CURCUMIN AND ITS ANALOGS AS POTENTIAL TREATMENT FOR PROSTATE CANCER

by

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ABSTRACT

Cancer is one of the leading causes of death in the United States. This disease is due to mutations that cause fast growth in some cells. Environmental, genetic and chemical factors can promote the development of cancer. Inflammation can also cause tumors to arise, suggesting that the immune system is involved as well in this process. Current chemotherapy treatments are inefficient or excessively toxic, which weakens the immune system. One way to address this problem is to find more effective and less toxic compounds with anticancer properties without destroying healthy cells. Among the compounds that are being evaluated as anticancer agents, curcumin has demonstrated to have anticancer properties. Curcumin is a polyphenol derived from *Curcuma spp* whose anti-proliferative properties have been demonstrated. Although preclinical and clinical studies have shown that curcumin is not toxic against normal human cells, several pharmacokinetic studies disadvantages-such as poor bioavailability, fast metabolism and requiring of repetitive oral doses have been reported, which limits its pharmacological applications. Novel curcumin analogs were synthesized to increase curcumin bioavailability. Our hypothesis is that curcumin analogs are more effective than curcumin increasing the cytotoxicity against the PC-3 prostate cancer cell line. In this study we tested the cytotoxic effect of curcumin and its analogs against the human prostate cancer cell line PC-3.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
COX2	Cyclooxygenase-2
CRPC	Castration resistant prostate cancer
HIF-1α	Hypoxia-inducible factor protein
HNSCC	Head and neck squamous cell carcinoma
IL-8	Interleukin-8
MAPK	Mitogen activated protein kinases
MTT	Thiazolyl blue tetrazolium bromide
NFKb	Nuclear factor kappa beta
NP	Nanoparticle
PBMC	Peripheral blood mononuclear cells
PCa	Prostate cancer
P-gp	P-glycoprotein
PLGA	Poly (lactic-co-glycolic acid)
PSA	Prostate specific antigen
TRAMP mice	Transgenic adenocarcinoma of the mouse prostate
Tregs	Regulatory T-cells
US	United States
VEGF	Vascular endothelial growth factor
3b-NP	3b- nanoparticle

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I want to thank God and my family, who were my strength and main motivation during this journey.

I also want to express my gratitude to:

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Chapter 1: Introduction

Prostate cancer is the predominant cancer in men in the United States. Like other cancers it can invade other organs such as the liver, lungs, brain and the bones (June-Kang et al., 2011). It has four stages, being stage IV the metastatic stage (metastasis). Patient's death can occur years after chemotherapy during metastasis (Figure 1.1).

Figure 1.1 The natural history of prostate cancer.

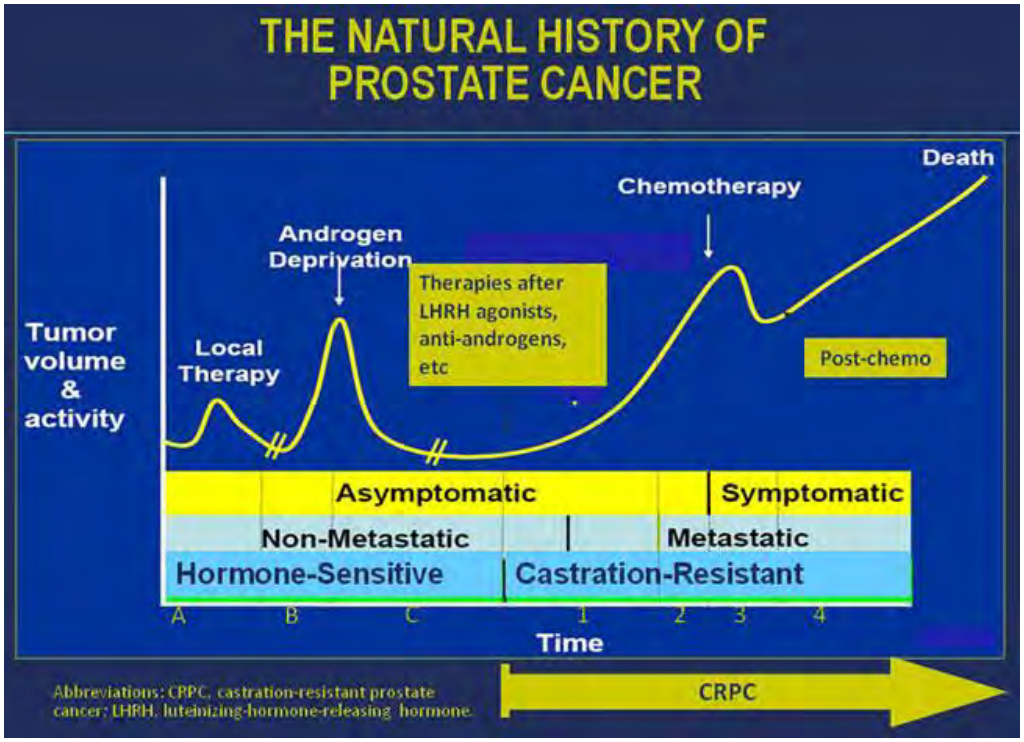


Figure 1.1 The natural history of prostate cancer. Prostate cancer treatments start by surgery or local therapy. Then hormonal therapy is given to patients, followed by chemotherapy. Years after chemotherapy an increase in death can be seen, suggesting the need for new therapies. Besides these treatments, cancer related deaths still occur (Freedland et al., 2007).

Prostate cancer incidence

In 2013, prostate cancer was the cancer with the highest percent of estimated new cases among all cancers (Figure 1.2).

Figure 1.2 Estimated new cancer cases in the US in 2013.

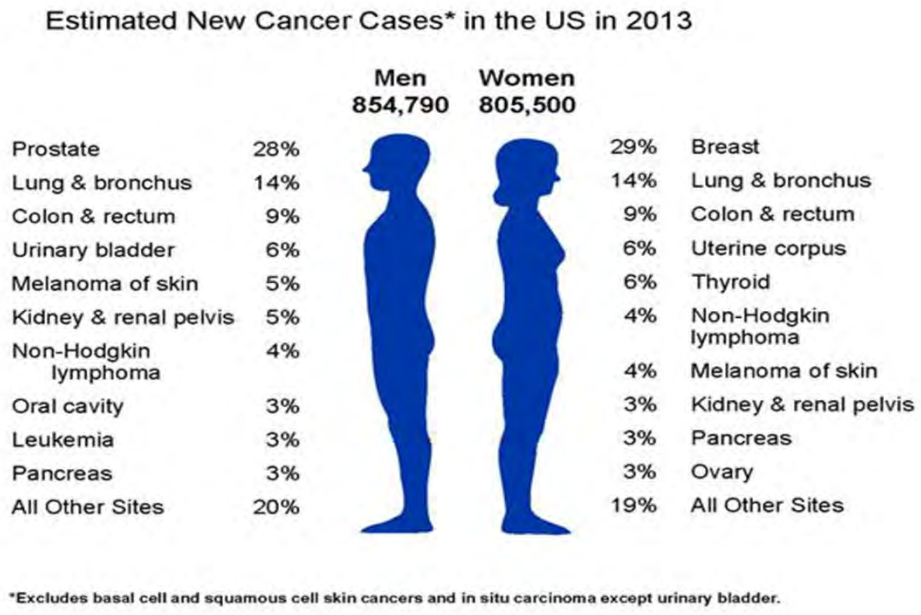


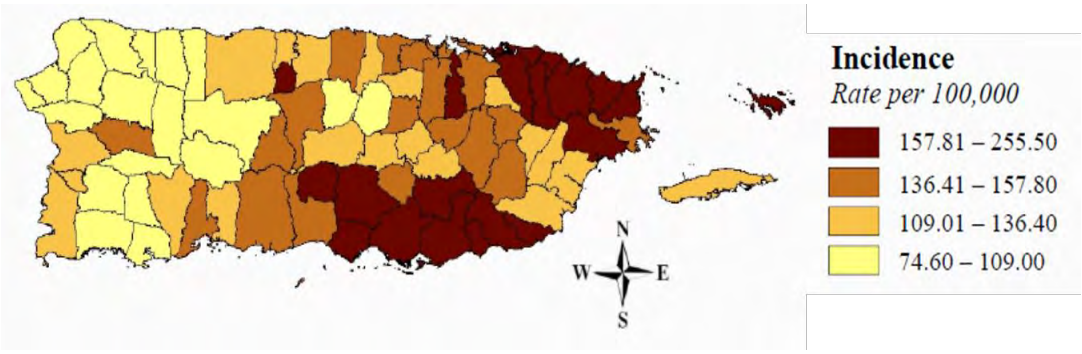
Figure 1.2 Estimated new cancer cases in the US in 2013. Prostate cancer is leading the estimated new cancer cases list. It represents the 28% of the estimated new cancer cases in men. Following prostate cancer are lung and bronchus, colon and rectum, urinary bladder, melanoma skin, and others. A total of 854,790 new cases of different types of cancers, were estimated to occur in men (Cancer Statistics 2013 A presentation from the American Cancer Society, 2013).

An estimated 15,400 Hispanic were expected to be diagnosed with prostate cancer in 2012, making it the most commonly diagnosed cancer among Hispanic men (American Cancer Society, 2012). An estimated 1,600 deaths from prostate cancer were expected among Hispanic men in 2012, making prostate cancer the fourth-leading cause of cancer death (American Cancer Society, 2012). Prostate cancer is the most common form of cancer among men in Puerto Rico. The median age of diagnosis was 68 years old (2006-2010), (Tortolero et al., 2013).

In the period of 2006-2010, prostate cancer was 40.6 percent of all cancer-specific deaths (Tortolero et al., 2013). Distribution of prostate cancer cases is shown in Figure 1.3.

Figure 1.3 Case distribution of prostate cancer in Puerto Rico.

A)



B)

Figure 1.3 Case distribution of prostate cancer in Puerto Rico. Prostate cancer cases are found in all municipalities. A) The highest incidence rates are at the south and northeast of the island. B) The highest mortality rates are found at the south and east of the island (Soto-Salgado et al., 2012).

Uncontrollable growth of cells can cause tumor in the prostate gland. The prostate specific antigen (PSA) blood test is used to detect prostate cancer, but it could bring false positives. PSA is a glycoprotein expressed in normal tissue and it is an indicator of mature protein that has been inactivated. On the contrary, the

proteolytic cleavage of PSA decreases in prostate cancer. This leads to low PSA levels in prostate cancer patients (Carter et al., 1997). Prostate cancer prevention, detection, and treatment must be addressed in order to decrease the amounts of deaths due to this disease.

Current cancer treatments such as chemotherapy have the advantages of slow the cancer growth and kill cancer cells but the disadvantage of killing normal cells. Curcumin the major component of turmeric, has antioxidant and anti-inflammatory properties which converts it in a promising compound to treat cancer (Figure 1.4).

Figure 1.4 Chemical structure of curcumin.

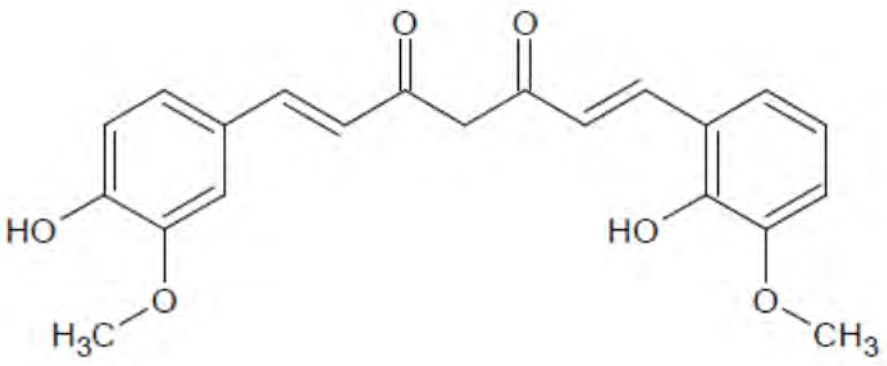


Figure 1.4 Chemical structure of curcumin. A phenolic compound (diferuloylmethane), which has chemopreventive and antioxidant properties (Mohammad et al., 2003).

