Report on the Development of HP8 a Natural Herbal Formulation for Prostate Health

Prepared for InterHealth Biosciences Australia (IBA) by Professor P. G. Waterman DSc, PhD, FRS(E)*[*]

Background

This report covers the major research undertaken by the Centre for Phytochemistry, Southern Cross University, Lismore, NSW, Australia to support the development of IBA's HP8 formula.

Starting Point

During the development phase, the HP8 formulation was provided to the Centre for Phytochemistry during the development phase for medical and scientific evaluation. The biological activity of HP8 was first evaluated against a number of standard cancer cell lines. Specifically, P_{388}, HL60 and a prostate cancer (PC-3) cell-lines to establish whether it had the capability to influence the proliferation of these cell-lines in vitro at the recommended dosage. This experimental method represents a standard bioassay for the detection of cytotoxic and/or cytostatic activity against different types of cancer cells. The procedure is explained in detail in Method 1.

Method 1. Cells were split into 10 cm^2 tissue culture tubes at a low concentration and allowed to grow till confluent. Media was DMEM with 10% horse sera (GIBCO) with glutamine and pen/strep (GIBCO) added tubes placed in a 10% CO_2 incubator. Cells are then transferred to tissue culture plates in 99 µL media. Samples for testing (in triplicate) are then added, 1 µL into each well ranging from 1 mg/mL to 0.001 mg/mL. The ATPLite (Packard) assay is based on the presence of ATP in living cells and is measured using luminescence readings on the Wallac Microbeta.

HP8 was observed to inhibit cell growth, to some degree, at the doses tested except the lowest dose (0.001 mg mL^{-1}) and at a level of 1 mg mL^{-1} (≡ 0.1%). The dose of 1 mg mL^{-1} resulted in the complete inhibition of cell growth in the cell lines tested. The inhibition is linked to the disruption of the mitotic spindle, which prevents cell division. The ATPLite assay, (an analysis of ATP production), cytotoxicity, and as a marker of cell death, indicated that HP8 in concentrations equivalent to recommended dosages produced cell cycle arrest at the G_2/M phase and eventual apoptosis (cell death) within 24 hours.

[*] Professor Waterman was formerly Director of the Centre for Phytochemistry, Southern Cross University and before that Head of Natural Products Research of the Strathclyde Institute for Drug Research in Glasgow, Scotland. He is an international expert on the chemistry and biological activity of medicinal plants and has over 400 refereed research publications.

HP8 Mechanism for Cell Growth Inhibition

The initial study confirmed that HP8, at higher dose levels, inhibited cell growth and the next logical step was to establish the mechanism of cell growth inhibition. The process by which cells divide and DNA is replicated conforms to a standard set of events in human body cells and involves a number of clearly identifiable phases.

Cancerous cells are fast dividing cells and so will exhibit a well-defined cell cycle, which is almost always separated into the several distinct phases illustrated in Figure 1. It starts at the G_1 phase or the first gap phase. If the cells are permanently arrested in G_1, as in non-dividing cells, we call this the G_0 phase. At G_1 the cell contains two copies of each chromosome. As the cell progresses from G_1 phase it enters the synthesis or S phase, and during this phase DNA is replicated. DNA replication represents a critical stage in the total cell division process.

When the replication is completed the cell enters the second gap phase or G_2 phase. At the completion of G_2 phase the cell is ready to enter mitosis and at this point the cell divides to form two new cells. Using cell cycle analysis we can identify the events into major phases, such as G_0-G_1 and G_2-M cell cycle inhibition. Many drugs used in "the fight against cancer" influence the cell cycle at these critical points.

