthermore an authentic synthetic sample of DIBOA was found structurally indistinguishable from V-7. The in vitro comparative inhibition curves obtained with V-7 and DIBOA were virtually superimposable (Figure 1).

DIBOA is the enzyme hydrolysis product of the glycoside derivative. It was recently shown that the parent glycosides of DIBOA and its methoxy derivatives occur in significant amount in some members of the Gramineae family of plants. Cernitin T-60 extract, which contains up to 95% pollen (w/w), appears to comprise at least one member of the DIBOA family. The cyclic hydroxamic acids have attracted much attention in agronomic research because of their role as phytotoxic agents, even though the glycoside derivatives appeared to be less potent.

Until now, there were no reports in the literature concerning the role of DIBOA and its dihydroxy, methoxy, and glucoside derivatives as potential agents in the treatment of human neoplastic diseases. To the best of our knowledge, this is the first report on the use of DIBOA to inhibit human prostate cancer cell growth in culture. In this context, evaluation and antitumoral activities of hydroxamic acids have been reported. It was suggested that a series of polyhydroxy-substituted benzohydroxamates act as inhibitors of ribonucleotide reductase activity. It remains to be seen whether the mechanism of action of DIBOA in the human prostate is similar. It is tempting to speculate that cell growth inhibition may result from the chelating or radical scavenger properties of DIBOA, which in turn may be the rate-limiting step of nucleotide biosynthesis; this is a possibility, which we are at present investigating.

## Experimental Section

**Cernitin T-60.** Cernitin T-60 is a pollen extract preparation from AB Cernelle, Helsingborg, Sweden. The water-soluble T-60 fraction accounted for more than 90% of the pollen extract.

**Purification of Active Natural Product(s) from T-60 Fraction.** This was carried out by a combination of dialysis, gel filtration, and reverse phase HPLC steps, as outlined in ref.6. Dialysis of T-60 fraction was carried out against distilled water using Spectra/Por (cutoff, 1 kDa) porous membranes. Contents inside and outside the bag were lyophilized. Twenty milliliters of the concentrated diffusate at a concentration of 200mg/mL was loaded onto a Sephadex G-25 superfine column (2.6x140cm) developed with distilled water at a flow rate of about 15mL/min, and the effluent was monitored at 280nm. The pooled biologically active material (20-30mg) was further fractionated through a Sephadex G-10 column (2.0x100cm) using distilled water as eluent and monitoring at 280 nm. Biologically active subfraction from Sephadex G-10 chromatography was further purified to homogeneity by reverse phase HPLC on a semi-preparative Nucleosil C18RP column (1x25cm); the eluting system was 0.1% aqueous TFA at pH 2.0 and an acetonitrile gradient was from 0 to 40% at a flow rate of 0.7mL/min. Monitoring was done at 254nm; 500-700 $\mu$ g of the active sub-fraction from the G-10 step was repeatedly loaded, and the practical recovery was about 30-40%.

**Physicochemical Characterization. Mass Spectrometry.** The mass spectrum was acquired by direct insertion on a MS 50S instrument (Kratos Ltd., Manchester, England) under conditions of electron impact ionization (70 eV) with a source temperature of 200 $^{\circ}$  C.

**Nuclear Magnetic Resonance.** The H- and C-NMR spectra of the active compound were recorded in deuterated dimethyl sulfoxide (DMSO-d<sub>6</sub>) on a Bruker AMX-400 spectrometer (9.4T) operating at 400 and 100 MHz, respectively. Assignments were ascertained by two-dimensional homonuclear and heteronuclear shift correlation spectroscopy.

**Chemical Synthesis of the Active Compound.** An authentic sample of the active compound was obtained by an independent synthesis in four steps following procedures properties of the synthetic and the natural compound isolated from the T-60 fraction was made by HPLC, UV, MS, and NMR.

**Biological Assay. Cell Culture.** To monitor and evaluate the inhibitory activity of each of the purified fractions from dialysis, gel filtration, and RP-HPLC steps, an assay employing the human prostate cancer cell line DU-145 was undertaken. Conditions for growth of these cells have been described previously as well as thymidine incorporation and statistical analysis of the data.

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## In Vitro Evaluation of the Pollen Extract, Cernitin T-60, in the Regulation of Prostate Cell Growth

F.K. HABIB, MARGARET ROSS, A.C. BUCK, L. EBELING and A. LEWENSTEIN

*University Department of Surgery (WGH), Western General Hospital, Edinburgh; Department of Urology, Royal Infirmary, Glasgow; Cernitin S.A., Lugano, Switzerland*

**Summary-** Nine human-derived cancer and non-cancer continuous cell lines were employed to evaluate the relative *in vitro* activity of the pollen extract, Cernitin T-60. Responses of the cell lines to the drug were assessed by measuring growth and cell survival as determined by cell count. The results demonstrated that of the 9 continuous cell lines tested, only those derived from the human prostate were growth inhibited by the pollen extract, whereas the non-prostate derived cells exhibited variable degrees of resistance to the T-60. The selectivity of the drug for the prostate cell lines was even more pronounced on the hormone-independent models, suggesting that there might be a place for the pollen extract in the control of abnormal growth in hormone-insensitive cells.