Curcumin can regulate a diverse range of molecular targets like the inhibition of NFk-ß, inhibition of AP-1, activation of Caspase-8, inhibition of STAT3, inhibition of COX-2, and inhibition of growth factors receptors like EGFR, also promotes apoptosis and growth inhibition of cancer cells (Aggarwal et al.,

2004). Noticing the properties of curcumin it could be a potential treatment for cancer. Due to its low bioavailability certain modifications to the molecule can be synthesized to obtain high bioavailability curcumin analogs. Novel curcumin chalcone analogs provided by Dr. David Sanabria Ríos laboratory, are going to be evaluated in this study to measure their cytotoxicity against highly metastatic prostate cancer cell line (PC-3).

Mono-carbonyl curcuminoids containing nitro moieties were prepared by reacting either an acetone or a cyclohexanone with a monosustituted nitrobenzaldehyde trough an Aldol reaction. Six curcumin analogs were obtained and were characterized by melting points (°C) and ^{13}C-NMR (400 MHz). The structures of the chalcone analogs are represented in Figure 1.5.

Figure 1.5 Curcumin chalcone analogs structures.

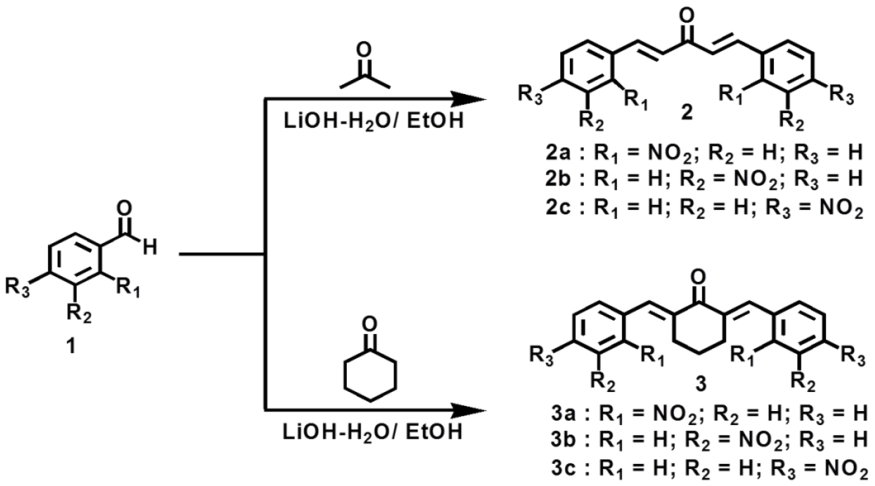


Figure 1.5 Curcumin chalcone analogs structures. The addition of a cetone or a cyclohexanone to the structure of curcumin, yields six curcumin analogs. The nitro groups, hydrogen and r groups have different orientations (ortho, meta and para) due to their positions in the structure of the curcumin analog.

(Bhattacharyya et al., 2010), reported that curcumin (50mg/kg) could block the destruction of circulating T cells by the tumor. Tumor bearing mice were treated with curcumin and showed an increased in CD8+ and CD4+ T cells in the tumor site. A study by (Barve, A., et al 2008), describes how mice that were fed with curcumin alone or in combination with another natural compound called Phenylethylisoathiocyanate (PEITC) can suppress prostate cancer tumors. The amount of proliferative cells decreased after 16 weeks of the treatment with Curcumin or PEITC. Low levels of PCNA, which is an antibody use to detect proliferative cells were observed in immunohistochemical analysis of TRAMP male mice after the treatment with curcumin or in combination with PEITC. High apoptotic cell percentages in the dorso-lateral prostates after the treatment with curcumin and/or PEITC were observed. After 16 weeks of dietary supplementation with curcumin alone, the highest percentage of apoptotic cells was observed. (Roy et al., 2013) demonstrated that the analog Difluorinated curcumin (CDF) reduces miR-21 in SW620 (a metastatic colon cancer cell line). MiR-21 regulates metastasis and it is elevated in colorectal cancer. The reduction in miR-21/Rnu1α, leads to the inhibition of colon cancer cell growth. Western-blots showed that the addition of CDF reduces pAKT which leads to the increase in PTEN. Low levels of PTEN are linked to resistance to therapy and reappearance of cancer. (Luo et al., 2011), reported that low doses of curcumin can increase the frequency and number of T-cell populations, among which IFN-γ-secreting CD8+T cells is the most especial. This previous studies suggests that curcumin or its analogs could be a potential treatment for cancer.

Prostate cancer

Despite the different prostate cancer treatment therapies it continues to be one of the leading causes of death in men. New treatment strategies are being developed to stop the progression of this disease. The prostate specific antigen (PSA) blood test is used during the diagnosis of prostate cancer. It has been reported that PSA is a serine protease protein, released to the blood as a result of changes in normal prostate architecture (Lilja et al., 2008). A patient with high levels of PSA undergo a biopsy to detect prostate cancer and after a Gleason score is used to sort tumor from most to least differentiated, usually from 1-5 (Epstein, 2010). The local extent of a tumor is named by four categories (T1-T4), lymph node involvement (N0 or 1) and metastasis (M0 and 1a-c) (Ohori et al., 1994).

Prostate cancer can spread to other parts of the body. A total of four stages are used for prostate cancer, briefly described as follow:

Stage I

Cancer is only present in the prostate. It is found after biopsy and PSA levels are high. In some cases cancer cannot be detected by digital rectal exam.

Stage II

Cancer still only in the prostate but this stage is divided in IIA and IIB. In IIA PSA levels are lower than 20, Gleason score is lower than 7. Cancer is present in one lobe of the prostate. In IIB PSA levels could be 20 or higher and

cancer is found in opposite sides of the prostate. Gleason score varies from 2 to 10.

Stage III

Cancer is spread to seminal vesicles. Gleason score is 2 to 10 and any level of PSA.

Stage IV

Cancer is found in other organs and distant parts of the body, such as the bones (Prostate Cancer, Prostate Cancer Treatment, 2015).

Castration resistance prostate cancer (CRPC) is defined as disease progression even with androgen deprivation therapy (Sharifi et al., 2005). “Castrate –resistant” is a term that describes the important role of intracrine and paracrine androgen production in the resistance of prostate cancer cells to testosterone suppression therapy (Mostaghel et al., 2007). A 90% of men with CRPC will have bone metastasis, affecting the bone marrow and could cause spinal cord compression. Anemia, fatigue and weight loss could be seen in these patients.

Prostate cancer cell line PC-3.

In vitro studies of prostate cancer are performed to prove different therapies. These studies can bring the idea of what a certain treatment could affect, up-regulate and down-regulate. Usually, cell lines require specific culture methods, temperature, growth factors as well as other specifications. Molecular

mechanisms studies can also be performed with cancer cell lines. Different prostate cancer lines are being used for the study of this disease Table 1.1. PC-3 and DU-145 cell lines are derived from human tumors and they lack androgen receptors. They also lack the expression of PSA and 5alpha-reductase.

Profile of Established Human Prostate Cancer and Immortalized Cell Lines

Cell line	Source	Media requirements[a]	Reference
PC-93	AD primary prostate cancer	A	*18, 86*
PC-3	Lumbar metastasis	B or D (ATCC[b] recommendation)	*19*
DU-145	Central nervous system metastasis	B or E (ATCC recommendation)	*20*
TSU-Pr1[c]	Cervival lymph node metastasis in Japanese male	B or F	*21*
LNCaP	Lymph node metastasis in Caucasian male	G or B	*24, 87, 88, 89*
LNCaP-FGC[d]	Clonal derivative of LNCaP	B	*24, 87*
LNCaP-LN-3	Metastatic subline of LNCaP cells derived by orthotopic implantation	H or I	*22*
LNCaP-C4	Metastatic subline of LNCaP, derived after coinoculation of LNCaP and fibroblasts	G	*29, 30*
LNCaP-C4B	Metastatic subline derived from LNCaP-C4 after reinoculation into castrated mice	G	*29, 30*
MDA PCa 2a	AI bone metastasis from African-American male	J or K	*34, 35*
MDA PCa 2b	AI bone metastasis from African-American male	J or K	*34, 35*
ALVA-101	Bone metastasis	L	*36*
ALVA-31[e]	Well-differentiated adenocarcinoma	M	*38*
ALVA-41[e]	Bone metastasis	L	*42*
22Rv1	Derived from CWR22R, an androgen-dependent prostate cancer xenograft line	B	*43*
ARCaP	Derived from ascitic fluid from a patient with metastatic disease	G	*44*
PPC-1[e]	Poorly differentiated adenocarcinoma	B	*45*

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PPC-1[e]	Poorly differentiated adenocarcinoma	B	*45*

Cell line	Source	Media requirements[a]	References
LAPC3	Derived from xenograft established from specimen obtained via transurethral resection of the prostate	N	*47*
LAPC4	Derived from xenograft established from a lymph node metastasis	N	*47*
P69SV40T	Immortalized cell line derived by transfection of adult prostate epithelial cells with the SV40 large T antigen gene	O	*58*
RWPE-2	Immortalized cell line initially derived by transfection of adult (Caucasian) prostatic epithelial cells with human papillomavirus 18, then made tumorigenic by infection with v-K-ras	P	*59*
CA-HPV-10	Immortalized cell line derived by human papilloma virus 18 transfection of prostatic epithelia cells from a high-grade adenocarcinoma	Q	*60*
PZ-HPV-7	Immortalized cell line derived by human papilloma virus 18 transfection of normal prostatic peripheral zone epithelial cells	Q	*60*

Table 1.1 Prostate cancer cell lines.

Major human prostate cell lines and media requirements (Russell and Kingsley, 2003). This table describes the different prostate cancer cell lines, their source and media requirements.

Media Requirements of Human Prostate Cancer and Immortalized Cell Lines

Designation	Components
A	Eagle's minimum essential medium (MEM) supplemented with 10% FBS and 2 m*M* L-glutamine
B	Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS)
C	Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS
D	Kaighn's modification of Ham's F-12 medium (F-12K), supplemented with 10% FBS and 2 m*M* L-glutamine, and adjusted to contain 1.5 g/L sodium bicarbonate
E	MEM supplemented with 10% FBS, 2 m*M* L-glutamine and Earle's balanced salt solution (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 m*M* nonessential amino acids and 1.0 m*M* sodium pyruvate
F	RPMI supplemented with 5% FBS
G	"T-medium": DMEM:F-12K, 4:1, supplemented with 5% FBS, 3 g/L sodium bicarbonate, 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL transferrin, 0.25 µg/mL biotin, 25 µg/mL adenine *(88,89)*
H	Medium "B" supplemented with sodium pyruvate, nonessential amino acids and vitamins
I	RPMI:F-12K, 1:1, supplemented with 10% FBS
J	BRFF HPC1 medium (Biological Research Faculty and Facility, Inc., Jamesville, MD) supplemented with 15% FBS
K	F-12K supplemented with 20% FBS, 10 mg/mL epidermal growth factor, 100 ng/mL hydrocortisone, 5 µg/mL insulin, 25 ng/mL cholera toxin, 5×10^{-6} *M* phosphoethanolamine, 3×10^{-8} *M* sodium selenite
L	RPMI supplemented with 5% FBS, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate
M	RPMI supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, buffered to pH 7.4 with 7.5% (w/v) sodium bicarbonate
N	Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 10 n*M* R1881 synthetic steroid
O	Serum-free RPMI supplemented with 10 ng/mL epidermal growth factor, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, and 0.1 µ*M* dexamethasone
P	Keratinocyte serum-free medium (KSFM) supplemented with 50 µg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor
Q	Keratinocyte serum-free medium (KSFM) supplemented with 50 µg/mL bovine pituitary extract

Table 1.2 Designation of media requirements of human prostate cancer and immortalized cell lines

Each letter in the table represents the different media components that are necessary for the cell line culture (Russell and Kingsley, 2003).