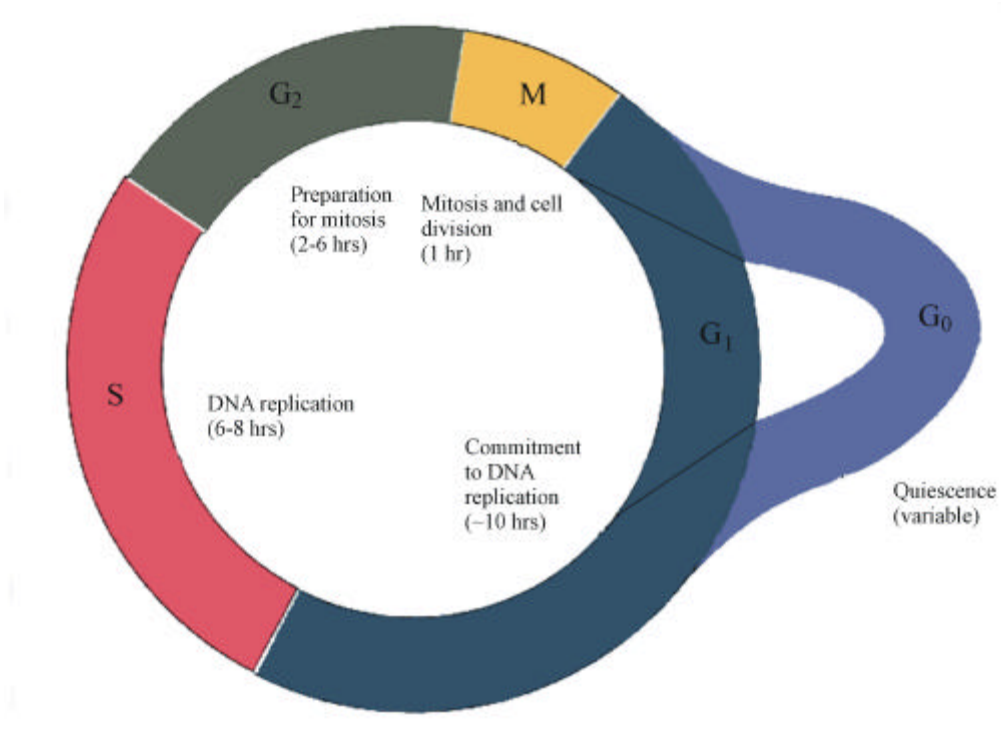


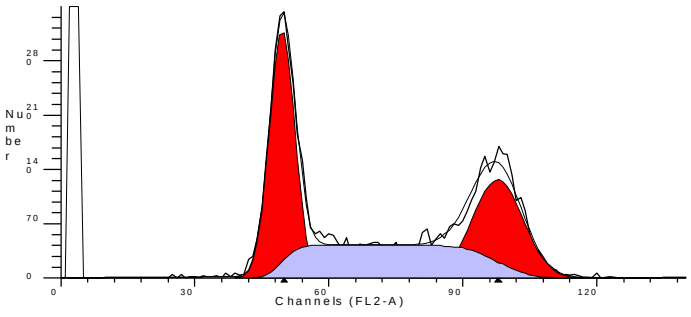
Figure 1. A model of the Cell Cycle.

The changes induced during cell division by a compound or product, such as HP8, can be studied using the technique of flow-cytometry. This experimental method can sort a cell population into the various phases in which they exist after a treatment by a specific compound. A standard experimental method using cell cytometry is explained in Method 2.

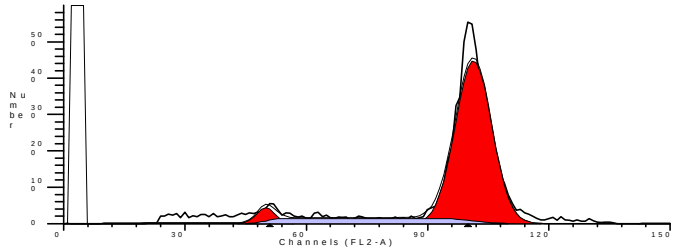
Method 2. A known weight of the test product was extracted with 5 mL of methanol. The extracts were sonicated for 15 minutes and then spun down at 4000 rpm. The supernatant was removed the extract was used in the cell cycle analysis. Cells were split into 16 x 25 cm^2 flasks at a low concentration and allowed to grow to 65-70% confluency (at the time of addition the cells were in log phase). Flasks were incubated for 24 hour and control flasks of Control media (no additions), a Control ethanol, a Control methanol and Taxol (10µL of 0.01 mg/mL=0.01ng/mL) were also run. Flow cytometry was performed on the fixed cells after washing in PBS and stained using propidium iodide (2% PI in 0.1% Triton X-100 containing 2 mg/mL Rnase A). A Becton Dickson FACSCaliber was used to assay the cells.

