PROSTATE SUPPORT:

GRAMINEX Flower Pollen Extract

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

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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 in 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 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, Cernilton, 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 Cernilton 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 (Horosewicz *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_2 and 95% air in 75cm^2 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 (100 µg/ml) and 1% (v/v) L-glutamine. At each transplant, cells from the confluent monolayer were removed by trysinisation (trypsin 0.05%, EDTA 0.025%, Gibco) and suspended at 5x10^4 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 5x10^4 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 studies, 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 17α-methyl-^{3}H-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 5a -reductase activity

5α-reductase was assayed at 37° C by following the conversion of (^{3}H) testosterone to (^{3}H) dihydrotestosterone and (^{3}H) 3α)β) androstanediol 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

Table 1 Details of Cell Lines

Cell line	*Tumour type*	*Source*	*Cell/well*	*Duration of drug exposure (days)*
HEP	Cancer of the larynx	Gifts from Dr Mary Norval, University Medical School, Edinburgh	5×10^4	1–4
CHANG	Cancer of the liver		5×10^4	1–4
HEF	Human embryo fibroblast		5×10^4	1–4
RT112	Cancer of the bladder	Dr J. R. W. Masters, Department of Pathology, St Paul's Hospital, London	5×10^4	1–4
SUZA	Cancer of the testis		5×10^4	1–4
DU145	Cancer of the prostate	Gifts from Dr D. Mickey, Department of Urologic Research, University of North Carolina, USA	5×10^4	1–4
1013L	Cancer of the prostate		5×10^4	1–4
LNCaP	Cancer of the prostate	Gift from Dr J. S. Horoszewicz, Department of Medical Virology and Oncology, Roswell Park Memorial Hospital, Buffalo, USA	5×10^4	1–4
MCF-7	Cancer of the breast	Gift from Dr W. R. Miller, Department of Clinical Surgery, University Medical School, Edinburgh	5×10^4	1–4

the 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 5α-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 a 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

Table 2 Effect of T-60 Concentrations on 5α - Reductase Activity of the Human Benign Prostate

Patient no.	T-60 concentration (mg/ml) 0	0.75	2	10
1	1.44 ± 0.2*	1.34 ± 0.23	1.38 ± 0.12	1.25 ± 0.09
2	1.55 ± 0.18	2.08 ± 1.10	0.98 ± 0.12	1.58 ± 0.29
3	6.29	6.98 ± 2.72	8.46 ± 1.29	9.89 ± 0.89
4	2.21 ± 0.15	2.18 ± 0.19	—	2.23 ± 0.23
5	2.98 ± 0.52	3.18 ± 0.21	4.45 ± 0.56	—
6	2.58 ± 0.26	2.4 ± 0.24	2.32 ± 0.04	2.28 ± 0.65

* Values expressed in pmol/mg protein/min ± SD.

Table 3 Effect of Cernitin T-60 (4mg/ml) on Nuclear Androgen Receptor Measurements in 6 BPH Specimens

Treatment	K_d (nmol/l ± SD)	Binding site (fmol/g tissue ± SD)
Control	2.95 ± 0.60	84.4 ± 27.5
T-60 added	2.80 ± 0.57	78.8 ± 32.1

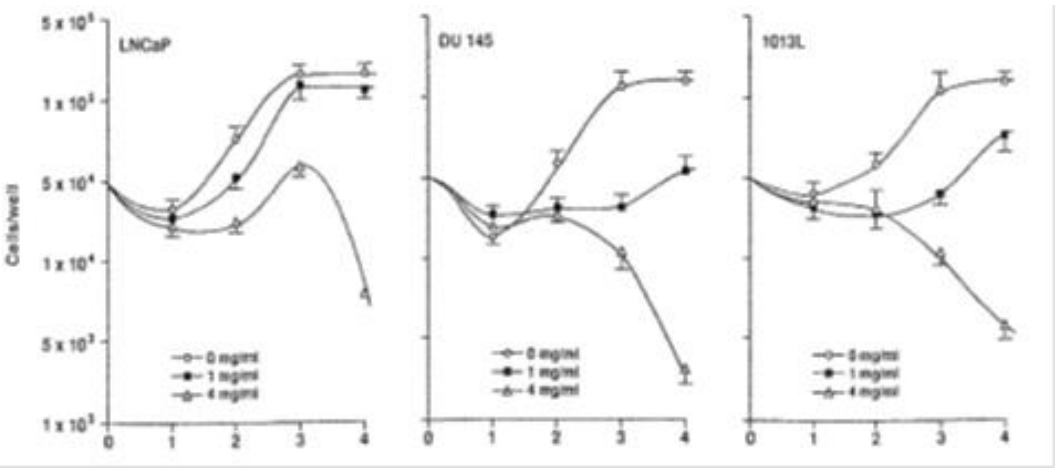

Fig 1. The effects of varying the concentrations of Cernitin T-60 on the growth of androgensensitive and androgen insensitive prostate cell lines. Each point represents the mean ± SD of 3 separate experiments each run 6 times.