Curcumin as chemotherapeutic agent

The most commonly used treatments for prostate cancer are: surgery radiation therapy, hormone therapy, chemotherapy, and targeted therapy (Cancer Treatment, Types of Treatment, 2015). In this study we were interested especially in chemotherapy, testing if our treatment could be used in combination with chemotherapy to reduce its toxicity. The advantage of chemotherapy is that it keeps the cancer from spreading, slows cancer growth and kills cancer cells that have spread to other parts of the body. The disadvantages are that chemotherapy kills normal cells, can cause pain, fatigue, nausea, vomiting and anemia (Cancer Treatment, Side Effects, 2015).

The majority of prostate cancer patients undergo androgen deprivation therapy but some could develop castration resistant prostate cancer (CRPC). Patients with CRPC, could receive Docetaxel as treatment. Docetaxel is a chemotherapeutic agent that prevents microtubule depolymerization. It binds to ß-tubulin, causing cell cycle arrest and apoptosis (Zhu et al., 2013). The most common side effects of docetaxel are: burning, numbness, tingling, or pain in the arms, tiredness, weakness, weight gain, and others (Drugs and supplements Docetaxel Intravenous Route, 2015).

In a study by (Kato, et al.2015), resistantce to docetaxel was due in part to P-glycoprotein (P-gp), which is encoded by multidrug resistance protein 1 (MDR1) gene. The taxane cabazitaxel is effective in docetaxel resistance of castration resistant prostate cancer (CRPC) because it has low affinity for (P-gp).

In the study the knockdown of MDR1, enhanced the sensitivity of docetaxel resistant PC-3 cells (www.urologiconcology.org).

Natural compounds can be used in addition to chemotherapy. As an example, Curcumin, a polyphenol isolated from *Curcuma longa*, sp. has demonstrated to inhibit cell proliferation, invasion and to promote apoptosis of cancer cells (Aggarwal et al., 2009). Curcumin has demonstrated to stop tumor development. It is really active against leukemias, lymphomas, multiple myeloma, brain cancer, melanoma and skin, lung, prostate, breast, colon cancer and others. Various mechanisms have been proposed to explain the action of Curcumin. Some pathways by which curcumin inhibit cancer progression are: inhibition of epidermal growth factors receptors (EGFR), nuclear factor-kappa B (NF-kB) and signal transducers and activators of transcription (STATS) (Mimeault and Batra, 2011) (Figure 1.5). Besides its ability to inhibit a variety of pathways, it has the disadvantage of low bioavailability and low stability which affects its effect in humans. Curcumin analogs are being synthesized to address this problem and are being used alone or in combination with others therapies.

Curcumin anticancer properties have been reported to be the inhibition of NF-kB, prevention of lipid peroxidation and DNA damage (Choudhary and Singh, 2012). Curcumin anti-inflammatory activity has been reported as the inhibition of COX-2 and cytokines like IL-6 and IL-8. In a study of advanced colorectal cancer patients, curcumin (3.6g/daily) showed a reduction in PGE_2 in peripheral blood as indicator of COX-2 activity (Sharma et al., 2004 and Plummer 2001).

Figure 1.6 Molecular pathways of curcumin.

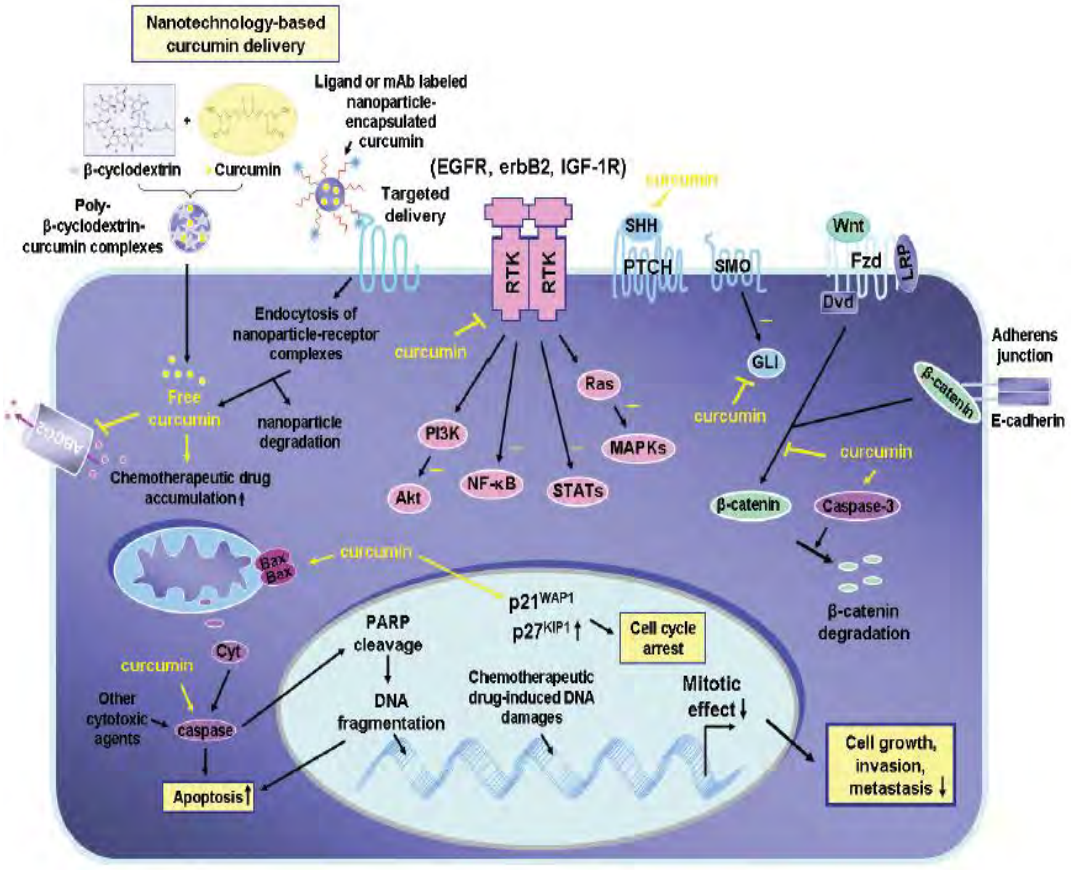

Figure 1.6 Molecular pathways of curcumin. This figure shows how Curcumin can inhibit or induce the expression of certain molecules like EGFR and p21, respectively (Mimeault and Batra, 2011). Curcumin analogs like curcumin nanoparticle, can cause the down-regulation of NF-kB and MAPKs. Analogs are very stable and can improve the delivery of curcumin to cancer cells.

Various studies have demonstrated that curcumin can cause the inhibition of mTOR a protein kinase that regulates cell growth, proliferation, survival and dysregulation in its pathway is found in cancer and diabetes (Riemenschneider et al., 2006). Curcumin is a potential agent to treat prostate cancer that can down regulate the androgen receptor and its cofactors. Reduction of serum PSA levels was observed in a study of 85 patients that were taken 0.1g/day of curcumin

during 6 months in combination with isoflavones (Ide et al., 2010). Previous studies have shown that curcumin can increase the levels of membranous B-catenin in prostate cancer cells leading to a decrease in the cell proliferation, both in vitro and in vivo (Sundram et al., 2012).

Curcumin analogs as chemotherapeutic agents

Besides the beneficial characteristics of curcumin as an anticancer agent it has the disadvantage of low bioavailability. Figure 1.7 represents the plasma and tissue distribution of curcumin on mice. Different methods have been used to solve curcumin poor bioavailability, for example: nanoparticles and liposomes (Sasaki et al., 2011 and Gota et al., 2010). Curcumin analogs have been developed from studies of the tautomeric forms of curcumin (Tamvakopoulos et al., 2007). Structural analogs and phospholipid complexes have been used also to improve the bioavailability of curcumin (Anand et al., 2007). The analog NanoDoxCur can overcome multidrug resistance (Pramanik et al., 2012). Cellulose nanoparticles are being used for prostate cancer treatment (Yallapu et al., 2012). Nanoparticles showed to increase curcumin bioavailability in hydrophilic environment, and it also improves the dispersion of curcumin (Bisht et al., 2007).

Figure 1.7 Plasma and tissue distribution of curcumin on mice.

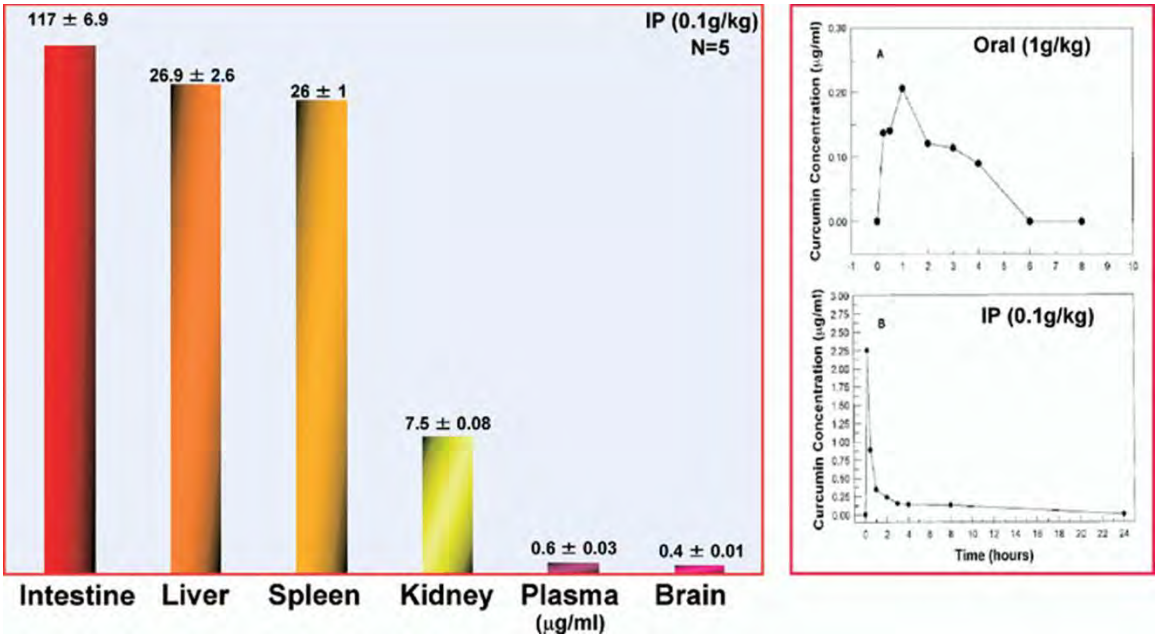

Figure 1.7 Plasma and tissue distribution of curcumin on mice. The bar graph shows the distribution of curcumin in tissue, after two hours of oral administration. The higher absorption of curcumin was found in the intestine, followed by liver and spleen. In the upper right panel, the graph shows that curcumin concentration starts to decreased two hours after oral administration (Pan et al., 1999).

Figure 1.8 Structures of curcumin and its synthetic analogs.

