



### Identification of a Prostate Inhibitory Substance in a Pollen Extract

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**ABSTRACT:** Recently, much attention has focused on the treatment of BPH with the pollen extract, Cernilton. The present investigation was designed to identify the active component in this agent which might be responsible for the symptomatic relief of BPH as previously reported [1,2]. Sequential purification of the active component present in the pollen extract was carried out by a combination of dialysis, gel filtration, and reverse phase chromatography. To monitor the biological activity of each of the purified fractions, a biological assay employing the human prostate cancer cell line DU145 was undertaken.

While we have identified a number of constituent components in the pollen extract, only one fraction designated V-7 (FV-7) maintained a strong inhibitory effect on the growth of DU145 cells. The inhibition was time- and dose-dependent, and the concentrations of FV-7 required to reduce the cell numbers by 50% (IC<sub>50</sub>) after 2 days of exposure was 5 µg/ml. FV-7 was also inhibitory towards the primary culture of prostate stroma and epithelial cells, with the stroma/fibroblast showing greater sensitivity towards the HPLC-purified component. However, it should be noted that this inhibitory activity measured in the primary culture cells was only achieved at higher concentrations of FV-7. Preliminary characterization of the active ingredient identified FV-7 as DIBOA which is a cyclic hydroxamic acid. FV-7 and DIBOA induce similar inhibitory effects on the growth of DU145 cells.

**KEY WORDS:** BPH, primary culture, Cernilton, fraction V-7, DIBOA

#### INTRODUCTION

Attention has recently focuses on an extract from rye pollen which was found to be most effective in the treatment of prostate diseases with no untoward side effects [1-3]. The pollen extract know as "Cernitin" is obtained by microbial digestion of the pollen followed by extraction with water and an organic solvent in a two-step process. Two fractions are consequently obtained: "T-60," containing the water-soluble substances and accounting for more than 80% of the total extracted material and "GBX," containing the fat-soluble substances. The two fraction T-60 and GBX are mixed in the final product designated "Cerniton" in a ration of 20:1 respectively.

Earlier studies on the water-soluble fraction, T-60, have shown that T-60 was inhibiting the growth of prostate cancer cell lines and primary cultures from BPH specimens [4,5]. In the

primary cultures, the inhibition was time- and concentration-dependent, with the fibroblast stomal component showing greater sensitivity to the pollen extract than the epithelial cells derived from the same BPH tissue [5].

The results from the in vitro studies seem to be backed up by pharmacological and clinical data. Pharmacological investigations have demonstrated a significant reduction (P<0.05) in the ventral and dorsal lobes of rat prostates after Cernitin was administered orally for 21 days [6]. Furthermore, in a double-blind placebo-controlled study, there was a significant decrease in residual urine in patients with Cernilton (P<0.025) and in the antero-posterior and transverse diameters of the prostate on ultrasound (P<0.025) following 6 months' treatment [2].

In an attempt to identify the growth inhibiting factor in T-60, fractionation was carried out

employing gel filtration and reverse phase chromatography. The eluted fractions were subsequently tested for their inhibiting effects on the prostate cancer cell line (DU145), and the active fractions singled out for further characterization and comparison with a known synthetic compound. Finally, the biological activity of the identified active substance was tested in primary cultures from BPH specimens.

## MATERIALS AND METHODS

### Chemicals and Purification Procedures

Cernitin T-60 was a gift from Cernitin SA, Lugano, Switzerland. The purification of the active compound present in T-60 was carried out by a combination of dialysis, gel filtration, and reverse phase chromatography steps. Details of the fractionation steps and of the chemical properties of the constituent product are the subject of a separate report [7; manuscript in preparation]. However, a brief summary of the strategy used is outlined in Figure 1. The synthesis of the active DIBOA compound was carried out by Professor U. Burger, Department of Organic Chemistry, University of Geneva, as detailed previously [8].

### Cell Culture

To monitor and evaluate the biological activity of each of the purified fractions detailed in Figure 1, a biological assay employing the human prostate cancer cell line DU145 [9] was undertaken. This was based on the earlier experiments which demonstrated an inhibition in DU145 growth following exposure to the pollen extract [4]. The conditions employed for the growth of these cells have previously been described [4,10,11].

