The effect of statins on proliferation of PC-3 prostate cancer cell line in the presence of DHT.

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JANTUNEN ERIKA: STATIINIEN VAIKUTUS ETURAUHASSYÖVÄN PC-3 SOLUJEN KASVUUN DHT:N LÄSNÄOLLESSA.

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Eturauhassyöpä on miesten yleisin syöpä ja johtaa vuosittain 900:aan kuolemaan Suomessa.

Androgeenit ovat eturauhasen solujen pääasiallisia kasvun säätelijöitä. Androgeeneista testosteroni on elimistön pääasiallinen hormoni, mutta eturauhasessa testosteroni muutetaan 5-alfa-reduktaasin avulla dihydrotestosteroniksi, jonka sitoutumiskyky soluliman androgeenireseptoriin on monikymmenkertainen verrattuna testosteroniin.

Eturauhassyövän pääasiallisena hoitomuotona tällä hetkellä kemiallinen kastraatio LHRH analogeilla. Useimmat levinneet eturauhassyövän muodot kuitenkin mutatoituvat myöhemmin resistenteiksi LHRH analogeille toistaiseksi tuntemattomasta syystä.

Androgeenireseptorin on havaittu mutatoituvan syövän edetessä usealla tavalla, mm. kehittävän herkistymistä aktivoiville aineille. Lisäksi on epäilty että eturauhassyöpä solut kehittävät lopulta kyvyn tuottaa androgeeneja itsenäisesti, eli de novo androgeenisynteesin.

Statiinien eli HMG-CoA reduktaasin estäjien on havaittu estävän eturauhassyövän etenemistä kastraatioresistentiksi. Syytä tälle ilmiölle ei kuitenkaan tunneta, mutta erään teorian mukaan ne estävät androgeenireseptorin aktivaatiota androgeenien läsnäollessa, sekä mahdollisesti myös de novo androgeenisynteesiä.

Tutkimuksessa käytetty PC-3 solulinjan tiedetään yleisen käsityksen mukaan olevan androgeeneistä riippumaton solulinja, joka ei enää ekspressoi androgeenireseptoria. Tutkimuksen tarkoitus on selvittää lähtötilanne DHT.n ja statiinien yhteisvaikutuksista soluissa, jotka eivät enää ekspressoi androgeenireseptoria.

Tutkimuksessamme havaitsimme, että Statiineilla ja DHT:llä on yhteisvaikutusta myös PC-3 soluissa, sillä Statiinit kykenivät estämään DHT:n solutoksisen vaikutuksen korkeissa konsentraatioissa (100nM). Tämä ilmiö vaatii kuitenkin tarkempaa selvittelyä myös PCR-tekniikalla, jolla voidaan selvittää tarkemmin, mistä vaikutus johtuu.

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1 Introduction

1.1 Prostate cancer

Prostate is a part of the male reproductive system. The primary function of which is to secrete components of the seminal fluid such as PSA (prostate specific antigen)(1). In a fetus the gland is formed from the urogenital sinus. (2) The prostate contains neuroendocrine cells and epithelial cells which are divided into basal and luminal cells.(3)

Prostate cancer is currently the most common cancer in male population in developed countries. In Finland the incidence rate is 85.6/100 000 per year. It is also the second deadliest cancer, resulting in a yearly death toll of almost 900 people. (4) There are currently secondary treatment methods, but none for primary prevention. Established risk factors for prostate cancer include age, race and family history, none of which are modifiable. Thus primary prevention of prostate cancer is currently not possible. (4)

Prostate cancer has been shown to arise from epithelial cells, however it remains unclear whether the tumor arises from luminal or basal cells (3). Prostate cancer has a unique tendency to develop multiple tumor foci within the gland, hence making it more difficult to separate the primary tumor cell line. As the tumor develops, the tumor mass becomes unorganized, and eventually penetrates the surrounding structures and thus metastasizes easily. The main sites for metastases are lymph nodes and bones of the pelvis and spine. (5)

Treatment of prostate cancer depends on tumor stage. Currently the curative treatment options are radical prostatectomy, radiation therapy combined with luteinizing hormone releasing hormone (LH-RH) analogs and androgen receptor antagonists (chemical castration). For metastatic prostate cancer curative treatment is no longer possible, but the standard treatment is castration. Chemical castration with anti-androgens such as casodex and enazalutamide or surgical castration often produces good results initially, because the growth of prostate cancer depends on androgens (6). This treatment is very effective in most patients initially, but within a few years, usually 18 months, a large part of patients become refractory to the treatment, developing an ability to progress despite castration treatment, a stage of cancer called castration resistant prostate cancer (CRPC)(7).

Several mechanisms have been suggested for the development of castration resistance including mutations within the AR receptor itself and its expression in the cell cytoplasm. It has been proven the CRPC is still AR dependent and the androgen signaling retains important for cancer progression even at this stage. (7)

1.2 Androgen receptor

Arising from urogenital sinus mesenchyme, the prostate cells express a receptor called the androgen receptor. (2) The androgen receptor is classified as NR3C4 (nuclear receptor subfamily 3, group C, member 4).(8) In a normal prostate, the unliganded AR is inactive and binds to cytoplasmic chaperone proteins, while the activated receptor transfers information into the nucleus and effects the expression of the target genes.(9)(10)(8)

The most abundant androgen in the sera is testosterone, produced in the male testes. However, in the prostate testosterone is converted into dihydrotestosterone (DHT) by a residential enzyme called 5α-reductase.(7) While both DHT and testosterone bind to the AR, DHT has much higher affinity, hence surpassing testosterone as the main effective hormone in the prostate. (10) DHT is required for the normal functions of the prostate as it serves as the main ligand for AR. Currently the main treatment for advanced prostate cancer is the aforementioned chemical castration, or androgen deprivation therapy, in which the 5α-reductase enzyme is inhibited by finasteride, thus blocking the reduction of testosterone into DHT, and hence depriving prostate cancer cells of stimulants in the entire body. (7)

Once a ligand is bound to the AR, the chaperones dissociate and the nuclear localization signal of the receptor is revealed. The dimerizised AR, bound to a hormone then translocates into the nucleus, where it binds to the DNA and regulates target gene expression, such as the production of PSA. There are hundreds of target genes known to AR with several combined down- and upregulators. (11)(12)

AR plays also an important role in the cell proliferation and differentiation of the prostate cell. This happens through interactions with several different proteins (RB,D-cyclins, Cdk6, and Cyclin E etc. (1)), hence resulting in different effects depending on phase of the cell cycle. This way AR can be both growth inhibiting and promoting. (1) Because of these various effects of the AR, it is still required for the cell proliferation also in the castration resistant prostate cancer, although its functions change while the prostate cancer cells mutate from normal cells to cancer cells and further on into CRPC cells. (13) Due to its importance in prostate cancer cell growth, most mechanisms that have been identified in the development of CRPC activate the AR even in the low-androgen milieu.