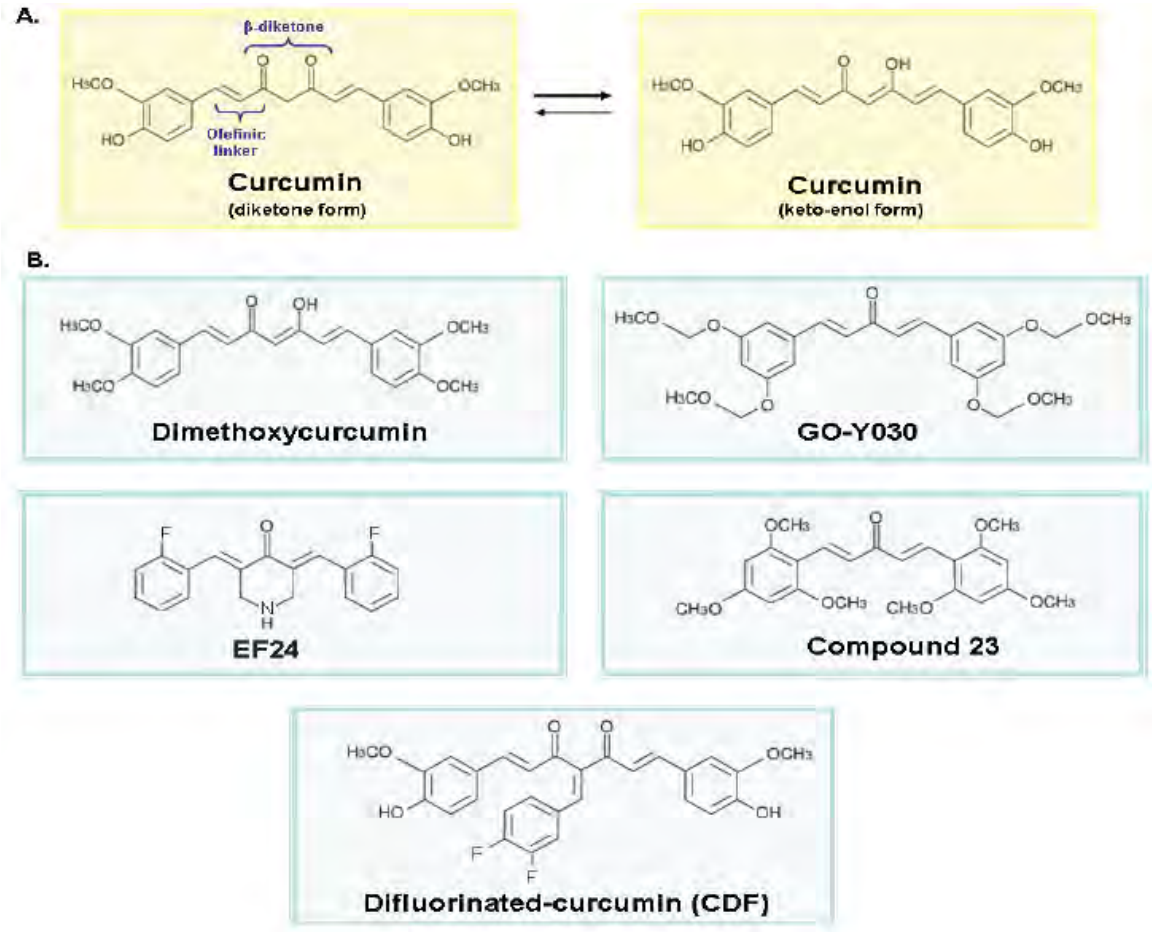

Figure 1.8 Structures of curcumin and its synthetic analogs. The diagram demonstrates the diketone and keto-enol forms of curcumin (A). The enol form is the most stable and favored form of curcumin in solution. B) Novel synthetic analogs of curcumin that had shown to have anticancer properties (Tamvakopoulos et al., 2007).

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Analogs with benzyl piperidone had demonstrated higher inhibitory effects than Curcumin (Table 1.3).

Compound	IC_{50} (μM)			
	PC-3	BxPC-3	HT-29	H1299
Curcumin	19.98±2.4	18.25±2.2	18.74±2.2	18.93±2.1
P1	9.13±1.3	6.67±0.9	7.95±1.1	5.61±0.7
P2	1.35±0.2	0.91±0.1	0.86±0.1	1.05±0.2
P3	10.18±2.1	8.93±1.2	9.16±1.2	10.95±1.6
P4	1.75±0.2	1.13±0.2	1.37±0.3	1.45±0.2
P5	5.05±0.7	2.64±0.3	4.97±0.6	1.58±0.2
P6	4.12±0.6	1.97±0.3	2.79±0.4	2.01±0.3
P7	1.82±0.3	1.09±0.2	1.55±0.2	1.31±0.2
PFBr1	17.67±2.6	15.59±2.4	16.43±2.1	17.23±2.7
PFBr2	1.78±0.2	1.70±0.2	2.38±0.4	2.16±0.3
PFBr3	1.75±0.2	1.51±0.2	1.63±0.3	2.59±0.4
PFBr4	0.49±0.1	0.50±0.1	0.47±0.1	0.41±0.1

Table 1.3 Inhibitory effects of curcumin related compounds containing benzyl piperidone on the growth of different cancer cell lines.

This table shows the effect of the compounds on the growth of different cancer cells. These values were obtained by MTT Assay after the treatment with (0.1-30 μM) of the compounds for 72 hours. Compound PFBr4 has the lowest EC50 against PC-3 cells in comparison with other compounds (Dai-Ying et al., 2013).

Structures of curcumin-related compounds with linker, aromatic ring and steric hindrance are significant for activity. Benzyl piperidone as linker increases the cytotoxicity of curcumin-related compounds.

Curcumin against prostate cancer

Prostate cancer represents a major cause of cancer related deaths. It encompasses a variety of genetic abnormalities, inhibition of tumor suppressor

genes and impairment of oncogenic pathways. Curcumin can stop tumor growth by the inhibition of cyclin-dependent kinase, MAPK and other molecules. Curcumin can suppress androgen dependent and androgen-independent prostate cancer. Curcumin and its analogs MS17 and MS13 have demonstrated that they can decrease cell viability of PC-3 cells and DU-145 cells as shown in the table 1.4 (Citalingam et al., 2015).

Compounds	EC_{50} Values (μM) of Compounds on Cell Lines			
	PC-3	DU 145	WI-38	WRL-68
MS13	7.5 ± 0.1	7.4 ± 2.6	9.4 ± 2.3	8.7 ± 0.2
MS17	4.4 ± 0.3	4.1 ± 0.8	5.2 ± 1.2	5.5 ± 0.5
MS40E	28.0 ± 7.8	30.3 ± 1.9	28.4 ± 1.7	30.3 ± 2.0
MS49	14.5 ± 1.2	12.3 ± 2.3	19.4 ± 7.0	13.1 ± 0.4
* Curcumin	35.9 ± 2.9	32.5 ± 1.4	27.9 ± 2.9	28.7 ± 3.2

Table 1. 4 EC50 values of curcumin and its analogues on PC-3 and DU-145 cell lines and normal cell lines.

Curcumin and its analogs increased cytotoxicity against prostate cancer cell lines PC-3 and DU-145. The highly metastatic PC-3 cell line and the DU-145 cell line growth can be inhibited by analog MS17 and curcumin. The analog MS17 was the most effective against the prostate cancer cell lines. *Curcumin was used as the positive control.

The cytotoxic effect could be seen in a dose-dependent manner. Curcumin and its analogs can also decrease proliferation of prostate cancer cells. In this study we aim to evaluate the chemotherapeutic effect of curcumin and its analogs against prostate cancer cells.

Curcumin analogs against prostate cancer

Some pharmacokinetic drawbacks have been attributed to curcumin including low bioavailability, fast metabolism, and demanding repetitive doses, which limit its pharmacological applications in vivo (Anand et al., 2007). When

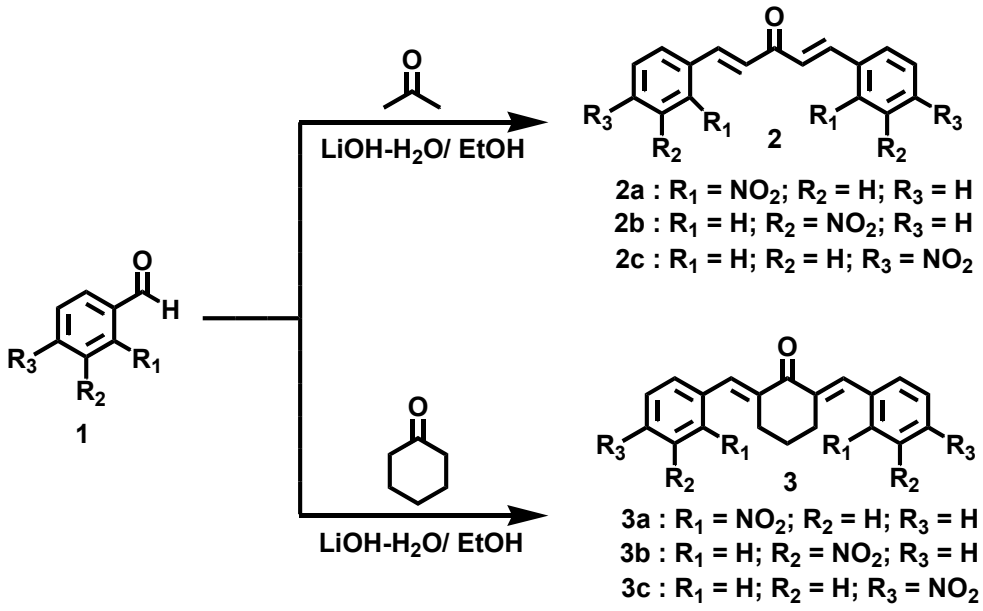
curcumin is incubated in 0.1mol/L phosphate buffer and serum-free medium (pH 7.2) at 37°C, it is degraded within 30 minutes into ferulic acid and vanillin (Wang et al., 1997). Both, ferulic acid and vanillin have anticancer properties. Ferulic acid can stop cyclooxygenase-1 and cyclooxygenase-2 (Jayaprakasam et al., 2006). On the other hand, vanillin could down-regulate NF-kB activation (Murakami et al., 2007). Another product of the degradation of curcumin is 4-vinyl guaiacol which augments the transactivation of the redox-regulated transcription factor Nrf2, associated to antioxidant defense mechanisms.

In order to address the pharmacokinetic problems of Curcumin, new curcumin analogs are being synthesized aimed at improving its bioavailability. The curcumin analog EF24 which is a fluorinated substance, has demonstrated to inhibit vascular endothelial growth factor (VEGF) (Shoji et al., 2008). In PC-3 cells the analog EF24 (1μM) has shown to down-regulate the alpha subunit of the hypoxia-inducible factor protein (HIF-1α), contrary to curcumin which needs to be administered at higher concentration (20-50 μM) to obtain this effect (Thomas et al., 2008). During hypoxia, HIF-1α is in the nucleus and activates hypoxia response elements. EF24 effect on HIF-1α is post-transcriptionally, as seen in tubulin inhibitors. The analog EF24 contains flour and nitrogen elements as well as cyclic structures.

In a study by (Lin, Li., et al 2006), 40 curcumin analogs were designed to evaluate their effect against prostate cancer. These analogs were tested in PC-3 and LNCaP cell lines. The four most effective compounds of this study were: 3, 4, 40 and 44.

In our study the curcumin analogs were synthesized by aldol condensation. Cetone or cyclohexanone spacers were incorporated to the curcumin molecule and six analogs were obtained (Figure 1.9.).

Figure 1.9 Curcumin analogs synthesized by aldol condensation.



After testing the cytotoxicity against PC-3 cell line of the six analogs synthesized in Dr. David Sanabria laboratory, analog **3b** demonstrated the highest cytotoxicity against PC-3 cell line in comparison with the other five analogs. It also showed that less amount of the analog **3b** in comparison with the other analogs should be added to the cells to obtain the cytotoxic effect. Analog **3b** was the most effective against PC-3 cell line of all the analogs, converting it into the analog that was chosen to be encapsulated in a nanoparticle.