In spite of the considerable advances in our understanding of the processes leading to the growth and proliferation of the human prostate, the management of prostate, the management of prostate diseases still remains a major clinical problem (Chisholm, 1989). Cancer of the prostate is the second most common cause of death due to cancer in males in the United Kingdom (Cancer Research Campaign, Factsheet 10.1, 1988) and the death rate is increasing. Clearly, the traditional forms of treatment such as surgery at the primary site, orchiectomy, hormone treatment and radiation are not as effective as Huggins might have originally perceived (Huggins and Hodges, 1941) and there is now every reason to find an alternative form of treatment.

Recently, there have been several reports suggesting that the pollen extract, Cernitin, is an effective agent in the treatment of prostate disease (Ito *et al.*, 1986; Buck *et al.*, 1989). The pollen extract is a preparation produced by AB Cernelle in Sweden and is essentially a microbial digestion of a mixture of pollens which have been extracted first in water and subsequently with an organic solvent (Kimura *et al.*, 1986).

In an attempt to assess the selectivity and specificity of these pollen extracts, we undertook a number of experiments to compare the *in vitro* activity of Cernitin towards a wide range of human-derived cancerous and non-cancerous continuous cell lines of prostate and non-prostate origin. We confined our experiments to the water-soluble fraction T-60 component, which accounts for approximately 60% of the pollen extract. In addition, we also undertook a

few experiments on benign hyperplastic prostates to test the impact of the pollen extract on testosterone metabolism and the binding of androgens to their receptors.

### Materials and Methods

#### Chemicals

Cernitin T-60 was a gift from AB Cernelle, Helisingborg, Sweden.

#### Tissues

Specimens of benign prostatic hyperplasia (BPH); obtained by transurethral resection, were transferred to the laboratory and either used immediately or snap frozen in liquid nitrogen and stored at -70° C.

#### Cell cultures

The epithelial and fibroblastic cell lines were all derived from human cancerous and non-cancerous tissue and details of their sources are given in Table 1. Of the 3 human prostate cancer cell lines investigated, the LNCaP model is the only one which is hormonally responsive (Horosiewicz *et al.*, 1983), whereas the other 2 cell lines, the DU145 (Stone *et al.*, 1978) and the 1013L (Williams, 1980) were all hormone-insensitive. All cell lines were maintained at 37°C under a humidified atmosphere at 5% CO<sub>2</sub> and 95% air in 75cm<sup>2</sup> tissue culture flasks (Corning, New York, USA). The culture medium used was RPMI-1640 (Gibco, Paisley) supplemented with 10% (v/v) fetal calf serum, 20 mM HEPES, penicillin (100

units/ml), streptomycin (100µg/ml) and 1% (v/v) L-glutamine. At each transplant, cells from the confluent monolayer were removed by trypsinisation (trypsin 0.05%, EDTA 0.025%, Gibco) and suspended at 5x10<sup>4</sup> cells/ml in the growth medium.

### Growth assays

Dose-response curves of Cernitin T-60 treatment were determined using the following method. Triplicate determinations for each treatment were performed in 24 well culture plates (Cell-Cult, Sterilin, Teddington). Each well was seeded with 5x10<sup>4</sup> cells and incubated overnight in the medium under incubation conditions as described above for routine cell culture. The following day, the T-60 stock solution was serially diluted in supplemented RPMI 1640 medium to yield concentrations of 1-4 mg/ml. Controlled cultures receive medium alone. For the dose-response curve studied, the cells were exposed to Cernitin T-60 for a total period of 4 days, with changes of freshly diluted T-60 in medium every 2 days. For the time course study, cells were treated in the presence and absence of T-60 for 1, 2, 3, or 4 days. Experiments were terminated by the removal of cells from the monolayer by 2 successive trypsinisations and the pellets of harvested cells were subsequently suspended in 0.5 ml of Dulbecco A Medium (Oxoid Ltd, Basingstoke). The counting of cells was achieved on a haemocytometer slide after a 1-2 dilution with trypsin/ glutamine.

### Nuclear androgen receptors

Method used for the preparation of nuclear fractions and measurements of androgen receptors followed those previously published (Habib *et al.*, 1986). For androgen receptor determinations, the competition binding assay was with 17 $\alpha$ -methyl-3H-methyltrienolone (R1881) in the presence of triamcinolone acetonide. Dissociation constants (Kd) and number of binding sites were determined by the Scatchard (1949) method.

### Assay for 5 $\alpha$ -reductase activity

5 $\alpha$ -reductase was assayed at 37°C by following the conversion of (3H) testosterone to (3H) dihydrotestosterone and (3H) 3 $\alpha$  androstenediol as previously detailed Habib *et al.*, 1985).