In hormone-dependent primary prostate cancer, the AR has already undergone some mutations. These mutations have been found to develop during anti-androgen treatments as coping mechanisms to the changing hormonal environment. (13) It has been discovered that in low androgen levels the AR promotes prostate cancer cell growth, but at high androgen levels the growth is inhibited.(13) When prostate cancer develops into CRPC the AR gains multiple new functions, important mutations can occur in the stereochemistry of the receptor making it nonspecific and hence able to activate from non-androgenic ligands, such as estrogens and progesterone and anti-androgens. (14) Thus antiandrogens that are used in hormone deprivation therapy are used to promote growth instead.

The androgen receptor can also become hypersensitized being able to achieve the same effect with lower androgen levels. This can happen by gene amplification where AR gene expression is enhanced, resulting in higher transcription rates and hence in more receptors being produced. (15) The AR undergoes changes in stability where a smaller amount of activated receptors is required for the same action to take place. (16) The receptor also requires less DHT in order to be activated in the first place (17,18). Other types of steroid hormones, such as glucocorticoids, may become ligands for androgen signaling. (19)

In addition to changes in the AR activity, sensitivity and structure, the tumor cells also undergo changes in metabolism. CRPC tumors have been proved to have increased expression of the enzymes required for androgen metabolism. (20) This hints that prostate cancer cells are capable of producing their own androgens or at least their precursors. It has been discovered that despite androgen deprivation therapy the intraprostatic androgen levels increase in castration resistant prostate cancer.(7) This usually coincides with an increase of PSA, suggesting increase in the AR activity, hence being involved in the development of castration resistance. It has been suggested that the prostate cancer cells gain a capability to synthesize enzymes required for androgen metabolism hence being able to produce their own androgens, thus providing prostate cancer cells the necessary androgen stimulus despite ADT (14). In fact Locke et al. (2008) were able to prove that the tumor cells are capable of conversing acetic acid into dihydrotestosterone and possibly producing six other steroids with the uptake of progesterone. (21)

1.3 Statins

3-hydroxy-3-methylglutaryl CoA reductase inhibitors, more commonly known as statins are the most common cholesterol-lowering drugs used at the moment. (22) They are the recommended treatment for hypercholesterolemia, and for primary and secondary prevention of coronary heart disease [2].

3-hydroxy-3-methylglutaryl CoA (HMG-CoA or in short) is a precursor to fatty acid pathway. Reduction of HMG-CoA is needed in order to the fatty acid pathway to proceed. Statins (HMG-CoA-reductase inhibitors) have a rigid hydrophobic structure, which is bound covalently to a ring-like structure chemically similar to HMG-CoA. Thus statins perform as ligands to the enzyme in the fatty acid pathway, the HMG-CoA-reductase (HMGCR), hence inhibiting the pathway and preventing the formation of the end results, cholesterol. (22) Simvastatin and atorvastatin have a different structure, the atorvastatin being more complicated and completely synthetic, whilst simvastatin is a more natural compound. (22) This results also in atorvastatin having higher abundance to the HMG-CoA reductase, thus making it a more powerful inhibitor. (22)

It has been shown in several studies that the incidence of advanced prostate cancer is significantly lower in males taking statin medication.(23)(24)(25) This is supported by decreased prostate cancer mortality among statin users. (25) The effects of statin use on the overall risk of prostate cancer however remain inconclusive. (26) Statin usage also lowers PSA in men. (27)The most effective statins are simvastatin, atorvastatin and rosuvastatin due to their hydrophobic tendencies and/or their efficacy in HMGCR inhibition (28).Suggested mechanisms for the cancer preventive effect of statins include direct inhibition of cell growth, interference with cell cycle regulatory proteins, inhibition of p53-oncogene and also via indirect effects due to lower cholesterol concentration in the sera.

It has been hypothesized that statins inhibit androgen synthesis by reducing availability of circulating cholesterol by inhibiting production in the liver. Cholesterol is the needed prerequisite for steroid synthesis, including androgens. Also prostate cancer cells produce cholesterol locally. However Hall et al. (2007) demonstrated that there was no change in the circulating androgen levels in men taking statins. (29) The effects of statins on local cholesterol and androgen biosynthesis in prostate cancer cells are unknown. According to Hoque et al. (2008) statins induce cell growth arrest and eventually apoptosis in cancer cells by inactivating RhoA (30) Toepfer et al. (2011) suggested that especially atorvastatin induces autophagy in PC-3 cells by activating LC3 transcription. (31) Sekine et al. (2008) demonstrated that simvastatin works by down-regulating the insulin-like growth factor 1 receptor thus inhibiting the proliferation of the same PC-3

cell line. (32) However the reason as to why statins are capable of preventing the progression of prostate cancer remains unclear.

2 Aims of the study

We studied the effects of atorvastatin and simvastatin on growth of androgen independent PC-3 cell line alone and in combination with DHT. This is a preliminary control study which is a part of a larger study designed to explore the mechanisms by which statins might prevent the growth of androgen dependent cells. The main target action site for our study was thought to be de novo androgen synthesis, mechanism described more in detain in chapter 5, discussion.

3 Materials and methods

3.1 PC-3 cell line

PC-3 cells are a prostate cancer cell line, which has been harvested from a bone metastasis and have been used in studies for several years. (33) It has been the belief for years, that these cells no longer express the androgen receptor at all, and are therefore completely androgen independent types of cancer, AR-negative cells to be exact. (34) However recently it has been proposed in some studies that these cells not only express the AR protein, but the receptor and mRNA as well to a detectable extent. (33)In fact, Alimirah et al were actually able to prove that in the cell line AR is able to locate to the nucleus after DHT treatment, hence suggesting that not only does the cell line express the AR, the receptor is in fact important to the function of the cell, and the cell line should hence be considered AR-positive. (35) However since the studies that have emerged lately are not yet enough to conclusively prove the androgen dependency of the PC-3 cell line, the cells are still considered to be AR negative and were here used as a control study in the preliminary phase.