The analog **3b** was introduced in a Poly (lactic-co-glycolic acid) (PLGA) nanoparticle in order to improve its delivery and stability. It was selected to use PLGA as drug carrier because in the United States PLGA is a Food and Drug

Administration approved co-polymer that can be used in pharmacological applications, since the metabolic conversion of PLGA results in water and carbon dioxide in a tricarboxylic acid cycle (Su et al., 2012). Endocytosis of nanoparticles results in their release to the cancer cell (Figure 1.10). In studies performed by (Tripathi et al., 2010) they demonstrated that the newly-designed PLGA nanoparticle encapsulating Rifampicin enhanced it anti-tuberculosis activity when compared to Rifampicin alone, when administered intravenously. They demonstrated that the percent of drug entrapment increased from 38.5% to 71.6% and the size of the nanoparticle changed from 920nm to 430nm. In that same study, PLGA nanoparticles demonstrated to have spherical shape and a polydispersity index with a distribution range from 0.000 to 0.500. A polydispersity index higher than 0.5 means the aggregation of particles. This study demonstrated that PLGA nanoparticles are slowly degraded, which means that the release of the drug mostly depends in the drug diffusion. This suggests that PLGA nanoparticles could be given in a single dose and it would maintain active drug levels for extended time. Nanoparticles maintain the stability of the drug and improve their delivery. More recently, Dr. David J. Sanabria-Ríos and his research group used the PLGA Nanoparticle approach for encapsulating **3b**. In this study, Dr. Sanabria-Ríos gathered preliminary results from Scanning Electron Microscopy (SEM), Zeta potential, and mobility measurements that demonstrated that PLGA NPs encapsulating **3b** has a spherical shape with a mean diameter of 116 nm, displays stability in water, and have a negative charge that avoids flocculation, which is in agreement with findings reported by (Tripathi

et al., 2010). Once **3b** was encapsulated into a PLGA NP, it was performed several experiments aimed at determining its cytotoxic activity against both PBMC and PC-3.

Figure 1.10 3b- Nanoparticle model and scanning electron microscopy (SEM) micrograph.

(a) **(b)**

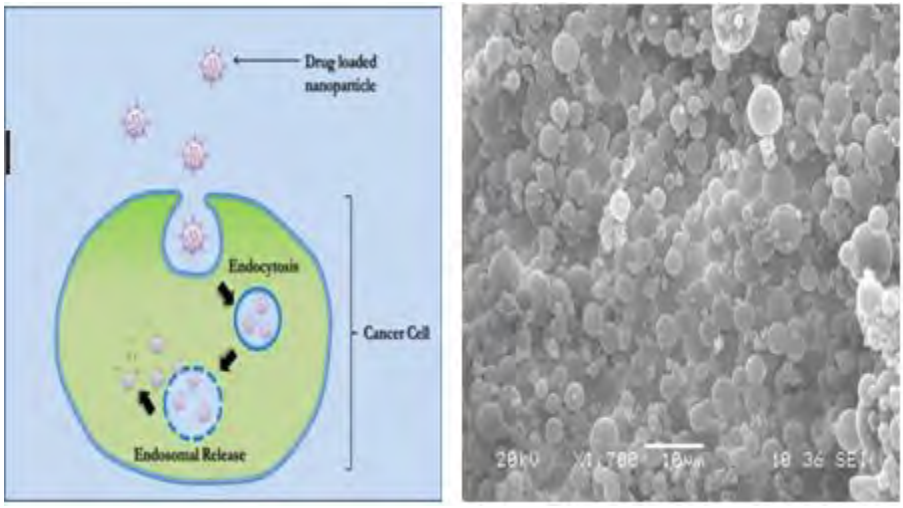


Figure 1.10 3b- Nanoparticle model and scanning electron microscopy (SEM) micrograph. Endocytosis of nanoparticles resulting in their release to the cancer cell (a); (SEM) micrograph showing nanoparticles (b).

In this study, the commercial Super-Biocurcumin, BCM-95 CG (Biocurcumax) from Life Extension was also tested against PC-3 cell line to measure cytotoxicity against the cells. This curcumin analog is 7-10 times more bioavailable than curcumin. In a study by (Antony et al.,2008) with healthy volunteers, BCM-95 CG Biocurcumax) demonstrated a rapid absorption after one hour (mean 315.8 ng/g), then after four hours the absorption was higher (456.88 ng/g). On the contrary, curcumin reached its peak of absorption at two hours (149.8 ng/g), and after that, it disappeared from blood. These values were

obtained from the concentration time profile of control curcumin and BCM-95 CG (Biocurcumax). BCM-95 CG (Biocurcumax, is made completely from turmeric. It does not contain piperine being less toxic to experimental animals.

Chapter 2: Chemotherapeutic effect of curcumin and its analogs against prostate cancer cell line PC-3

Introduction

Treatment of PC3MM2 cell lines with high doses (10-40 μM) of CMC2.24, a polyenolic zinc-binding curcuminoid showed the highest cytotoxicity (Botchkina et al., 2013). In that study, CMC2.24 in combination with a new generation taxoid SBT-1214 are more effective together in promoting cell death of tumor initiating cells (CD133+ cells). CMC2.24 in combination with SBT-1214 causes the total inhibition of c-Myc and Sox2 in CD133+ and they also increase the P53 and P21 genes which are tumor suppressor and regulators of apoptosis.

Another curcuminoid that has demonstrated to be effective against prostate cancer is Demethoxycurcumin (DMC) (Xiaochen et al., 2012). The difference between curcumin and DMC is that the last one does not has a methoxy group directly linking to the benzene ring. DMC demonstrated the reduction of PCNA leading to the inhibition of cell proliferation. This curcuminoid also showed an increased in the G2/M phase of cell cycle arrest of PC-3 cells. MMP-2 activity was inhibited by DMC which causes the suppression of migration and invasion.

The curcumin analog 27 (ca27) has demonstrated to down regulate the expression of the androgen receptor (AR) (Fajardo et al., 2012). This analogs in contrast to curcumin had a shorter 5-carbon unsaturated linker with a single carbonyl group and its α,ß-unsaturated ketone gives anti-proliferative effects. Cells (LNCap, C4-2, LAPC-4) were treated with ca27 per 12 hours from 1to 5μM

showed a reduction of AR protein expression to approximately 30% of control. Curcumin, on the other hand, did not showed reduction of the AR protein expression in C4-2 cells, even at high concentrations of 20μM curcumin for 72hours. In this study, ca27 mediates the down-regulation of endogenous AR independent of proteasomal degradation or its effect on AR mRNA transcription. When cells (LNCap, C4-2) were exposed to ca27 the loss of AR protein expression occurs at lower concentration suggesting that this could contribute to cell growth inhibition and death. The ca27 can inhibit AR activation and can also lower prostate specific antigen (PSA at 12 hours). LNCap cells treated for 1hr with (3μM) ca27, increased the production of ROS. The critical cellular redox sensor Nrf2, showed a significant activation after LNcaP cells were treated with ca27 (5μM). In this study we aimed to evaluate the cytotoxic effect of Docetaxel, curcumin and its analogs against the prostate cancer cell line PC-3.

Materials and methods

Human cancer cell line PC-3 (prostate cancer) was purchased from ATCC. The cell line was cultured in 19ml of F-12K medium in culture flasks. Trypsin EDTA (2ml) (Hyclone) was used to detach cells from cultured flasks. Cancer cells were seeded 10,000 cells/ 200 μL/ well in a 96 well plate (BD Falcon). The PC-3 cells were incubated with the different concentrations of curcumin and its analogs: 2μg/ml, 5μg/ml,10μg/ml, 15μg/ml at 37°C for three days in humidified 5% CO2 incubator. The cell viability was evaluated by MTT (methyl thiazolyl tetrazolium) assay from Sigma. The absorbance was measured with a spectrophotometer (Tecan). Experiments were performed in triplicates.

Chemicals

BCM-95 (Biocurcumin) 500 mg capsule were obtained from Life Extension. Curcumin was purchased from Sigma. Analog **3b** was synthesized by Dr. David Sanabria (Inter American University of Puerto Rico) by aldol condensation reaction. Docetaxel and MTT (methyl thiazolyl tetrazolium) were purchased from Sigma (St. Louis, MO, USA).

Cell culture

PC-3 cells were purchased from ATCC. Cells were grown in F-12K medium (ATCC), supplemented with 10% fetal bovine serum (Thermo Fisher) and 1% of penicillin-streptomycin. Cells were grown in a 5% CO2 humidified incubator at 37°C.

Citotoxicity assay of curcumin and its analogs

Cell viability was measured by MTT Assay. Cells (20,000 cells/well) were seeded in 96 well plates (Thermo Fisher) and in 200µl completed F12-K medium. Compounds were added to the cells (1-25µg/ml) and incubated for three days. The third day MTT (20 µl per well) were added and the plate was incubated for four hours at 37^0C. After the incubation with MTT, the medium was removed and 100 µl of DMSO (Dimethyl Sulfoxide from Sigma) were added. The plate was placed on a shaker for 15 minutes. The cell viability was measured by Tecan 200 Infinite Pro plate reader using a wavelength of 560nm.

Statistics

Values were analyzed using the program GraphPad Prism 6. Logaritmic fit was performed to obtain IC 50 values.

Results

Figure 2.1 Dose response curve of docetaxel in PBMC.

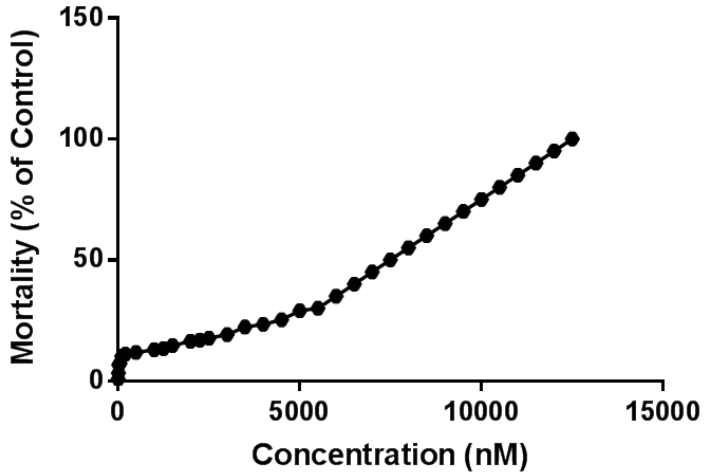


Figure 2.2 Dose response curve of docetaxel in PBMC. Docetaxel was added to the peripheral blood mononuclear cells at different concentrations (0-5000nM) for three days. Cytotoxicity was measured by MTT assay. The mortality for of PBMC after the addition of Docetaxel started at approximately 5000nM. Data is based on the mean of three experiments.

Figure 2.3 Dose response curve of docetaxel in PBMC.