### Primary Culture of Prostate Epithelial and Fibroblast Cells

Human BPH epithelial and fibroblast cells were cultured from prostate chips removed by transurethral resection. The epithelial and fibroblast cells were released from prostate tissue following overnight digestion in collagenase solution (600 IU/ml in 5% FCS RPMI 1640), and sub- and primary cultures were grown by plating onto plastic culture flasks and incubated at 37°C in a 95% air and 5% CO<sub>2</sub>-humidified atmosphere. By using this system it was possible to establish and serially culture

pure populations of both epithelial and fibroblast cells in well-defined media as detailed previously [5,12,13]. Verification of the cultures as prostatic fibroblast and epithelial cells has been confirmed by immunocytochemical staining employing a variety of antibodies, and also by phase contrast microscopy as described in our earlier work [5].

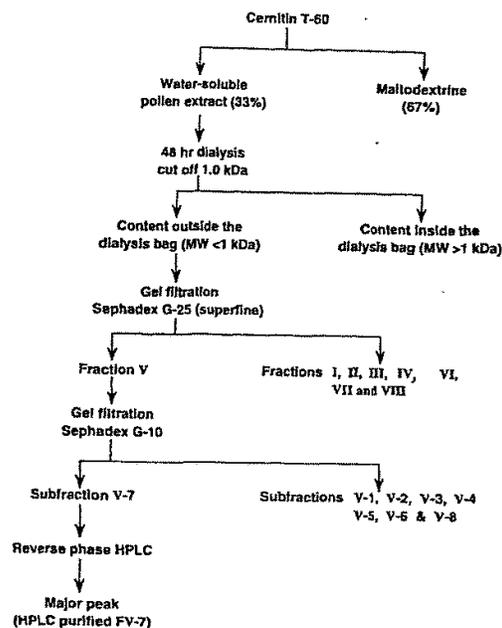


Fig. 1. Details of the fractionation steps and the strategy employed to isolate and purify the active ingredients in Cernitin T-60.

### Cell Growth and Thymidine Incorporation

Cell growth was monitored using thymidine incorporation, backed up well cell counting using the trypan blue exclusion method. Confluent DU145 as well as stroma and epithelial cells from 75-cm<sup>2</sup> tissue culture flasks were harvested and plated at a density of 1.5 x 10<sup>3</sup> cells/well in 96-well plates. After plating the cells, the Cernitin fractions (1-100 µg/ml) were added for periods of up to 6 days, with media changes on day 3; control wells received no pollen extract fractions. Following the incubation, cells were plated with thymidine and harvested, and radioactivity was counted as published previously [5,10,11]. The patterns obtained were also confirmed by cell count using the trypan blue exclusion method. In parallel experiments, the activity of the synthetic DIBOA compound was tested for its effects on the growth of DU145 cells and compared to the activity of the natural component isolated from Cernitin T-60.

## Statistical Analysis

Differences between control and test groups were examined for statistical significance by Student's *t* test.

## RESULTS

### Localization of the Active Ingredients in Cernitin T-60

At each step of the purification procedure (Fig. 1), aliquots of the fractionated substances were removed and tested for their DU145 inhibitory activity. Dialysis of the water-soluble component demonstrated that the activity was merely confined to the diffusate with an apparent molecular weight < 1 kD; the inert dialysate was therefore discarded. The diffusate was subsequently lyophilized and chromatographed on a G-25 column yielding eight well-resolved fractions, of which only fraction V (FV) exhibited potent inhibitory activity (Fig. 2; Table 1). FV was, in turn, eluted on a G-10 chromatography column resulting in eight subfractions of which only subfraction 7 (FV-7) manifested a significant inhibitory activity towards the DU145 cells (Fig. 3; Table 1). Further purification of subfraction FV-7 was carried out on a reverse-phase high-performance chromatography column resulting in one major peak which was strongly inhibitory towards DU145 cells (Fig. 4). This peak accounted for approximately 90% of the material loaded on the HPLC column [7].