Previous tests with PC-3 cell line have been done regarding statins effects on the cell line. According to Brown et al. especially lipophilic statins (i.e. simvastatin, atorvastatin) are capable to reduce the migration and colony formation of PC-3 cells in to the human bone marrow, thus preventing prostate cancer metastasis.(36) According to them, statins work by inhibiting the conversion of HMG-CoA to mevalonic acid via inhibition of the HMG-CoA-reductase. This is however only the theory regarding statins capability of inhibiting invasion, not suppressing the growth of the tumor itself.

PC-3 cells represent an aggressive prostate cancer cell model. The cell line was obtained from American Type Culture Collection (ATCC).

3.2 Cell cultivation

The cells were grown in a solution of Ham's F-12 with L-Glutamine (Bio-Whittaker® Cat. N°: BE12-615F)and serum. For cell cultivation FBS (Bio-Whittaker® Cat. N°: DE14-801F) serum was added to achieve a concentration of 10%. For the drug treatments the serum was substituted by a characterized Fetal bovine

serum (Hyclone® Cat N°: SH30071,03), which has reduced hormone concentration due to the charcoal-dextran treatment. In addition to serum, extra L-glutamine was added to the medium to a concentration of 2mmol/l. The L-glutamine used was 200mM in 0,85% NaCl solution by Bio-Whittaker® (Cat. N°: BE17-605E).

Prior to adding any exposure agents, the cells were left to grow in the stripped medium for 3-4 days in order to remove any remaining steroids left inside the cells from the growth medium. The cells were grown in sterile 75cm^2, 250ml, Ps,cell culture flasks with red standard cap (5 cell star® Cat. N°: 658-170). Trypsin EDTA 200mg/l Versene 170 000U Trypsin L (Bio-Whittaker® Cat. N°: BE17-161F) was used in order to detach the cells from their growth flasks and transfer them into the cell culture plates. The plates were greiner 24-well cell culture plates (Cellstar® Cat. N°: 662-160).

The amount of cells per well was 7000, derived from earlier test where we drew growth graphs with different initial cell numbers, thus finding eventually the ideal number of initial cells where there is just enough cells to induce growth signaling but not too many to drive the cells to a confluent state within the 8 growing days required for the results, where mutations and cell death would eventually occur. The amount of cells in a cell solution was calculated using Bürker cell counting chamber with depth of 0,100mm. From the concentration of cells in the solution the amount of solution needed in order to get 7000 cells per well, was then calculated from the value obtained by Bürker cell counting chamber.

Each combination of DHT and statin was repeated 6 times

3.3 Treatments

The DHT concentrations were chosen to be 1nM, 10nM and 100nM. 10nM DHT is usually considered to mimic the concentration of DHT in the sera. However since the actual concentrations of the hormone inside the prostate remain unknown, factors of 10 were included as well, although 100nM DHT is known to be relatively toxic to prostate cancer cells.

As for the statins the concentrations were chosen to be 1nM, 10nM, 100nM, 1uM and 10uM. 10nM represents the concentrations at which the statins are used in treatment of hypercholesterolemia. Thus for each DHT concentration there were altogether 6 concentrations of statin including 0nM to be used as a control study.

Below (table 1 and 2) are visual representations of different concentrations of exposure agents used and where in the result figures you can find each combination. The page numbers of the figures can be found in the figure index.

Table 1 Different concentrations of Simvastatin and DHT with their respective figures, which can be found in the results chapter.

	0nM Simvastatin	1nM	10nM	100nM	1uM	10uM
0nM DHT	Figure 1, 2, 3	Figure 2	Figure 2	Figure 2	Figure 2	Figure 2
1nM DHT	Figure 1, 4	Figure 4	Figure 4	Figure 4	Figure 4	Figure4
10nM DHT	Figure 1, 6	Figure 6	Figure 6	Figure 6	Figure 6	Figure 6
100nM DHT	Figure1, 8	Figure 8	Figure 8	Figure 8	Figure 8	Figure 8

Table 2 Different concentrations of Atorvastatin and DHT with their respective figures that can be found in the results chapter.

	0nM Atrovastatin	1nM	10nM	100nM	1uM	10uM
0nM DHT	Figure 1, 2, 3	Figure 3	Figure 3	Figure 3	Figure 3	Figure 3
1nM DHT	Figure 1, 5	Figure 5	Figure 5	Figure 5	Figure 5	Figure 5
10nM DHT	Figure 1, 7	Figure 7	Figure 7	Figure 7	Figure 7	Figure 7
100nM DHT	Figure 1, 9	Figure 9	Figure 9	Figure 9	Figure 9	Figure 9

Simvastatin and Atorvastatin were added with and without the DHT into the cell medium solutions. The statins were purchased from Calbiochem (Gibbstown, NJ, USA), while the DHT (5α-androstan-17-β-ol-3-one) was obtained from Sigma-Aldrich (Cat. N°:A8380).
Each experiment was repeated 6 times.

The amount of cells needed to draw the growth curves, were calculated at time points 1,2,4,6 and 8 days from the original cultivation into culture plates, which was analyzed by first photographing the plates at random points using the Olympus IX71 inverted research microscope with 4x magnification and QImaging Fast 1394, Retiga 2000R camera to take the shots. The microscope and camera were controlled using the Surveyor version V.8.0.0.0. automated specimen scanning for the oasis automation control system software.

3.4 Analysis and statistics

The photographs were then analyzed using the ImageJ cellular area measurement- software using settings "Strong". From the data ImageJ provided, mean, average and standard deviation of the concurrent tests were calculated and the graphs were drawn from the standard deviations using Microsoft Excel 2007.

The program provides mean values of cells per area and from these values an average was calculated. In order to analyze the data and the reliability of the results also standard deviations were concluded for each time and concentrations. From these results tables were drawn so the standard deviation can be easily applied to the average values.

Because getting the exactly same numbers of cells per frame each time would have been impossible, from the averages we were able to calculate from the mean values, proportional graphs dividing each time point by the value at time point 1 were drawn thus eliminating the random factor of initial cells per frame.

4 Results

DHT alone didn't induce PC-3 cell growth at physiologic 1nM and 10nM concentrations indicating that despite some recent studies it is in fact androgen independent. However DHT surprisingly suppressed growth slightly at 100nM concentration [Figure 1], possibly due to its toxic tendencies in high concentrations.