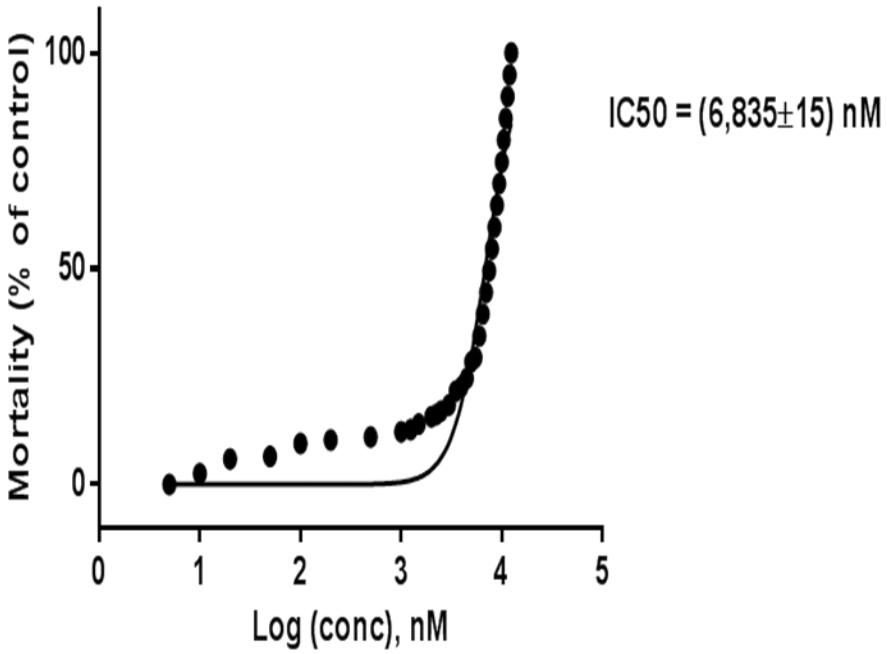


Figure 2.3 Dose response curve of docetaxel in PBMC. Docetaxel was added to the peripheral blood mononuclear cells (PBMC) at different concentrations (0-5000nM) for three days. Cytotoxicity was measured by MTT assay. The IC50 for Docetaxel on PBMC was 6,835±15nM. Data is based on the mean ± SEM of three experiments.

Figure 2.4 Dose response curve of docetaxel in PC-3.

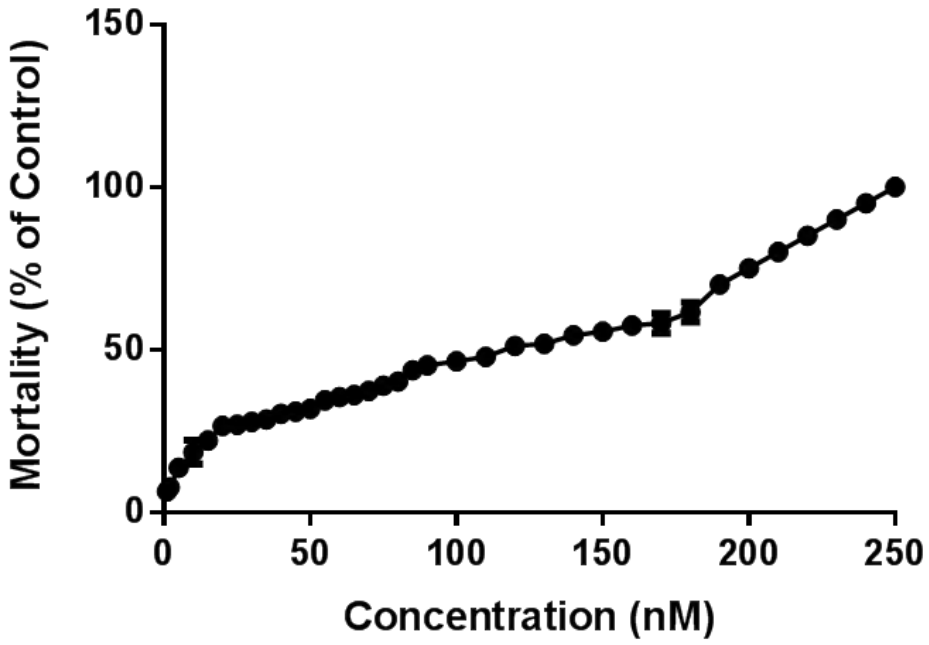


Figure 2.4 Dose response curve of docetaxel in PC-3. Docetaxel was added to PC-3 cells at different concentrations (0-250nM) for three days. Cytotoxicity was measured by MTT assay. The mortality for of PC-3 cells after the addition of Docetaxel started below 50nM. Data is based on the mean of three experiments.

Figure 2.5 Dose response curve of docetaxel in PC-3.

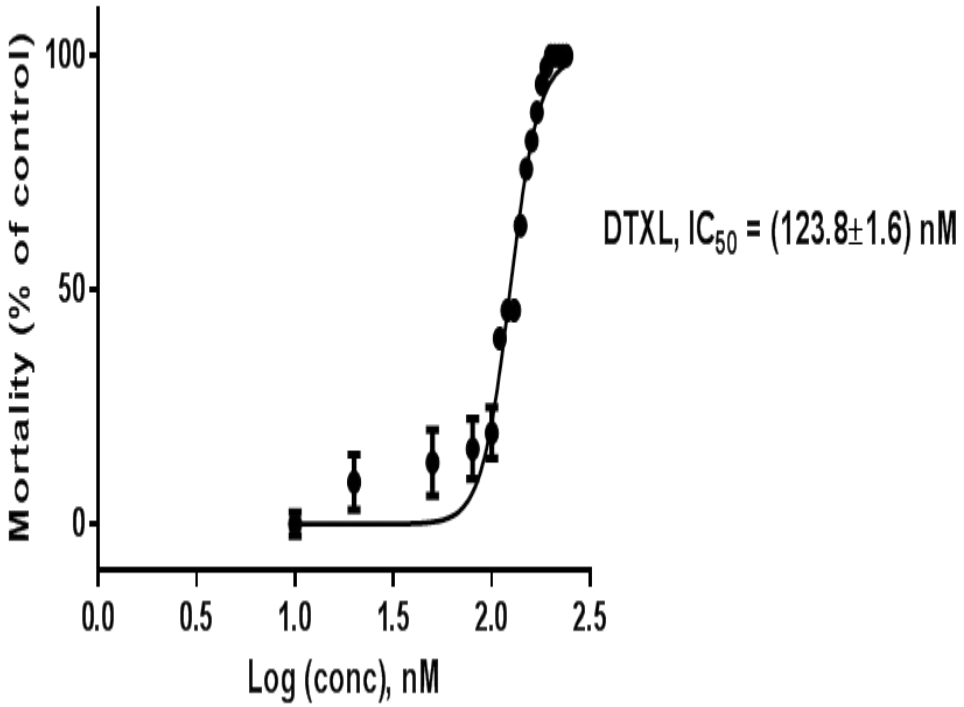


Figure 2.5 Dose response curve of docetaxel in PC-3. Docetaxel was added to PC-3 cells at different concentrations (0-250nM) for three days. Cytotoxicity was measured by MTT assay. The IC50 for Docetaxel on PC-3 cells was 123.8±1.6nM. Data is based on the mean ± SEM of three experiments.

Figure 2.6 Dose response curve of curcumin and its analogs in PBMC.

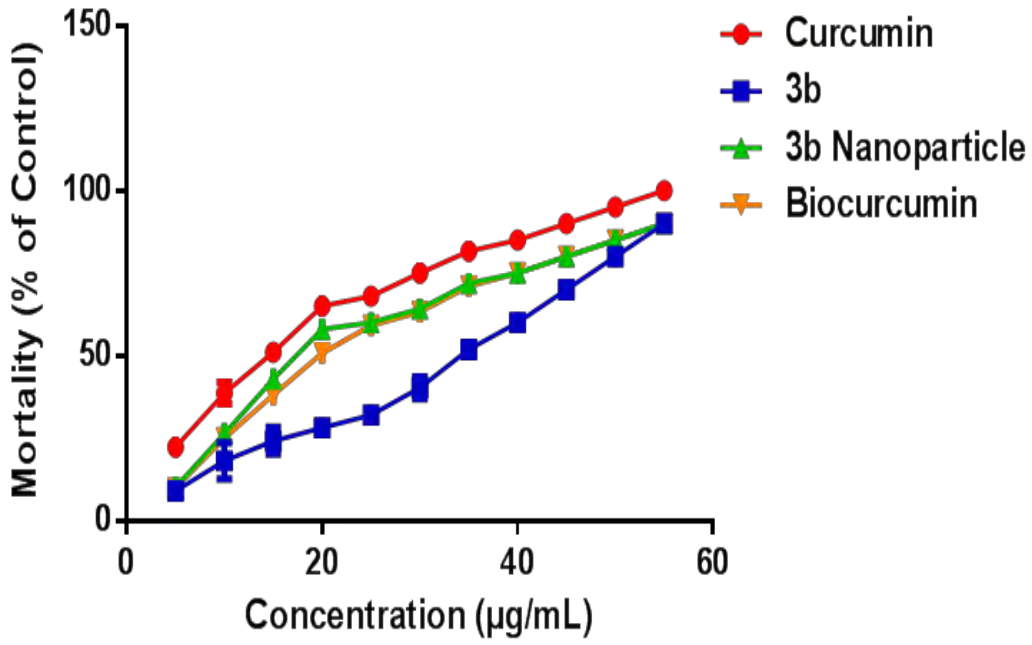


Figure 2.6 Dose response curve of curcumin and its analogs in PBMC.
Curcumin or its analogs were added to the peripheral blood mononuclear cells (PBMC) at different concentrations (0-50µg/ml) for three days. Cytotoxicity was measured by MTT assay. The mortality for of PBMC after the addition of curcumin, 3b Nanoparticle and Biocurcumin started at approximately 20µg/ml. Mortality of PBMC after the addition of **3b** started approximately at 35µg/ml. Data is based on the mean of three experiments.

Figure 2.7 Dose response curve of curcumin and its analogs in PBMC.

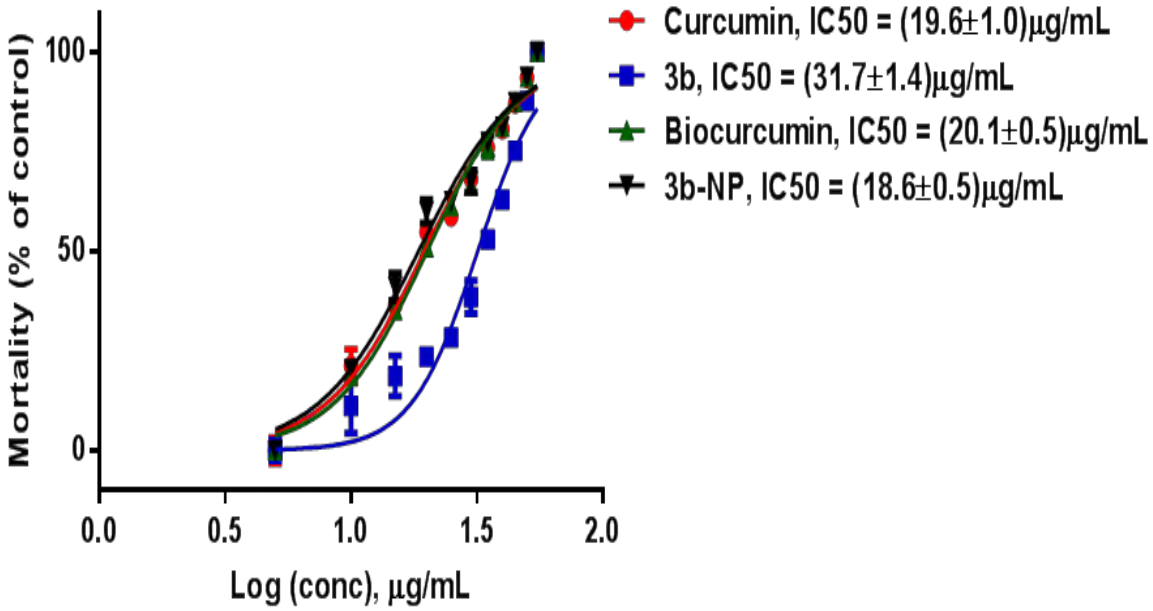


Figure 2.7 Dose response curve of curcumin and its analogs in PBMC. Curcumin or its analogs were added to the peripheral blood mononuclear cells (PBMC) at different concentrations (0-50µg/ml) for three days. Cytotoxicity was measured by MTT assay. The IC50's for Curcumin, **3b**, Biocurcumin, 3b Nanoparticle were: 19.6 ± 1.0µg/ml, 31.7±1.4 µg/ml, 20.1 ± 0.5µg/ml and 18.6±0.5 µg/ml, respectively. Data is based on the mean ± SEM of three experiments.