The biological potency of each of the active fractions was compared to the starting T-60 material, and the IC<sub>50</sub> for each fraction was determined (Table 1). It is apparent from the data in Table 1 that the potency of the active substances increases markedly with each purification step, yielding a final product (FV-7) which is roughly 200 times more active than the starting T-60 product, and showing inhibitory activity at concentrations as low as 5 µg/ml (Table 1).

### Effect of Fraction FV-7 on DU145

The results depicted in Figure 4 demonstrate the impact of increasing concentrations of FV-7 (Fig. 4a) and DIBOA (Fig. 4b.) on the growth of DU145 cells at different days of incubation. While both FV-7 and DIBOA at 1µg/ml demonstrated no effect on cell growth,

increasing the concentration of either FV-7 or DIBOA to 10 µg/ml induced a strong inhibitory effect which was significantly different from control values ( $P < 0.001$ ) even after one day of exposure to either compound. However, the inhibitory activity of the natural product at 10 µg/ml appeared to be slightly more potent than that of the synthetic compound. Further incubation of the cells for longer periods and/or with higher concentrations of the extract totally inhibited growth and depleted cell numbers.

### Effects of Fraction FV-7 on Primary Culture of Prostate Epithelia and Fibroblast Cells

In addition to the studies on DU145, we have also examined the impact of the HPLC-purified FV-7 at various concentrations on the growth of primary culture of prostate epithelial and fibroblast cells obtained from patients with BPH. These studies were carried out over a period of 6 days.

The results depicted in Figure 5a,b demonstrate that FV-7 maintains a time- and concentration-dependent effect on both stroma and epithelial cells. At concentrations of 1 µg/ml, FV-7 stimulated DNA synthesis in the epithelial cells, including a 300% increase in thymidine incorporation ( $P < 0.001$ ) after 5 days' exposure. However, a dose-dependent decrease in DNA synthesis was also noted with concentrations >1 µg/ml. This was particularly evident in FV-7 at concentrations of 100 µg/ml, with the inhibition of the epithelial cells increasing with time of exposure and demonstrating an 80% inhibition following 4 days' treatment ( $P < 0.001$ ).

Experiments with primary culture of fibroblast cells yielded similar results to those described for the epithelium. Initially at a low concentration of FV-7 (1 µg/ml), the fibroblast cells were stimulated and thymidine incorporation increase by 90% after 5 days; treatment ( $P < 0.001$ ). However, at concentrations >10 µg/ml, FV-7 inhibited the growth of the fibroblast cells, with maximum inhibition being reached after 4 days' exposure.

## DISCUSSION

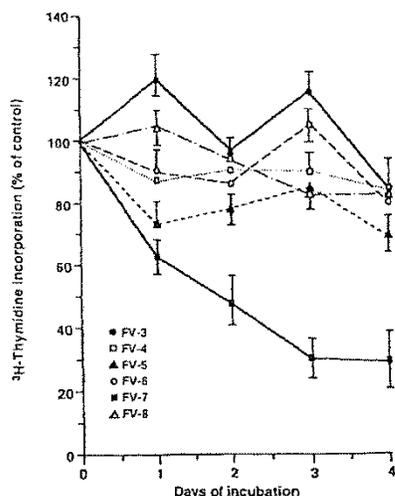
The commercial preparation, Cernilton, contains a pollen extract of which the water-soluble fraction, designated Cernitin T-60, is exceeding heterogeneous and comprises mainly low

**TABLE I. Identification of the Biologically Active Products in Cernitin T-60 Following Fractionation**

Method for fractionation	Active fraction <sup>a</sup>	Weight of active fraction (% of T-60)	IC <sub>50</sub> <sup>b</sup>
Initial product	T-60	100	1.0 mg/ml
Dialysis (cut off <1 kD)	Diffusate	60	0.8 mg/ml
Sephadex G-25	Fraction V	3.6	100 µg/ml
Sephadex G-10	Fraction V-7	0.3	10 µg/ml
Reverse phase HPLC	Fraction V-7 (HPLC-purified)	0.2	5 µg/ml

<sup>a</sup>As monitored by the DU145 cell proliferation test.

<sup>b</sup>Concentrations of active ingredient causing 50% growth inhibition of DU145 after two days of exposure.