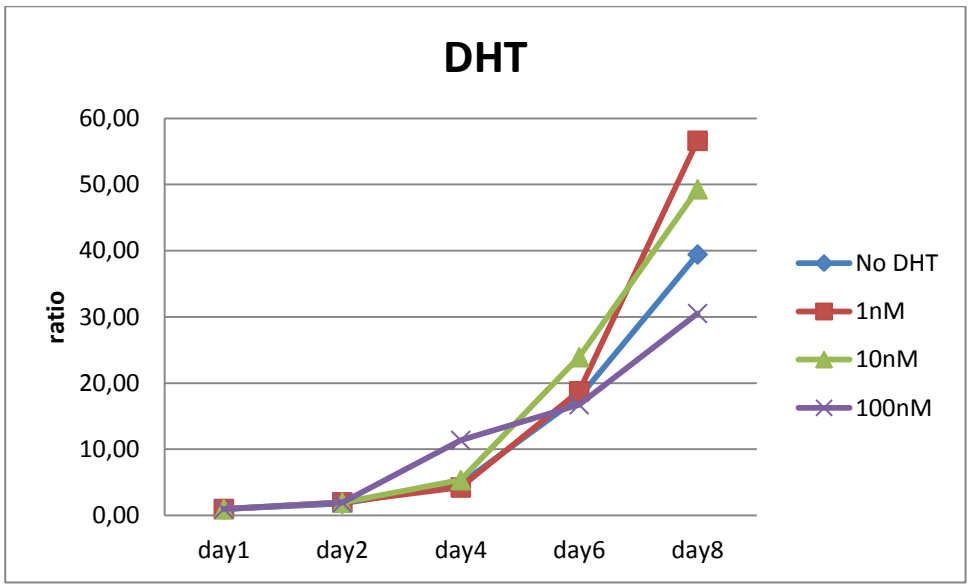

Figure 1 **Change in proportional cell growth with varying concentrations of DHT. There are no statins present in the growth medium and hence the graph indicates the control growth rates of the cells. The darker blue graph indicates the PC-3 cell growth in neutral environment, with only the growth medium. The 100nM DHT surprisingly appears to hinder cell growth, possibly due to its toxic tendencies at higher concentrations.**

Table 3 **Showing the averages of the mean values of cells per frame calculated by the Image J program. Below each average value is its standard deviation. From these averages the ratio shown in graphs is calculated by dividing each day's average by day 1 average. Standard deviations are here to indicate, how much variation there was in the results from the 6 replicate experiments conducted**

DHT	day1	day2	day4	day6	day8
No DHT	3,22	5,85	15,49	57,63	127,21
S.D.	0,59	1,56	4,96	13,09	77,17
1nM	3,52	7,06	14,99	66,12	199,34
S.D.	0,93	1,90	8,35	33,98	59,48
10nM	3,21	6,30	17,25	76,87	158,31
S.D.	0,52	0,38	6,03	47,01	70,71
100nM	6,20	12,15	70,55	103,94	189,42
S.D.	0,79	2,23	13,83	16,93	18,07

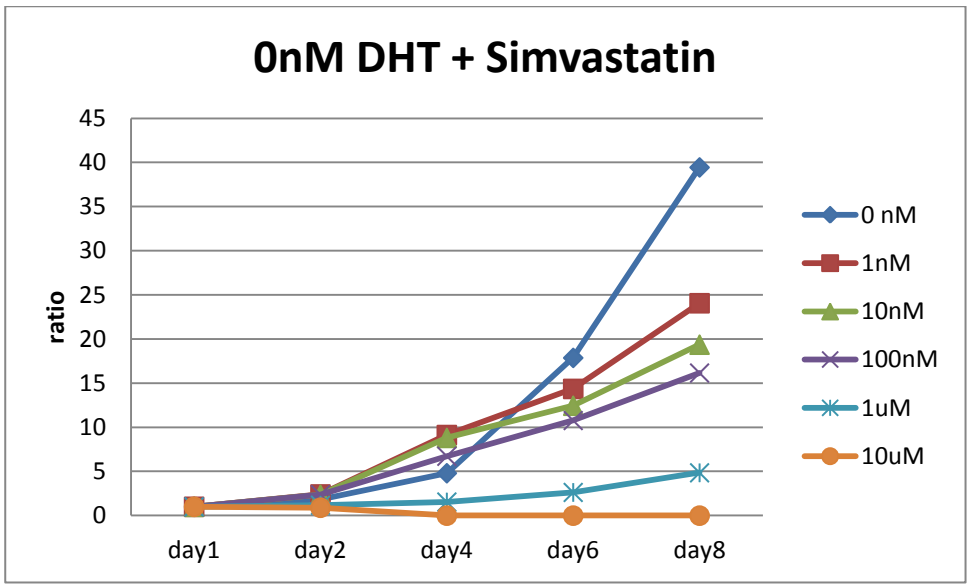

Figure 2 **Change in proportional cell growth in varying concentrations of simvastatin without any DHT present. The darker blue graph indicates the PC-3 cell growth in neutral environment, with no simvastatin present either. Notice how 10uM Simvastatin is very toxic to the cells, resulting in cell death at day4**

Table 4 **The averages of the mean values of cells per frame calculated by Image J program and their respective standard deviations. In 0nM DHT with varying concentrations of Simvastatin.**

0 DHT, Simvastatin	day1	day2	day4	day6	day8
0 nM	3,22	5,85	15,49	57,63	127,21
S.D.	0,59	1,56	4,96	13,09	77,17
1nM	8,74	20,97	79,84	125,52	210,39
S.D.	1,07	3,46	9,14	22,89	18,04
10nM	9,59	22,70	84,44	119,37	185,71
S.D.	1,81	7,61	21,53	33,99	36,05
100nM	10,91	25,86	73,40	117,58	176,31
S.D.	0,87	3,47	11,80	18,06	33,22
1uM	8,99	10,58	13,85	23,62	43,63
S.D.	1,29	1,49	3,11	5,06	14,35
10uM	8,31	7,42	0,00	0,00	0,00
S.D.	1,20	0,91	0,00	0,00	0,00

The growth trend in the control test, with only statins and no DHT present, followed the regular growth pattern for PC-3 cells. Simvastatin suppressed growth of PC-3 cells without the presence of DHT [Figure 1],

the highest concentration (10uM) drove all cells to death by day 2. Atorvastatin appears to have a stronger growth suppressing effect as can be seen from figure 3.