### Results

#### The effect of T-60 on cell growth

Proliferation curves of the hormone-sensitive and hormone-insensitive prostate cell lines in the  
**Table 1** Details of Cell Lines

Absence and presence of increasing concentrations of T-60 for periods of up to 4 days are shown in Figure 1. Although the growth of each of these

prostate cell lines was slowed following the addition of the pollen extract, the results show that the inhibition was much more marked in the case of the androgen-insensitive cell lines. Indeed, at 1mg/ml the pollen had no effect on the growth of the LNCaP cells, which exhibited an identical profile to that of the control, whereas the androgen-insensitive 1013L and DU145 cells demonstrated significant inhibition, particularly on day 4. By contrast, at the higher pollen concentrations (4mg/ml) the growth of all 3 prostate cell lines was arrested and the cell numbers were rapidly depleted with the time of exposure. After 4 days, cell counts had been reduced by an average of 94% compared with controls.

Parallel experiments on the non-prostate derived cell lines showed no response to pollen extract (1mg/ml) even after 4 days' exposure (Fig.2). However, at the higher concentrations (4mg/ml) the pollen induced some inhibition with the HEF and RT112 cells ( $P < 0.01$ ) following a 4-day incubation (Fig. 2), although this was not as marked as in the prostate cells. Significantly, none of the other non-prostate derived cells showed any significant response ( $P > 0.5$ ).

The effect of T-60 on androgen metabolism and steroid receptors

We also tested the impact of increasing concentrations of Cernitin T-60 (0-10mg/ml) on the 5 $\alpha$ -reductase activity of tissue obtained from 6 separate BPH patients. As demonstrated in Table 2, there was no change in the activity of the enzyme with increase in T-60 even at concentrations as high as 10mg/ml.

In addition, we undertook several experiments to measure nuclear androgen receptor levels in the absence and presence of the pollen extract at 4mg/ml. The results summarized in table 3 indicate that there was no significant difference between the control and test groups with regard to the number of binding sites ( $P > 0.5$ ) and dissociation constants ( $p > 0.5$ ).

**Fig 1** The effects of varying the concentrations of Cernitin T-60 on the growth of androgen-sensitive and androgen-insensitive prostate cell lines. Each point represents the mean  $\pm$  SD of 3 separate experiments each run 6 times.

**Fig. 2** The effect of Cernitin T-60 on the growth of 6 non-prostate derived cell lines after 4 days' exposure to the drug. Results are the mean  $\pm$  SD of 3 separate experiments each run 6 times ( $P > 0.01$ )

### Discussion

These data represent the first report of the *in vitro* evaluation of the water-soluble fraction of the pollen extract, Cernitin T-60, using a panel of human prostate tumor-derived continuous cell lines. In addition, parallel *in vitro* experiments were also

undertaken on 6 other cell lines derived from non-prostatic sources essentially to assess the specificity and efficacy of pollen extract.

Attempts to minimise variations between experiments were made by standardising experimental conditions with regard to the same medium, fetal calf serum concentrations, and narrow range of cell passages. Furthermore, we observed a little variation in drug response with repeated experiments for each particular cell line. Nonetheless, the results of this study suggest that the responses induced were varied and these were predominantly a function of the cell lines: high in the case of the prostate, low or non-existent in the non-prostate derived cells. Of interest also is the heterogeneity in responses of the prostate cell lines to the agent. The hormone-insensitive cells demonstrated a greater sensitivity to the pollen extract than the androgen-dependent line and this was particularly evident at the lower pollen concentrations

We are not yet sure of the mechanism of action of this drug but quite obviously it is not mediated via the androgen delivery system of the cell, since the pollen had no effect on either the 5 $\alpha$ -reductase activity of the tissues or its steroid receptors. There have also been reports suggesting that Cernilton might be a potent inhibitor of the cyclo-oxygenase and lipoxygenase enzymes which are needed for leucotrine and prostaglandin synthesis (Loschen, personal communication) but these reports have not been extended to the prostate and will require verification.

However, it is gratifying to note that the selectivity of the pollen extract for the prostate, as demonstrated in the present study, was also supported by the work carried out by Ito *et al.* (1986). Following an intake of Cernilton over a period of 21 days, the rats in the latter study showed significant reductions in the weight of the ventral and dorsal prostate but there was no change in any of the other major organs. Following these encouraging results, a double-blind trial was undertaken on a group of patients with BPH, the results of which are described by Buck *et al.* (1990).

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#### The Authors

- F.K. Habib, PhD, Cchem, FRSC, Senior Lecturer in Biochemistry, University Department of Surgery, Western General Hospital, Edinburgh.
- Margaret Ross, BSc, Research Technician, University Department of Surgery, Western General Hospital, Edinburgh.
- A.C. Buck, PhD, FRSC, Consultant Urologist, Royal Infirmary, Glasgow.
- L. Ebeling, MD, Medical Associate, Hamburg, Germany.
- A. Lewenstein, PhD, General manager, Cernitin S.A., Lugano, Switzerland.

Requests for reprints to F.K. Habib, University department of Surgery (WGH), Western General Hospital, Edinburgh EH4 2XU.