**Fig. 3.** Growth of the androgen-insensitive DU145 human prostate cell line following treatment with subfractions of FV (100 µg/ml). Experiments were carried for periods of up to four days, and the results are expressed as the percentage of <sup>3</sup>H 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.

molecular weight components, most of which have not yet been identified. All of the biological investigations to date have been conducted using the whole unfractionated Cernitin T-60 extract. This extract was recently reported to inhibit in vitro the growth of various prostatic cancer cell lines and primary cultures of fibroblast and epithelial cells [4-5]. The main objective of the present investigation was to extend those studies by identifying the active agent(s) present in the pollen extract, and to investigate the biological activity of the pure substance(s).

The strategy of combining the biological assays with the fractionation techniques enabled us to pinpoint the precise component responsible for

inhibiting the prostate growth in vitro. The fraction designated FV-7 was shown to be inhibitory at a concentration as low as 5 µg/ml, and this is closely comparable to the concentrations of most other drugs used in in vitro assays. It was also of interest to note that FV-7 accounts for only 0.3% (w/w) of the total T-60 pollen extract, a value based on the combined material recovered from all fractions following the initial lyophilization. However, because of the losses incurred after every fractionation step, estimated at around 30% for each of the gel permeation chromatography and HPLC steps, it would be more realistic to assume that the concentration of FV-7 in the whole pollen extract may be close to 1% (w/w). Such a percentage is compatible with the growth inhibition data obtained with T-60 where inhibition >50% was recorded in the presence of 1 mg/ml of the original material [4].

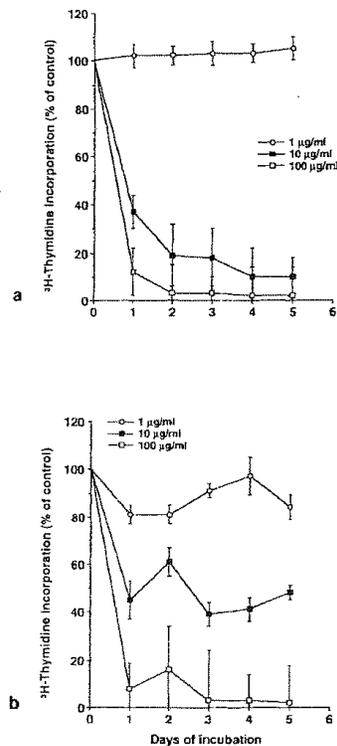
The inhibitory effects of FV-7 on prostatic tumor cell growth appear to be dose- and time-dependent. After the initial exposure to FV-7, DU145 cells stop growing and dividing, an effect which can persist for at least nine days. Following reverse-phase HPLC, purified FV-7 at concentrations as low as 10 µg/ml induced significant inhibition of the DU145 cells, even after two days' exposure. The structure of FV-7 has been elucidated by mass spectrometry and nuclear magnetic resonance. The bulk of FV-7 (over 95%) was identified as DIBOA (2,4-dihydroxy-2H-1,4-benzoxazine-3(4H)-one; Fig. 6), a cyclic hydroxamic acid [7] which was originally found in most members of the Gramineae family of plants [14]. Up to now, the physiological properties of DIBOA had not been clearly elucidated, although its role as a phytotoxic agent has been suggested [15,16]. Furthermore, several laboratories have evaluated the antitumor activity of hydroxamic acid. It has

been shown that these may act as inhibitors of ribonucleotide reductase activity [17-19], but whether this is their mode of action in the human prostate still remains to be established. However, it was interesting to note that the inhibitory activities of FV-7 towards the prostate DU145 cells mimicked those of the synthetic DIBOA.