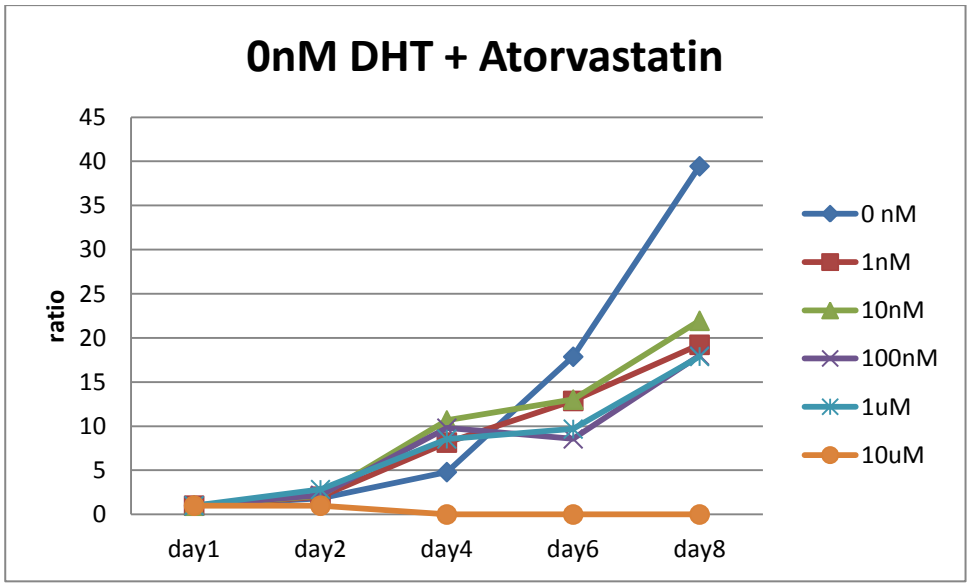

Figure 3 Relative growth ratio of atorvastatin only without any DHT present. Atorvastatin appears to have stronger growth hindering effect when compared to the simvastatin control graph (Figure 2).

Table 5 Averages and their respective standard deviations of 0nMDHT in the presence of varying concentrations of simvastatin.

0DHT, Atorvastatin	day1	day2	day4	day6	day8
0 nM	3,22	5,85	15,49	57,63	127,21
S.D.	0,59	1,56	4,96	13,09	77,17
1nM	10,36	20,93	84,43	133,34	199,23
S.D.	2,16	5,06	19,66	26,73	25,96
10nM	8,61	18,31	91,82	112,16	189,08
S.D.	1,23	3,41	15,57	30,10	35,08
100nM	9,09	19,80	88,93	77,80	163,69
S.D.	1,76	3,78	19,40	29,23	30,02
1uM	8,44	23,92	72,10	81,64	151,00
S.D.	2,62	7,19	31,08	48,01	42,36
10uM	9,57	9,55	0,00	0,00	0,00
S.D.	1,65	2,00	0,00	0,00	0,00

In 1nM DHT simvastatin had no major effect on the growth patterns. 10nM DHT could not prevent growth inhibition by 1nM to 1uM simvastatin or the complete cell death by 10uM simvastatin [Figure 4].

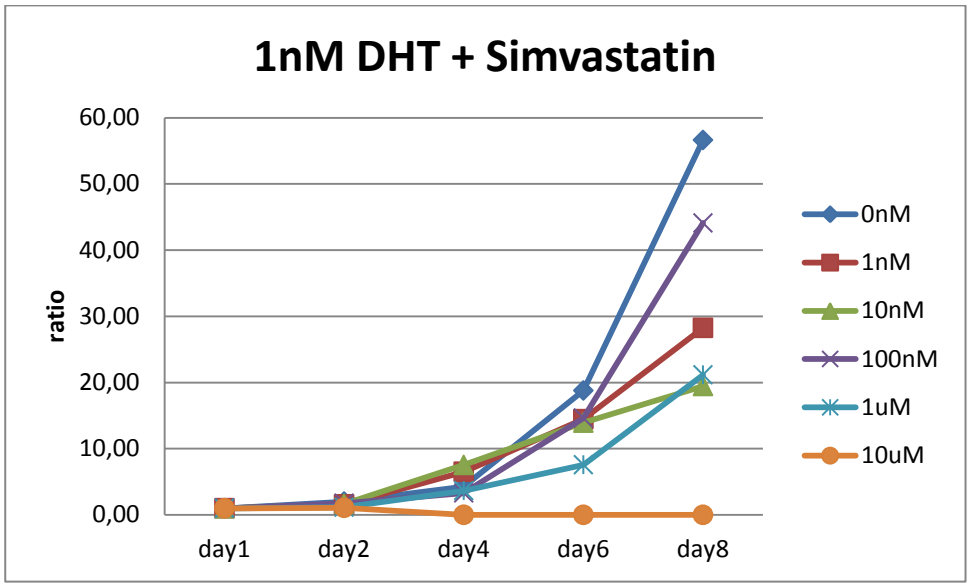

Figure 4 1nM DHT with varying concentrations of Simvastatin. Surprisingly the higher concentration of Simvastatin 100nM appears to accelerate growth instead of slowing it down as could be extrapolated from the other curves representing lower concentrations. However the overall curve shapes coincide with the control curves (Figure 2).

Table 6 The averages and standard deviations of 1nM DHT and varying concentrations of Simvastatin.

1nM DHT, Simvastatin	day1	day2	day4	day6	day8
0nM	3,52	7,06	14,99	66,12	199,34
S.D.	0,93	1,90	8,35	33,98	59,48
1nM	7,61	12,02	49,56	110,00	215,01
S.D.	1,59	2,23	14,53	29,26	24,21
10nM	8,18	13,57	61,67	113,84	159,14
S.D.	1,87	3,72	14,95	49,90	20,27
100nM	3,41	5,80	21,86	59,84	124,51
S.D.	1,02	2,45	7,44	22,17	26,99
1uM	7,75	8,93	28,37	58,66	163,90
S.D.	1,45	1,34	4,56	11,90	18,36
10uM	3,00	3,21	0,02	0,00	0,00
S.D.	1,29	1,03	0,02	0,00	0,00