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).

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 minimize variations between experiments were made by standardizing 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 5α-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 leukotriene 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).

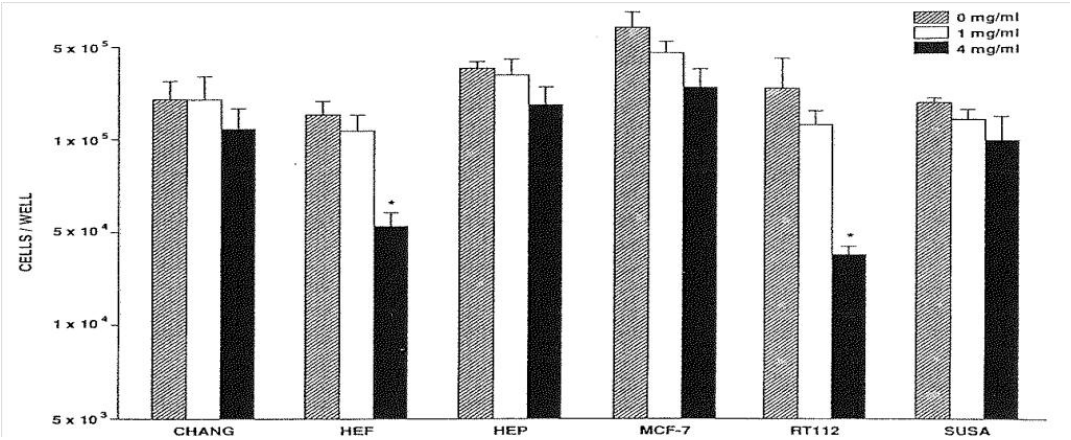

Fig. 2 The effect of Cernitin T-60 on the growth of 6 non-prostate derived cell lines after 4 days' exposure to the drug. Results are the mean ± SD of 3 separate experiments each run 6 times (P> 0.01).

References

1. Buck, A.C. Cox, R., Rees, R. W. M. et al. (1990). Treatment of outflow tract obstruction due to benign prostatic hypertrophy with the pollen extract, Cernilton. A double-blind, placebo controlled study. Br. J. Urol., 66, 398-404.

2. Buck, A.C., Rees R.W. M. and Ebeling, L. (1989). Treatment of chronic prostatitis and prostatodynia with pollen extract. Br. J. Urol., 64, 496-499.

3. Chisholm, G.D. (1989). Benign prostatic hyperplasia. The best treatment. Br. Med. J., 299, 215-216.

4. Habib, F.K., Odoma, S., Busuttil, A. et al. (1985). 5a-reductase activity in human prostate cancer is related to the histological differentiation of the tumour. Clin. Endocrinol., 23, 431-438.

5. Habib, F.K., Odoma, S., Busuttil, A. et al. (1986). Androgen receptors in cancer of the prostate, correlation with the stage and grade of the tumor. Cancer, 57, 2351-2356.

6. Horoszewicz, J. S., Leong, S. S., Kawinski, E. et al. (1983). LNCaP model of human prostatic carcinoma. Cancer Res., 43, 1809-1818.

7. Huggins, C. and Hodges, C.V. (1941). Studies on prostatic cancer. I. The effect of castration, of estrogen, and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Res., 1, 293-297.

8. Ito, R., Ishi, M., Yamashita, S. et al. (1986). Cernitin pollen extract (cernilton): antiprostatic, hypertrophic action of cernitin pollen extract. Pharmacometrics, 31, 2-14.

9. Kimura, M. Kimura, I., Nakese, K. et al. (1986). Micturition activity of pollen extract: contractile effects on bladder and inhibitory effects on urethral smooth muscle of mouse and pig. Planta Medica, 2, 148-151.

10. Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann N.Y. Acad. Sci., 51, 660-672.

11. Stone, K.R., Mickey, D.D., Wunderli, H. et al. (1978). Isolation of a human prostate carcinoma cell line (DU145). Int. J. Cancer, 21, 274-281.

12. Williams, R.D. (1980). Human urologic cancer cell lines. Invest. Urol., 17, 359-363.

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