The results for control cells (which provide an experimental reference point), cells treated with Taxol (10 ng mL^{-1}), and cells treated with HP8 are illustrated and compared in Figure 2. The area under the curves (in red) indicates the proportion of cells in the sample, which are present in G_1/G_0 and G_2/M phases.

a) Control **G_1/G_0** **G_2/M**



b) Taxol **G_1/G_0** **G_2/M**



c) HP8 **G_1/G_0** **G_2/M**

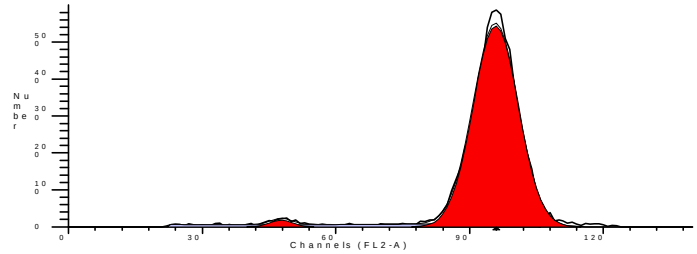


Figure 2. Cell Cycle analysis results.

Note: Taxol concentration: 0.1ng/10ml of cell culture; HP8 concentration: Extract equivalent to 5.88mg of tablet/10ml of cell culture.

The results shown in Figure 2 indicate in a) control, the normal proportion of cells to be found in the G_1/G_0 and in the G_2/M phases of the cell cycle; b) Taxol, a widely used drug for the treatment of breast cancer, works by arresting cell division in the G_2/M phase and this effect is demonstrated clearly by the graph; and c) results for HP8 clearly indicate that almost all cells were exhibiting cell cycle arrest at the G_2/M phase (>95%). This demonstrates that HP8 has a specific mode of action that is similar to Taxol.

Comparison of HP8 with PC-SPES

As HP8 would compete with PC-SPES in the marketplace at the time when this research was commissioned, a direct comparison of HP8 with PC-SPES was requested by IBA using in vitro cancer cell lines.

Cell Cycle Analysis using Prostate Cancer Cell Line PC3

Extracts of HP8 and PC-SPES were examined following Method 2. These results this experiment are displayed in Figure 3 and Figure 4. The cell cycle result for HP8 is shown in Figure 3 and that of a comparable dose of PC-SPES in Figure 4. The graphs indicate clearly that mode of action for HP8 is very significant for G_2/M cell cycle arrest when compared to PC-SPES, and once again HP8's bioactivity is very similar to the curves produced by Taxol.

HP8

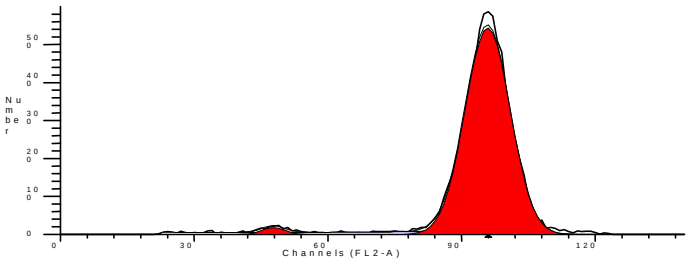


Figure 3. Cell Cycle analysis for HP8.

Note: HP8 concentration: Extract equivalent to 5.88mg of tablet/10ml of cell culture.

The curves for PC-SPES indicate the cell cycle arrest is distributed over both the G_0/G_1 and G_2/M phases with a slightly higher proportion of cells arrested at the G_2/M phase. This distribution for cell cycle arrest is very different from the curves produced by Taxol suggesting the mode of action for PC-SPES is different from this frequently used cancer drug.