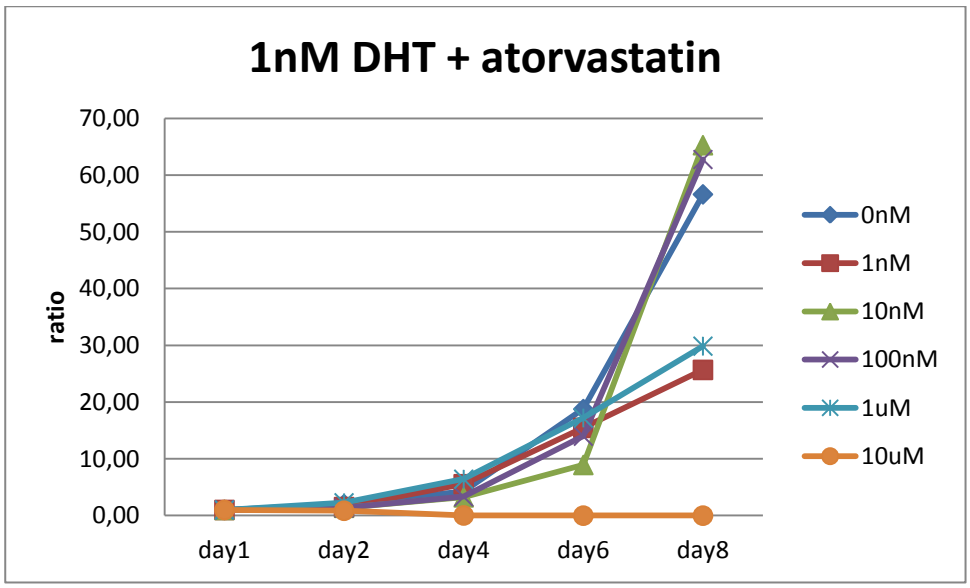

Figure 5 1nM DHT with varying concentrations of Atorvastatin. 100nM and 10nM Atorvastatin have accelerated the growth when compared to the curves in the control graph (Figure3)

Table 7 Averages and standard deviations of 1nM DHT with varying concentrations of Atrovastatin.

1nM	day1	day2	day4	day6	day8
0nM	3,52	7,06	14,99	66,12	199,34
S.D.	0,93	1,90	8,35	33,98	59,48
1nM	6,78	10,21	37,54	104,90	174,21
S.D.	3,45	3,42	12,92	29,34	82,05
10nM	3,21	4,44	10,34	28,65	209,38
S.D.	0,83	1,12	8,90	23,97	40,49
100nM	3,30	4,46	10,96	46,24	207,44
S.D.	0,37	0,71	7,34	25,48	24,52
1uM	6,40	14,54	41,10	110,14	191,19
S.D.	1,80	4,97	6,27	15,30	31,13
10uM	1,21	0,63	0,16	0,00	0,00
S.D.	3,38	2,92	0,11	0,00	0,00

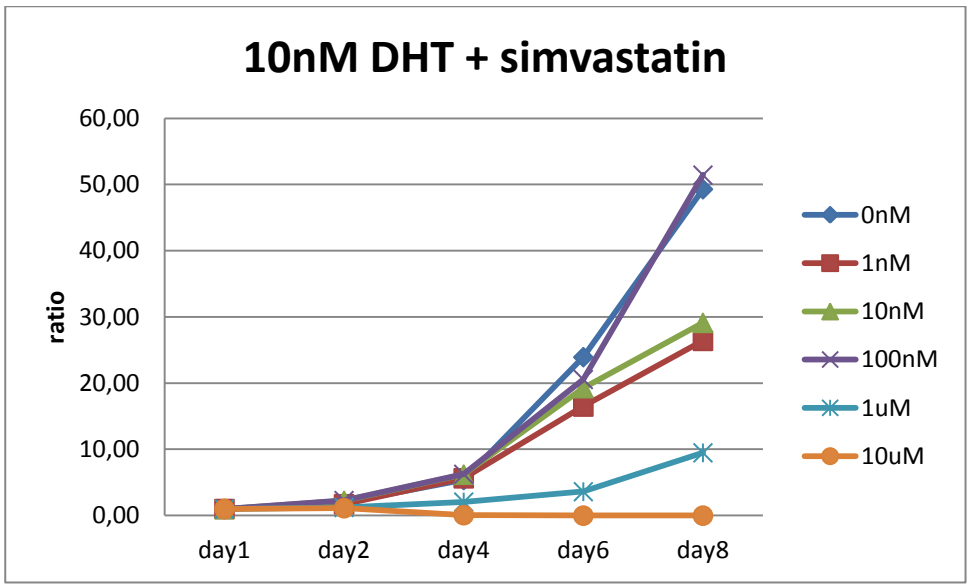

Figure 6 **Simvastatin in the presence of 10nM DHT. The curves are fairly similar to those shown earlier with lower concentrations of DHT. However 1uM simvastatin has increased its growth inhibiting effect by half.**

Table 8 **Averages and standard deviations of 10nM DHT with varying concentrations of Simvastatin.**

10nM	day1	day2	day4	day6	day8
0nM	3,21	6,30	17,25	76,87	158,31
S.D.	0,52	0,38	6,03	47,01	70,71
1nM	7,71	13,23	43,48	127,07	203,55
S.D.	1,82	2,45	10,93	27,36	27,49
10nM	6,85	15,35	42,46	132,03	199,59
S.D.	1,28	3,52	7,56	30,06	29,27
100nM	3,76	8,61	23,49	77,29	193,45
S.D.	1,16	2,37	11,19	36,10	39,04
1uM	8,67	10,67	17,81	31,37	82,31
S.D.	0,86	1,49	4,95	10,30	20,98
10uM	2,54	2,86	0,18	0,00	0,00
S.D.	0,39	0,46	0,08	0,00	0,00

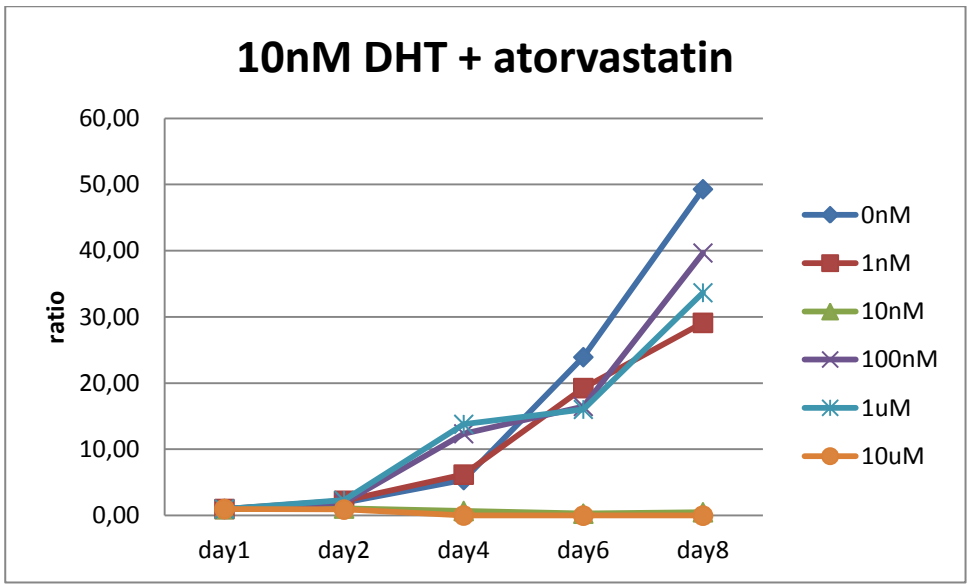

Figure 7 10nM DHT with varying concentrations of Atorvastatin. At concentrations 100nM and 1uM the curves accelerate after day 6, but no overall change in the coefficients can be seen.