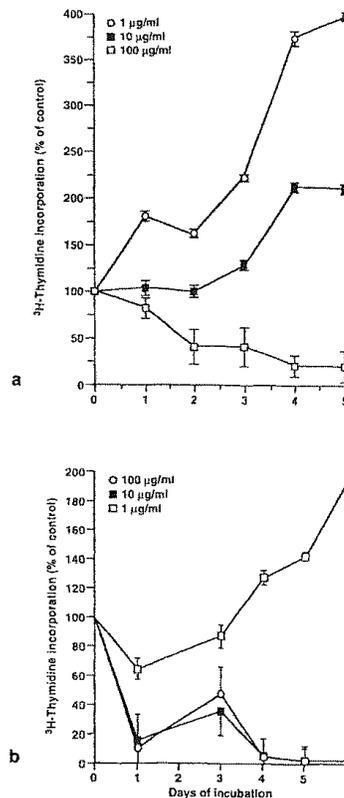
Although the usage of immortal cell lines has been most helpful in identifying the active inhibitory agent in the Cernitin T-60, their use is somewhat limited because of: a) the neoplastic nature of the continuous cells, while Cernilton is prescribed purely for BPH [3]; b) immortal cell lines are identical clones and do not therefore take account of the morphological heterogeneity of the prostate [20]; and c) continuous cell lines may undergo phenotypic changes and this might

render them distinctive from the cells of origin [21]. In view of these limitations, we have decided to continue our work with the HPLC-purified Cernitin T-60 subfraction FV-7, employing the well-established primary cultures of epithelial and fibroblast cells from human hyperplastic prostates [5,12-13]. Those studies were facilitated by our abilities to establish and serially culture pure populations of epithelial and fibroblast cells in a well-defined serum-free medium [5].

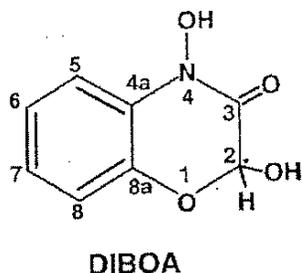
The results outlined in this manuscript demonstrate that the HPLC-purified subfraction FV-7 acts on both epithelial and stromal cells in a dose-dependent fashion. At low concentrations, we have observed a stimulatory



**Fig. 4.** The effect of time of exposure (1–6 days) to FV-7 (HPLC-purified, 1–100 µg/ml, a) and DIBOA (1–100 µg/ml, b) on DNA synthesis in the androgen-insensitive DU145 prostate cell line. The data are expressed as percent of <sup>3</sup>H-thymidine incorporation relative to the untreated control. Each point represents the mean of three separate experiments, each run eight times. Bars represent coefficient of variation.



**Fig. 5.** The effect of HPLC-purified FV-7 at various concentrations on the cell growth of primary culture of epithelial (a) and fibroblast (b) cells. The data is normalized relative to the untreated control (100%), and each point represents the mean of three separate experiments, each run eight times. Bars represent coefficient of variation.



**Fig. 6.** The structure and formula of DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one).

effect, but this is totally reserved at higher concentrations when the active factor induces an inhibitory effect on both cell types. The reasons for the initial stimulation of DNA synthesis at the lower doses of FV-7 (<1 µg/ml) is not very clear, but it is significant that similar patterns have been observed with other herbal medicines [22] and may be related to an increase in cells in the A<sub>0</sub> or D<sub>3</sub> regions of the cell cycle [23] at the lower doses of FV-7. Additional studies are currently underway to elucidate the exact mechanism(s) responsible for this phenomenon. However, the stroma cells appear to be far more sensitive to exposure to this factor than the epithelium, which requires 10 times the concentrations of FV-7 to induce a comparable inhibitory effect. Since the human BPH is predominantly a stromal hyperplasia, the greater susceptibility of the stromal component to the Cernitin factor highlights the potential usefulness of this drug in the management of BPH. Efforts are now directed at identifying its mode of action and at the possibility that this is mediated via growth factors known to induce BPH pathogenesis [24].