PC-SPES

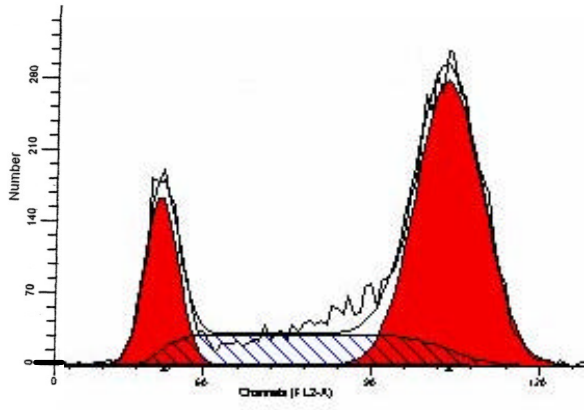


Figure 4. Cell Cycle analysis for PC-SPES.

Note: PC SPES concentration: Extract equivalent to 6.4mg of tablet/10ml of cell culture (figure 4).
The % G_2/M inhibitions from further experiments of HP8 and PC-SPES at the concentrations given below were:
PC-SPES - Extract equivalent to 12.80mg of tablet/10ml of cell culture: 59.64%
HP8 - Extract equivalent to 11.76mg of tablet/10ml of cell culture: 98.28%

Testing Bioactivity by Cell Cycle Analysis Against a Range of Cancer Cell Lines
Cell cycle analysis was carried out using HP8 in tablet form and PC-SPES on additional cancerous cell lines. Specifically, liver cancer (HepG2), human leukaemia (HL60) and an androgen-dependent prostate cancer (LNCap). The experimental methods used were slightly modified from those reported previously in Method 1 and Method 2, and the detail is provided in Method 3.

The results are presented Tables 1 to 3 using percentage changes in the cell cycle phases for each cancer cell line tested. The two products were tested using a series of four concentrations and the results are expressed in terms of the percentage of cells that are found in either the G_0/G_1 or G_2/M phase. Each experiment was performed in triplicate.

Method 3. Known weights were extracted with 50 mL of methanol. The extracts were sonicated for 30 minutes and then spun down at 4000 rpm . The supernatant was removed and dried and then dissolved in MeOH and used in the cell cycle analysis at concentrations of 0.4, 0.8, 1.6, and 3.2 mg/10 mL. Cells were split into 25 cm^2 flasks at a low concentration and allowed to grow to 65-70% confluency (at the time of addition the cells were in log phase). Flasks were incubated for 24 hour and control flasks of Control media (no additions), a Control ethanol (100 µL ethanol), a Control DMSO (100 µL DMSO) and Taxol (10µL of 0.01 mg/mL=10 ng/mL) were also run.

Flow cytometry was performed on the fixed cells after washing in PBS and dyed using propidium iodide (2% PI in 0.1% Triton X-100 containing 2 mg/mL Rnase A). A Becton Dickson FACSCaliber was used to assay the cells.

Table 1. Percentage Changes in G_0/G_1 and G_2/M for HP8 and PC-SPES on HL60 Cell Lines.

Treatment	Concentration (mg $10mL^{-1}$)	G_0/G_1	G_2/M
"HP8"	0.4	31.65	13.74
	0.8	32.44	12.07
	1.6	28.65	21.89
	3.2	12.34	52.68
PC-SPES	0.4	33.53	14.30
	0.8	2.40	62.73
	1.6	8.95	45.23
	3.2	45.78	26.99
Control		34.79	12.77

Table 2. Percentage Changes in G_0/G_1 and G_2/M for HP8 and PC-SPES on HepG2 Cell Lines.

Treatment	Concentration (mg $10mL^{-1}$)	G_0/G_1	G_2/M
"HP8"	0.4	61.97	14.74
	0.8	60.14	16.50
	1.6	58.94	13.70
	3.2	54.16	14.86
PC-SPES	0.4	58.63	16.50
	0.8	37.06	37.91
	1.6	16.12	72.46
	3.2	15.18	76.72
Control		61.05	16.04

Table 3. Percentage Changes in G_0/G_1 and G_2/M for HP8 and PC-SPES on LNCap Cell Lines.

Treatment	Concentration (mg 10mL^{-1})	G_0-G_1	G_2-M
"HP8"	0.4	62.72	10.83
	0.8	72.23	9.91
	1.6	10.88	79.69
	3.2	10.73	77.23
PC-SPES	0.4	77.36	13.24
	0.8	55.64	36.40
	1.6	53.49	38.51
	3.2	49.35	25.07
Control		80.79	5.31

The results displayed in Tables 1 to 3, indicate clearly extracts from HP8 and PC-SPES influenced the cell cycle of HL60 and LNCap cell lines, where there was an increase in G_2/M arrest and consequent decrease in G_0-G_1 arrest. However, only PC-SPES displayed a G_2/M effect on HepG2 cells.