Table 9 Averages and standard deviations of 10nm DHT and varying concentrations of atorvastatin.

10nM	day1	day2	day4	day6	day8
0nM	3,21	6,30	17,25	76,87	158,31
S.D.	0,52	0,38	6,03	47,01	70,71
1nM	6,85	15,35	42,46	132,03	199,59
S.D.	1,28	3,52	7,56	30,06	29,27
10nM	4,85	5,22	3,41	1,45	2,49
S.D.	0,76	0,52	0,78	0,57	1,52
100nM	4,96	9,55	61,34	81,73	196,81
S.D.	0,39	1,70	14,99	12,52	21,56
1uM	5,94	13,73	81,91	95,21	199,87
S.D.	1,14	3,06	25,55	36,11	28,45
10uM	2,94	2,69	0,06	0,00	0,00
S.D.	0,66	0,71	0,07	0,00	0,00

Also 100nM DHT was not able to prevent growth inhibition by 1nM to 10nM simvastatin. However growth of PC-3 cells was induced by the combination of 100nM DHT and simvastatin at 100nM to 1uM concentrations. Suggesting that simvastatin at these concentrations might prevent growth inhibition by 100nM DHT.

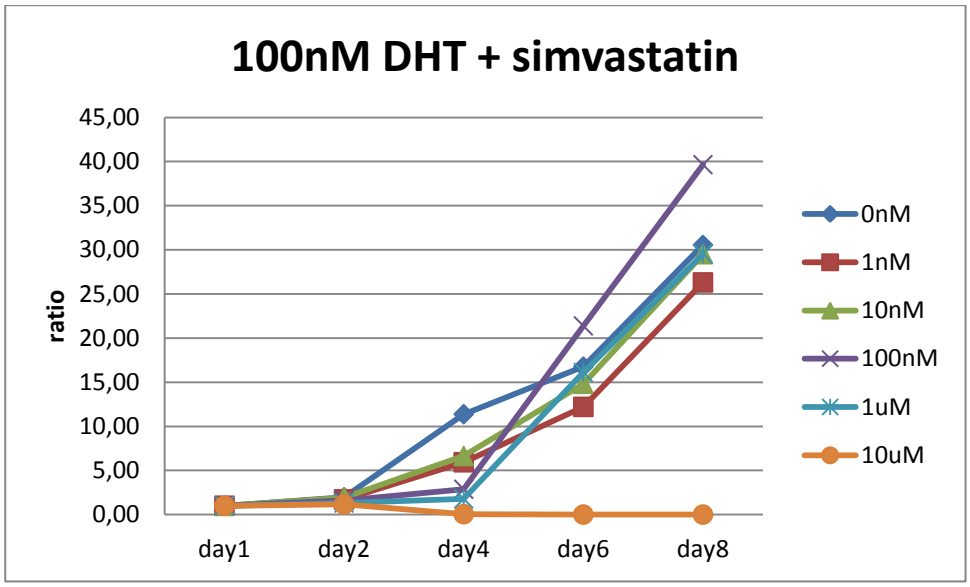

Figure 8 100nM DHT with simvastatin. 100nM simvastatin curve is very similar to the 100nM simvastatin curve in 10nM DHT. However, the growth suppression by 1uM simvastatin has been clearly cancelled out, resulting in an upward curve. The lower concentrations of statins resemble the plain 100nM DHT curve (here dark blue), indicating that the growth inhibiting effect seen here is caused by the toxic levels of DHT, which appears to be cancelled out by statins resulting in steeper curves.

Table 10 Averages and standard deviations of 100nM DHT and varying concentrations of simvastatin.

100nM	day1	day2	day4	day6	day8
0nM	6,20	12,15	70,55	103,94	189,42
S.D.	0,793	2,235	13,828	16,930	18,070
1nM	5,86	10,12	34,76	71,53	154,20
S.D.	1,101	1,795	10,595	13,561	14,493
10nM	6,91	13,31	45,84	102,36	203,82
S.D.	1,130	2,401	10,184	15,729	18,614
100nM	4,48	7,19	12,85	95,71	177,46
S.D.	1,548	2,206	4,949	47,714	51,769
1uM	4,74	6,20	8,42	76,30	139,97
S.D.	2,175	1,980	3,196	29,335	35,456
10uM	2,93	3,39	0,14	0,00	0,00
S.D.	0,623	1,092	0,091	0,000	0,000

1-10nM DHT had no effect on the growth inhibition by atorvastatin, which was true also for the combination of 100nM DHT and atorvastatin [Figure 6]. Unlike simvastatin, atorvastatin did not prevent growth inhibition by 100nM DHT.

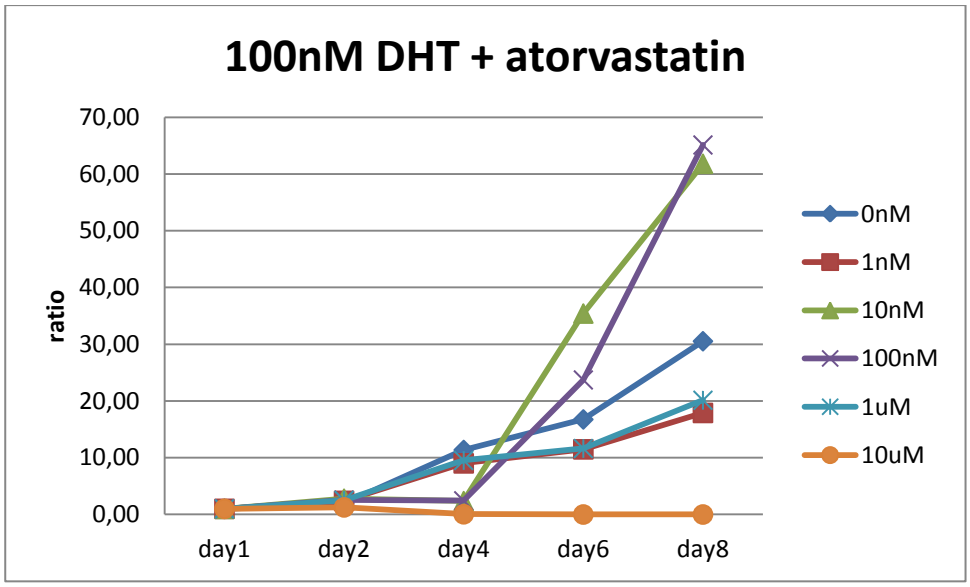

Figure 9 The curves are all very much like the curve for only 100nM DHT (Figure 1) with the exception of concentrations 100nM to 1uM, and slightly unexpectedly 1nM. Atorvastatins ability to cancel out the toxic effect of 100nM DHT appears to be much weaker than that of simvastatins.