Figure 2.8 Drug response curve of curcumin and its analogs in PC-3 cells.

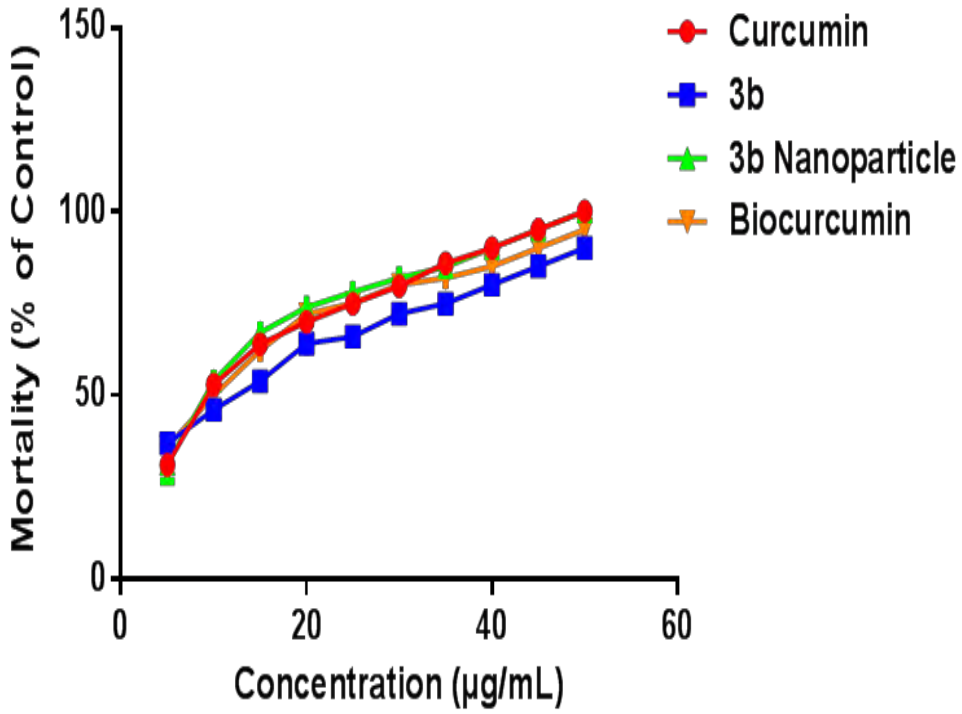


Figure 2.8 Drug response curve of curcumin and its analogs in PC-3 cells. Curcumin or its analogs were added to the PC-3 cells at different concentrations (0-50µg/ml) for three days. Cytotoxicity was measured by MTT assay. The mortality for of PC-3 cells after the addition of curcumin, **3b**, 3b-Nanoparticle and Biocurcumin started at approximately at 10µg/ml-15µg/ml. Data is based on the mean of three experiments.

Figure 2.9 Drug response curve of curcumin and its analogs in PC-3 cells.

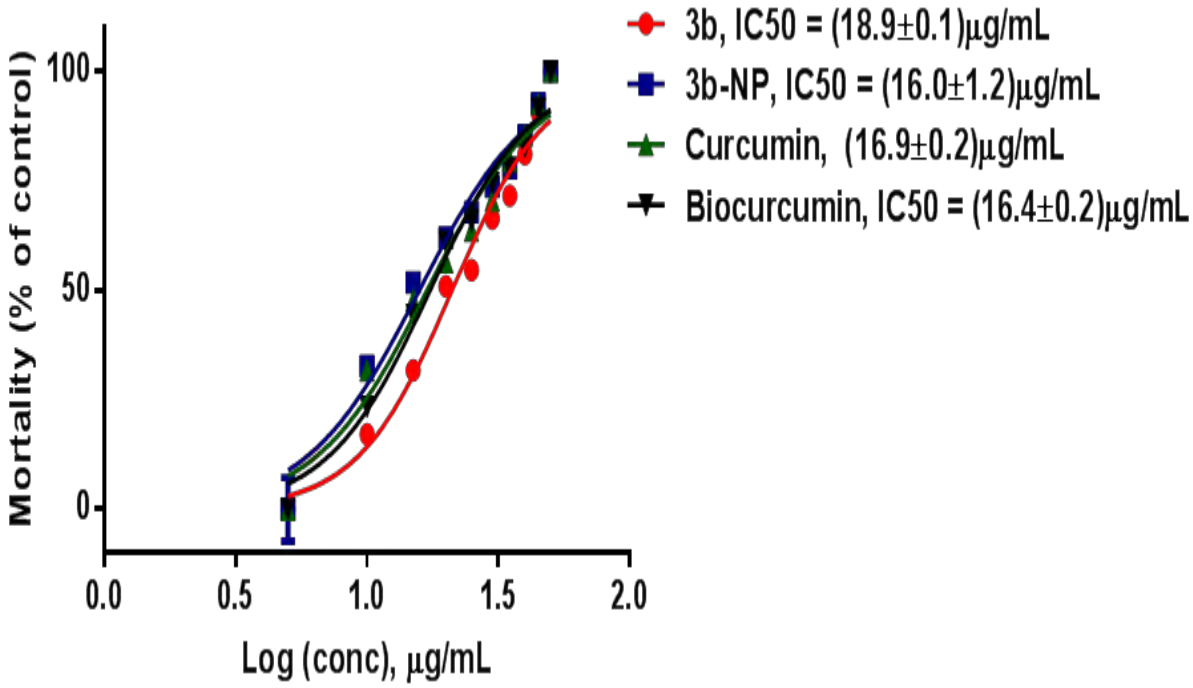


Figure 2.9 Drug response curve of curcumin and its analogs in PC-3 cells.
Curcumin or its analogs were added to the PC-3 cells at different concentrations (0-50µg/ml) for three days. Cytotoxicity was measured by MTT assay. The IC50's for **3b**, 3b-Nanoparticle, Curcumin and Biocurcumin, were: 18.9 ± 0.1µg/ml, 16.0±1.2 µg/ml, 16.9 ± 0.2µg/ml and 16.4±0.2 µg/ml, respectively. Data is based on the mean ± SEM of three experiments.

Discussion

Our experiments were performed in lower concentrations of Docetaxel (<5000nM), suggesting that it could be less toxic to normal cells (PBMC). Docetaxel IC50 was 6,835±15nM, which indicates that near fifty percent of the PBMC mortality was achieved at that concentration. Docetaxel below 50nM demonstrated that it can kill the PC-3 cells, indicating that higher doses of docetaxel could be used and will not be toxic to PBMC. Fifty percent of PC-3 cells mortality was achieved at 123.8±1.6nM of docetaxel .That concentration of docetaxel was effective against PC-3 cells and not toxic to PBMC.

The analog **3b** was less toxic than curcumin , biocurcumin and 3b-Nanoparticle against PBMC. It demonstrated the highest IC50 , indicating that it could be used at higher concentrations than curcumin, 3b-Nanoparticle and biocurcumin without being toxic to PBMC. Curcumin and its analogs demonstrated to increase the percent of mortality of PC-3 cells at approximately 10µg/ml-15µg/ml and these concentrations were not toxic to PBMC. The analog 3b-Nanoparticle showed the lower IC50, suggesting that it was more effective than curcumin, biocurcumin and **3b** against PC-3 cells. The analog **3b** is effective as curcumin. The therapeutic index of **3b** is better than the therapeutic index of curcumin.

Chapter 3: Effect of curcumin and docetaxel in combination therapy against PC-3 cell line

Introduction

Initiation, promotion and progression of cancer can be targets for the anticancer effect of curcumin (Cheng et al., 2001). Curcumin has demonstrated to regulate various cell signaling pathways in prostate cancer cells, including the down regulation of androgen receptor (AR) (Cheng et al., 2001) . Experiments with LNCaP and PC-3 cells showed that Curcumin can suppress the epidermal growth factor receptor (EGFR) expression which is related to cancer cells growth (Dorai et al., 2000).

Combination therapy experiments of Curcumin with other chemotherapeutic agents has showed to be more effective against cancer cells. Docetaxel is a product of 10-deacetylbaccatin III (*Taxus baccata*) which can be administered with combining agents to reduce chemoresistance (Bayet-Robert et al., 2010).The combination of curcumin (500 mg/d) with docetaxel (100mg/m^2) can reduce VEGF levels in patients with breast cancer and this could limit cancer progression (Figure 3.1) (Bayet-Robert et al., 2010).

Figure 3.1 Antiangiogenic VEGF marker as percentage of baseline across six cycles of treatment in eight patients with measurable lesions; *p <0.05.

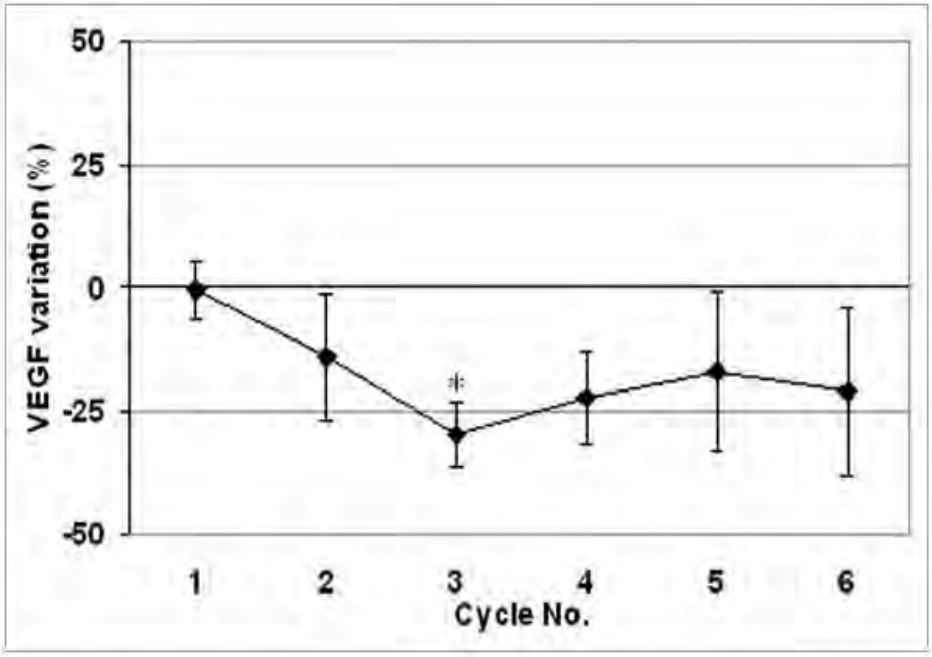


Figure 3.1 Antiangiogenic VEGF marker as percentage of baseline across six cycles of treatment in eight patients with measurable lesions; *p <0.05.
This figure demonstrates that the highest percent in VEGF variation was achieved after the third cycle of curcumin in combination therapy with docetaxel.

In a study with anaplastic thyroid carcinoma cells combination experiments of Curcumin (10μM) and Docetaxel (5nM) were performed and this resulted in lower viability of the cells after the combination treatment (Hong et al., 2014). In that same study they showed that combination therapy of curcumin and docetaxel could decrease the expression of protein related to cell proliferation like NF-kB and COX-2.