The results for the HL60 cell line (Table 1) indicate HP8 was effective at the highest concentration (3.2 mg/10 mL), producing a G_2/M effect of 52.68%. PC-SPES was effective at lower concentrations but did not give an equivalent G_2/M arrest at the higher concentration. A reasonable explanation for the observation of greater G_1/G_0 cells in highest concentration of PC-SPES is that at this level PC-SPES was causing non-specific cytotoxicity.

One of the interesting results was the lack of G_2/M effect by HP8 at all concentrations tested on the HepG2 cell line (Table 2). In contrast, PC-SPES interrupted cell division at all concentrations from 0.8 mg/10 mL upward. This may simply reflect a greater resistance by this cell line to compounds in HP8 and a more specific action by HP8 on the other cell lines tested, especially the prostate cancer cell lines.

An important finding is the very significant G_2/M affect (77.23 and 79.69 %) produced by HP8 on the LNCap androgen-dependent prostate cancer cell line at concentrations of 3.2 and 1.6 mg/10 mL (Table 3). This was not observed in the PC-SPES extracts, which exhibited only a very weak G_2/M effect. Since the LNCap is an androgen-dependent and an estrogen-dependent prostate cell line, it had been anticipated that PC-SPES would have had a more significant impact due to the greater estrogen binding activity observed in PC-SPES and which will be addressed in the following section. This result confirms that the formulation of HP8 has increased the susceptibility of the LNCap cell line to an interruption of the cell cycle at the G_2/M phase. This important result indicates a significant advantage for HP8, as it confirms that HP8 has a broader spectrum of activity against different types of prostate cancer cells.

Estrogenic Effects

The two primary side effects observed while taking PC-SPES were gynecomastia (tender and enlarged breasts) and reduction in libido. Both are very disturbing side effects for males. These were attributed to the presence of components causing enhanced levels of estrogenic activity. It had been reported previously that PC-SPES had significant estrogenic activity as determined by both ß-galactosidase assay in a yeast strain and the increase in uterine weights in ovariectomized CD-1 mice (Di Paola et al., 1998).

The Centre for Phytochemistry developed an in vitro bioassay (refer to Method 4) to determine if the herbal extracts that constitute PC-SPES had any binding activity towards the estrogen (E_2) binding site on the MCF-7 cell line. The MCF-7 is a breast cancer cell line and was chosen because of the ability to express E_2 binding sites, and therefore binding effects. A competitive binding of ^{3}H 17-ß-estradiol against cold 17-ß-estradiol was demonstrated by this assay (data not shown). As expected both diethyl stilboestrol (DES) (100 µM) and diadzein (197 µM) bind to the E_2 receptor, displacing over 85% and 60% of the ^{3}H E_2, respectively (refer to Table 4).

PC-SPES also causes inhibition of ^{3}H 17-ß-estradiol binding to E_2 receptors in MCF-7 cells. The binding response occurs in a dose dependent manner, with no inhibition occurring at 0.1 µg mL^{-1} and over 80% inhibition at 100 µg mL^{-1}. Di Paola et al. (1998) reported that PC-SPES had potent estrogenic activity in yeast, mice and humans. Although the nature of this experiment does not allow any claim on the in vivo estrogenic activity of PC-SPES, it can be report that it does inhibit binding of ^{3}H 17-ß-estradiol in MCF-7 cells at a similar concentration to that of the known estrogen DES (100 µM ~ 26.83 µg mL^{-1}).