Table 11 The averages and standard deviations of 100nM DHT and atorvastatin.

100nM	day1	day2	day4	day6	day8
0nM	6,20	12,15	70,55	103,94	189,42
S.D.	0,793	2,235	13,828	16,930	18,070
1nM	8,28	19,92	74,66	95,12	148,27
S.D.	1,020	3,006	6,962	12,794	23,320
10nM	2,22	6,19	5,25	78,81	137,54
S.D.	0,252	1,962	1,197	25,918	33,686
100nM	2,34	5,92	5,68	55,55	152,52
S.D.	0,307	1,310	1,022	8,427	35,762
1uM	8,432	21,000	80,303	98,112	169,771
S.D.	0,776	2,283	9,858	21,089	28,733
10uM	2,84	3,57	0,13	0,00	0,00
S.D.	0,977	1,233	0,097	0,000	0,000

Image 1 PC-3 cells in plain medium and with the presence of 1uM simvastatin. Very limited growth pattern visible.

As seen in image 1, in the presence of 1uM simvastatin the PC-3 cells appear to struggle to grow. The cells are capable of forming barely enough growth that's capable of sending the growth signals required to prevent cell death.

Image 2 Pictures of cell growth of PC-3 cells in 100nM DHT and 1uM Simvastatin at time points 1, 4, and 8 respectively. Notice how the growth is nearly exponential when compared to image 1. This proves that 100nM DHT clearly removes the growth inhibiting effect 1uM simvastatin has on PC-3 cells, seen in image 1.

In the presence of 100nM DHT cells look completely different with the capability to grow nearly exponentially. On day 8, the cells have grown to such extent that there will soon be too many of them, which will eventually lead to cell death as the cells will begin to struggle from the lack of space.

5 Discussion

As this study was designed to be a control study for a larger study, the hypothesis was that there would be no synergistic effect between DHT and statins was not proved. The effect however was minimal, and only occurred at high concentrations of DHT, much higher than the estimated concentration in the human sera is assumed to be. Simvastatin had an unexpected ability to prevent cell growth inhibition caused by 100nM DHT, which suggests that HMGCR (target of statins) and androgen metabolism might be connected in prostate cancer cells. The experiment demonstrates that the connection is more complicated than previously thought and should be studied further.

The cell growth curve followed the typical exponential growth trend of PC-3 cells hence indicating that there were no major systematic errors in the methods used. DHT affected PC-3 cell growth by suppressing their growth in high concentrations [Figure 1]. This might be due to DHT affecting androgen signaling by toxically overloading the PC-3 cells. This coincides with the conclusion that Alimirah et al. suggested, that PC-3 cell line should in fact be considered as AR-positive, since DHT effects the cells growth pattern. (35) DHT might also be toxic at high concentrations to any prostate cancer cell and thus suppress growth even in androgen independent cells.

However from the results no conclusions as to why the cells behaved as they did can be drawn since no closer inspection was directed at cell morphology nor PCR or androgen receptor blotting.

The standard deviations drawn from the mean values calculated by Image J-program did not vary greatly between different concentrations and hence random error could not explain the results we were able to draw at higher concentrations. [Tables 1-9]

Another speculation as to why the cell growth was promoted instead of prevented by high concentrations of DHT could be that as part of androgen independent coping mechanisms, PC-3 cell line could have developed the capability of de novo androgen synthesis and also has become sensitized to the effects of DHT. This phenomenon has been described by Nazareth et al. in 1996 (14). In such scenario high concentration of DHT could lead to profound toxic effects on cell growth. And if HMGCR inhibitors have been linked to prevention of de novo androgen synthesis, this could explain why statins were able to counter the growth preventing effect of toxic DHT levels.

As described in the paper by Shafi et al. (2013) (7) the androgen receptors become hypersensitized after a while resulting the cell being activated by other ligands such as glucocorticoids and cholesterols. In our experiment these factors were eliminated by growing the cells in hormone deprived environment, thus eliminating all other ligand that might affect the receptor.

Previous studies have suggested several different action sites for statins, including liganding with the AR receptor and affecting the production of other ligands among others (11, 12, 13). Our results propose a different mechanism because statins and DHT appear to have some synergistic effects in PC-3 cells, a cell line commonly known for its AR independence. This phenomenon, that we recorded, also fits the theory that statins would affect the de novo androgen synthesis or other mechanism of castration resistance within the cells that are usually androgen independent. If the growth suppressing effect of higher levels of DHT is caused by sensitization of the cells to the androgens produced by de novo synthesis, then the adding of statins should dilute the effects of DHT. Thus decreased intracellular production of DHT during statin therapy leads to stable DHT levels being available to the cells despite addition of extracellular DHT. This mechanism would explain why statins were able to prevent the growth inhibition by 100nM DHT.

Atorvastatin did not clearly have similar effect. Atorvastatin is more potent inhibitor of HMGCR than simvastatin, but simvastatin is more lipophilic, thus lipophilicity may be a deciding factor in statins' effects in prostate cancer cells. Hence the difference in molecular morphology described by Istvan et al. (22) could be the reason to the different results between the two statins. In epidemiological studies atorvastatin and simvastatin have been equally effective in preventing prostate cancer deaths. (9) According to Brown et al. (36) all statins except pravastatin are also similarly able to prevent metastasis of prostate cancer.

Since PC-3 cells used in our experiment are androgen independent, in order to study de novo androgen synthesis properly tn future studies the experiments should be repeated also with androgen dependent cell lines. The fitting candidate for these studies would be LNCaP cell line, for this cell line still expresses the androgen receptor and is therefore definitely androgen dependent. To find definite proof of statins blocking the de novo synthesis, future studies should also include progesterone and other hormones and cholesterols that have been shown to work as precursors for de novo androgenesis, and include direct immunohistochemical measurements of androgen synthesizing enzymes. (7)

6 Conclusions

Statins and DHT appear to have synergistic effects on prostate cancer cell growth at high concentrations. Further studies are needed to conclude the reason behind this synergism.

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