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Becker H, Ebeling L: Phytotherapy of BPH with Cernilton N—Results of a controlled prospective study. In Rutishauser G, Vahlensieck W (eds): "Benign Prostatic Disease." Stuttgart, New York: George Thieme Verlag, 1992, pp 175-179.
2. Buck C, Cox R, Rees RWM, Ebeling L, John A: 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, 1990.
3. Rugendorff EW, Weidner W, Ebeling L, Buck AC: Results of treatment with pollen extract (Cernilton) in chronic prostatitis and prostatodynia. *Br J Urol* 71: 433-438, 1993.
4. Habib FK, Ross Maragret, Buck AC, Ebeling L, Lewenstein A: In vitro evaluation of the pollen extract, Cernitin T-60, in the regulation of prostate cell growth. *Br J Urol* 66:393-397, 1990.
5. Habib FK: Regulation of prostate growth in culture with the pollen extract, Cernitin T-60, and the impact of the drug on the EGF tissue profiles. In Rutishauser G, Vahlensieck W (eds): "Benign Prostatic Diseases". Stuttgart, New York: George Thieme Verlag, 1992, pp 120-128.
6. Ito R, Ishi M, Yamashita S, Noguchi K, Kunikane K, Ohkubo Y, Tsushima K, Sato S, Akamatsu H: Cernitin pollen-extract (Cernilton), anti-prostatic hypertrophic action of Cernitin pollen extract. *Pharmacometries* 31:2-14, 1986.
7. Zhang X: Isolation and characterization of a cyclic hydroxamic acid from a pollen extract which inhibits cancerous cell growth in vitro. MD Thesis No. 1276, University of Geneva, 1992.
8. Sicker D, Praetorium B, Mann G, Mayer L: A convenient synthesis of 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one). *Synthesis* 3:211-212, 1989.
9. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF: Isolation of a human prostate carcinoma cell line (DU145). *Int. J Cancer* 21-274-281, 1978.
10. MacDonald A, Chisholm GD, Habib FK: Production and response of a human prostatic cancer line to transforming growth factor-like molecules. *BR J Cancer* 62: 579-584, 1990.
11. MacDonald A, Habib FK: Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *BR J Cancer* 65: 177-182, 1992.
12. Chaproniere DM, McKeehan WL: Serial culture of single adult human prostate epithelial cells in serum free medium containing low calcium and a new growth factor from bovine brain. *Cancer res* 46-819-824, 1986.
13. Peehl DM, Stamey TA: Growth responses of normal benign hyperplastic and malignant human prostatic epithelial cells in vitro to cholera toxin, pituitary extract, and hydrocortisone. *Prostate* 8:51-61, 1986.
14. Zuniga GE, Argandona VH, Niemeyer HM, Corcuera LJ: Hydroxamic acid content in wild and cultivated gramineae. *Phytochemistry* 22: 2665-2668, 1983.
15. Queirolo CB, Andreo CS, Vallejos RH, Niemeyer HM, Concuera LJ: Effects of hydroxamic acids isolated from Gramineae on adenosin 5'-triphosphate synthesis in chloroplasts. *Plant Physiol* 68: 941-943, 1981.
16. Queirolo CB, Andreo CS, Vallejos RH, Concuera LJ: Inhibition of ATPase from chloroplasts by a hydroxamic acid from the gramineae. *Phytochemistry* 22: 2455-2458, 1983.
17. Elford HL, Wampler GL, Riet BV: New nucleotide reductase inhibitors with antineoplastic activity. *Cancer Res* 39: 844-851, 1979.
18. Elford HL, Van't Riet B: Inhibition of nucleotide diphosphate reductase by hydroxybenzohydroxaminic acid derivative. *Pharmacol Ther* 29: 239-254, 1985.
19. Tihan T, Elford HL, Gory JC: Studies on the mechanisms of inhibition of L1210 cell growth by 3,4-dihydroxybenzamidoxime. *Adv Enzyme Regul* 31: 71-83, 1991.
20. Cunha JR, Donjacour AA, Cooke PS, Mees S, Bicksby RM, Higgins SJ, Sugimore Y: the endocrinology and

- developmental biology of the prostate. *Endocr Rev* 8: 338-363, 1987.
21. Mori H, Maki M, Oishi K, Jaye M, Igarashi K, Yoshida O, Hatanaka M: Increased expression of genes for basic fibroblast growth factor and transforming growth factor type  $\beta$ 2 in human benign prostatic hyperplasia. *Prostate* 16: 71-80, 1990.
  22. Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogaswara S, Momsaki S, Kojiro M: The herbal medicine sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. *Cancer Res* 54: 448-454, 1994.
  23. Tone S: Programmed cell death and cell cyce. *Cell* 21: 1290133, 1989.
  24. Merchant JD: Requirements of in vitro models systems. *Prog CLin Biol Res* 37: 3-7, 1980.