The HP8 formulation results indicated less than 30% inhibition of ^{3}H 17-ß-estradiol binding to E_2 receptors at the highest concentration tested (100µg mL^{-1}) compared to a significant 80% for PC-SPES. The other concentrations of HP8 had little or no effect on the inhibition of ^{3}H 17-ß-estradiol binding, whereas this response could be said for only the lowest dose of PC-SPES. From these results it cannot be predicted with any certainty whether HP8 will have estrogenic activity in whole animals or humans, however, it can be inferred that since there is little or no binding to the E_2 receptors (at the concentrations tested) it would seem unlikely that estrogenic activity would occur. These results also suggest strongly, that the majority of adverse side-affects seen in PC-SPES are due to its estrogenic activity, and it would follow that in HP8 such disturbing side effects (breast tenderness, breast enlargement and loss of libido) should be minimised.

Method 4. PC-SPES was extracted with methanol by sonication for 30 minutes, centrifuged and the supernatant removed. The extract was dried under vacuum and made up to a concentration of 100 mg mL^{-1}. MCF-7 cells were grown in a 175 cm^2 flask till 70% confluent. The cells were then trypsinized and centrifuged at 200g for 3 min. The pellet was resuspended in colour free RPMI media, containing charcoal stripped FBS (hormone free FBS). The cells were then plated out into 24 well plates at 750µL per well and incubated overnight at 37°C and 5% CO_2.

^{3}H 17-ß-estradiol (E_2) was used at a final concentration in the wells of 1 nM in media. Samples were incubated for 3 hours after which the wells were aspirated, washed 3 times with warm PBS. Cell bound radioactivity was extracted with 300µL ethanol which was added to each well and left to lyse for 20 min at room temperature. An aliquot was removed for counting, transferred to a counting plate and scintillant added and the plate counted in a Perkin Elmer MicroBeta. Diethylstilbestrol (DES) (100 µM) and diadzein (197 µM) were used as positive binding controls. Cold 17-ß-estradiol (non-radioactive) (0.051-12.5 nM) was used as a competitor for the E_2 binding sites.

Table 4. Comparison of the Estrogenic Activity for HP8 and PC-SPES.

Test Product	Concentration	Inhibition of estradiol binding (%)
DES	100μM	86
Diadzein	197μM	64
HP8	0.1 μG ml^{-1}	0
	1 μG ml^{-1}	2
	10 μG ml^{-1}	5
	100 μG ml^{-1}	27
PC-SPES	0.1 μG ml^{-1}	3
	1 μG ml^{-1}	30
	10 μG ml^{-1}	62
	100 μG ml^{-1}	82

Current Research, Quality Control and Quality Change

The most recent research and quality control data, using IBA's latest HP8 formulation and testing varied concentrations of HP8 on the PC-3 androgen independent prostate cancer cell line, confirmed the G_2/M mechanism of action. The results indicated extremely high bioactivity for the commercial batch of HP8, with a very significant cell cycle arrest of 98.22% at the G_2/M phase at concentrations of 1.6 and 3.2 mg/10mL. The bioactivity, which is very specific for prostate cancer cell lines (PC-3 and LNCap) can be confirmed when HP8 effects are evaluated against leukaemia (HL60) and liver cancer (HepG2) cell lines. This is presented graphically in Attachment A (attached at end of document).

Conclusion

HP8 was developed by using widely accepted scientific testing protocols and laboratory monitoring of a range of in vitro effects that are considered to be relevant in assessing bioactivity, potential toxicity, side effects and safety as well as potential therapeutic applications for enhancing prostate health. The findings of these in vitro experiments demonstrate clearly that HP8 is active against hormone dependent and hormone independent prostate cancer cell lines and displays minimal estrogenic side effects. The experimental methods developed and utilised by the Centre for Phytochemistry, in vitro bioassay methods, can now be applied to quality assurance procedures, which will ensure HP8 batch-to-batch consistency of activity within the HP8 formulation that is commercially available.

References

DiPaola, R.S., Zhang, H., Lambert, G.H., Meeker, R., Licitra, E., Rafi, M.M., Zhu, B.T., Spaulding, H., Goodin, S., Toledano, M.B., Hait, W.N. and Gallo, M.A. Clinical and Biology Activity of an Estrogenic Herbal Combination (PC-SPES) in Prostate Cancer. New England Journal of Medicine (1998) 339, 785-791.

Executive Summary

- HP8 shows clear evidence for in vitro activity in arresting and inhibiting the growth of prostate cancer cells, both hormone dependent and hormone independent cells.

- The mechanism by which HP8 exerts this effect is specific to the cell cycle and cell division mechanisms and not by indiscriminate toxicity.

A comparison between the product PC-SPES and HP8 revealed the following:

- Both products exhibited the same mode of activity in arresting the growth of prostate cancer cells, however PC-SPES exhibited indiscriminate cell toxicity at higher dose levels whilst HP8 did not. As levels tested reflected recommended dosage to be administered to people, this indicates that HP8 should have a greater margin of safety before any side effects are observed.

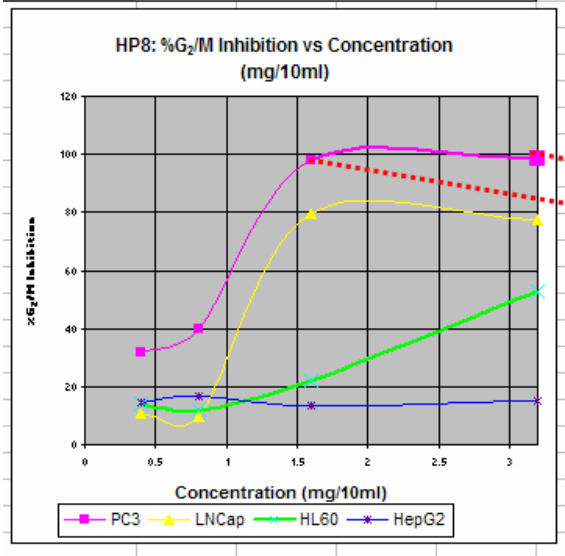
- A comparison of the estrogen-binding activity of PC-SPES and HP8 demonstrated that the HP8 binding activity is greatly reduced, whereas PC-SPES binding activity is very high. This suggests that HP8 will not exhibit the significant and disturbing hormone-based side effects in males that occur with PC-SPES.

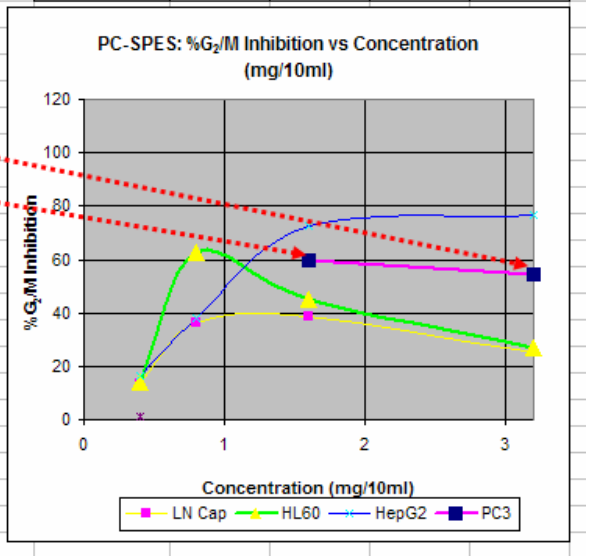
Attachment A

Comparison Between HP-8 and PC-Spes: Specificity Towards Prostate Cancer Cell Lines (PC3-hormone dependent and LNCap-hormone independent) in Comparison To Other Types of Cancer Cell Lines

Concentration mg/10ml	Cancer Cell Lines Used To Determine The Specificity of HP8 Towards Prostate			
	PC3	LNCap	HL60	HepG2
0.4	31.87	10.83	13.74	14.74
0.8	39.83	9.91	12.07	16.5
1.6	98.175	79.69	21.89	13.7
3.2	98.28	77.23	52.68	14.86

Concentration mg/10ml	Cancer Cell Lines Used To Determine The Specificity of PC-SPES Towards Prostate			
	PC3	LNCap	HL60	HepG2
0.4		13.24	14.3	16.5
0.8		36.4	62.73	37.91
1.6	59.64	38.51	45.23	72.46
3.2	54.31	25.07	26.99	76.42





Arrow Indicates The Huge Difference in Bioactivity At Equivalent Concentrations For HP8 (left) and PC-SPES (right)

Note:Values for PC-SPES using the PC3 cell line are based on very close approximation (at 1.08 times the HP8 concentration).