Docetaxel is an analog of paclitaxel which is another chemotherapeutic agent , but the advantage is that Docetaxel has lower systemic toxicity (Jones et al., 2005). Docetaxel causes microtubular stabilization leading to apoptosis in

cancer cells due to cell cycle arrest of the G_2M phase (Clarke et al., 1999). It is hydrophobic and enters into the nucleus via plasma proteins like lipoproteins, albumin and others (Clarke et al., 1999). After being oxidized in the nucleus a cyclic form of Docetaxel is formed and it causes the stabilization of microtubules that leads to cell cycle arrest (Verweij et al., 1994). Docetaxel resistance in prostate cancer cells is caused by the effluence of the drug outside the cell, with the help of P-glycoprotein (Ueda et al.,1987).

Nanotechnology is also used in the treatments against cancer. Nanoparticles have demonstrated that the delivery of compounds is more effective when encapsulated in nanoparticles under in vitro and in vivo conditions, an example are polylactic acid-polyethylene glycol (PLA-PEG) nanoparticles (Siddiqui et al., 2009). Another type of nanoparticle being effective against prostate cancer are polysaccharide nanoparticles which induces apoptosis (Rocha et al., 2011). Nanoparticles containing the chemotherapeutic agent Paclitaxel have demonstrated tumor regression in murine models of prostate cancer (Sahoo et al., 2004).

In this study we wanted to measure the effect of curcumin and docetaxel in combination therapy against PC-3 cell line.

Materials and methods

Chemicals

BCM-95 (Biocurcumin) 500 mg capsule were obtained from Life Extension. Curcumin was purchased from Sigma. Analog **3b** was synthesized by Dr. David Sanabria (Inter American University of Puerto Rico). Docetaxel and MTT were purchased from Sigma (St. Louis, MO, USA).

Cell culture

PC-3 cells were purchased from ATCC. Cells were grown in F-12K (ATCC) medium, supplemented with 10% fetal bovine serum (Thermo Fisher) and 1% of penicillin-streptomycin. Cells were grown in a 5% CO2 humidified incubator at 37°C.

Combination therapy experiments

PC-3 cells (20,000 cells/well) were seeded in 96 well plate (Thermo Fisher) in 200µl completed growth medium. Docetaxel (100nM) was added in the presence of Curcumin in 1µg/mL, 2µg/mL, and 5µg/mL for 72 hours. After incubation in 5% CO2 humidified incubator, MTT (20µl) was added and incubated for 4 hours. Then medium was removed and 100µl of DMSO were added. The plate was placed on a shaker for 15 minutes. The cell viability was measured by Tecan 200 Infinite Pro plate reader using a wavelength of 560nm measurement and 670nm reference. Two experiments were performed as described in Table 3.1 and Table 3.2.

Docetaxel	Curcumin and Analogs
20 nM	0-15 ug/ml
50 nM	0-15 ug/ml
100 nM	0-15 ug/ml

Table 3.1 Combination experiment #1

In this experiment curcumin and its analogs remained constant at 15 µg/ml. Docexatel was added to the PC-3 cells at different concentrations (20,50, and 100nM).

Curcumin and Analogs	Docetaxel
2 ug/ml	0-120 nM
5 ug/ml	0-120 nM
10 ug/ml	0-120 nM

Table 3.2 Combination experiment #2.

In this experiment curcumin and its analogs were added to the PC-3 cells at different concentrations (2, 5, and 10 µg/ml). Docexatel was added to the PC-3 cells at different concentrations (0-120 nM).

Figure 3.2 Mortality percent curve of docetaxel (50nM) in combination with various concentrations of curcumin and its analogs.

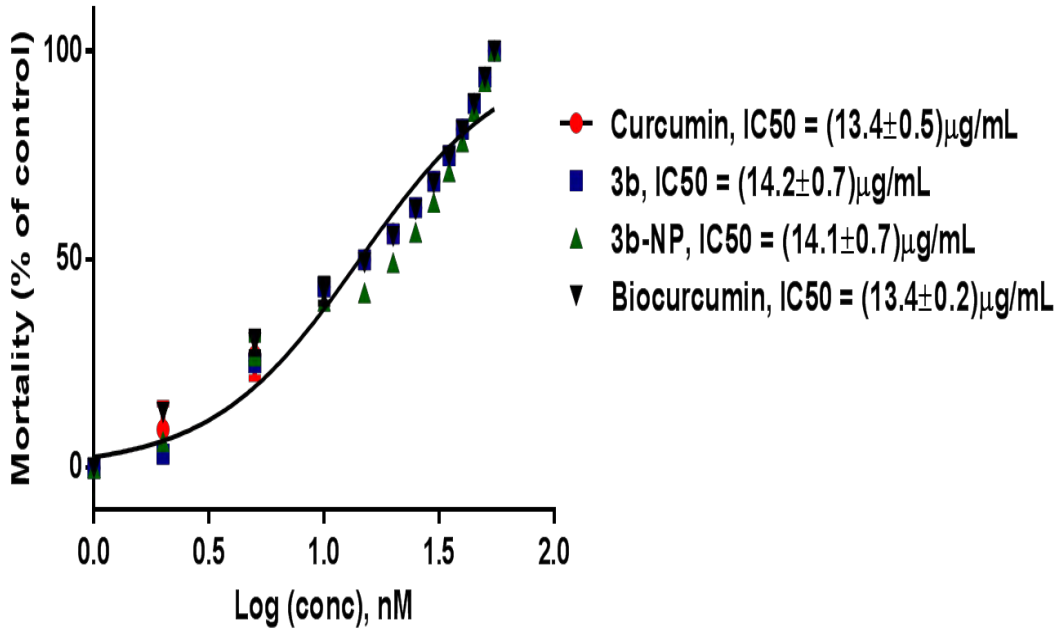


Figure 3.2 Mortality percent curve of docetaxel (50nM) in combination with various concentrations of curcumin and its analogs Curcumin or its analogs (0-10μg/ml) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The IC50 obtained for curcumin, **3b**, 3b-Nanoparticle and biocurcumin were: 13.4±0.5μg/ml, 14.2±0.7μg/ml, 14.1±0.7μg/ml and 13.4±0.2 μg/ml, respectively. Data is based on the mean ± SEM of three experiments.

Figure 3.3 Mortality percent curve of docetaxel (100nM) in combination with various concentrations of curcumin and its analogs.

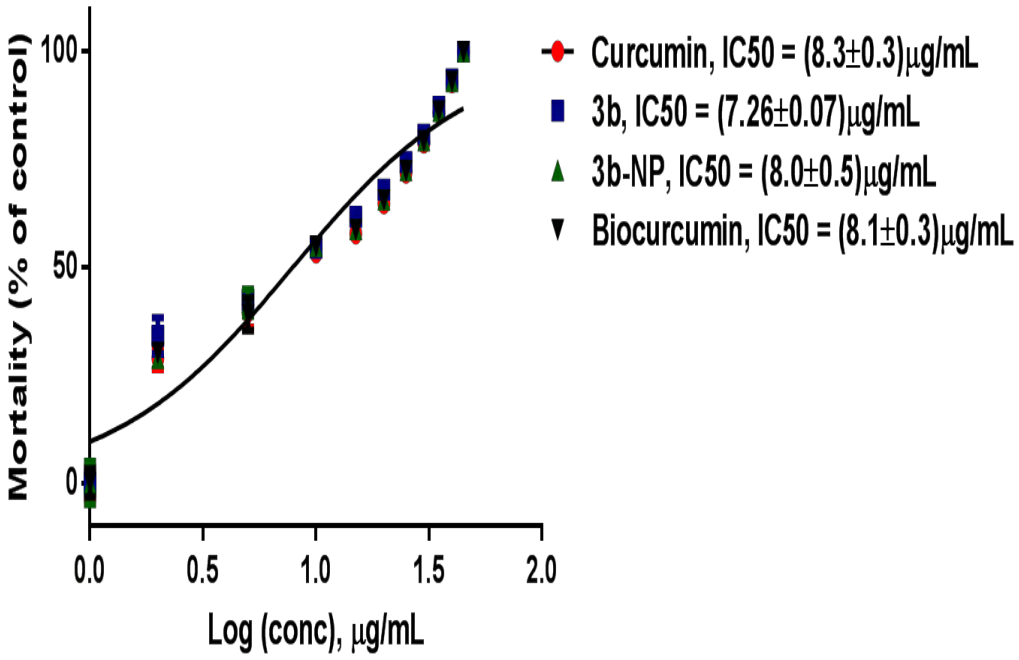


Figure 3.3 Mortality percent curve of docetaxel (100nM) in combination with various concentrations of curcumin and its analogs. Curcumin or its analogs (0-10µg/ml) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The IC50 obtained for curcumin, **3b**, 3b-Nanoparticle and biocurcumin were: 8.3±0.3µg/ml, 7.26±0.07µg/ml, 8.0±0.5µg/ml and 8.1±0.3 µg/ml, respectively. Data is based on the mean ± SEM of three experiments.

Figure 3.4 Mortality of PC-3 cells in combination therapy of curcumin with docetaxel (100nM).

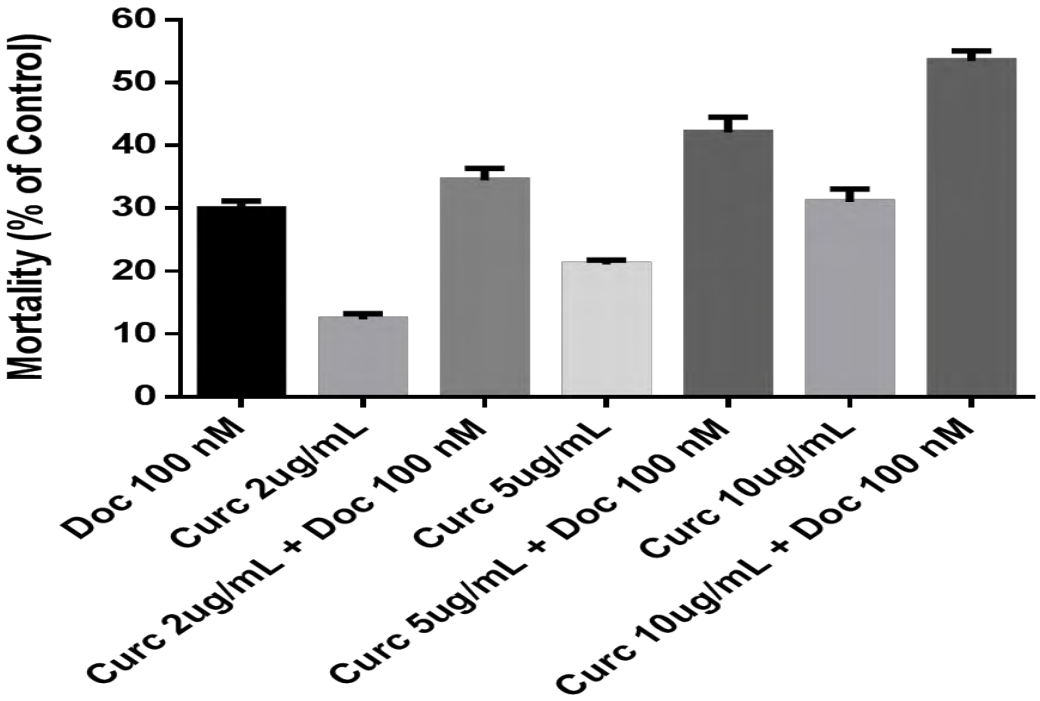


Figure 3.4 Mortality of PC-3 cells in combination therapy of curcumin with docetaxel (100nM). Curcumin (2, 5, and 10µg/ml) and docetaxel (100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The percent of mortality obtained for docetaxel alone was: 30%. The percent of mortality obtained for curcumin alone (2, 5, and 10 µg/ml) were: 10%, 20% and 30%, respectively. The percent of mortality obtained for curcumin (2, 5, and 10 µg/ml) in combination with docetaxel (100nM) were: 35%, 45% and 55%. Data is based on the mean of three experiments.

Figure 3.5 Mortality of PC-3 cells in combination therapy of the analog 3b with docetaxel (100nM).

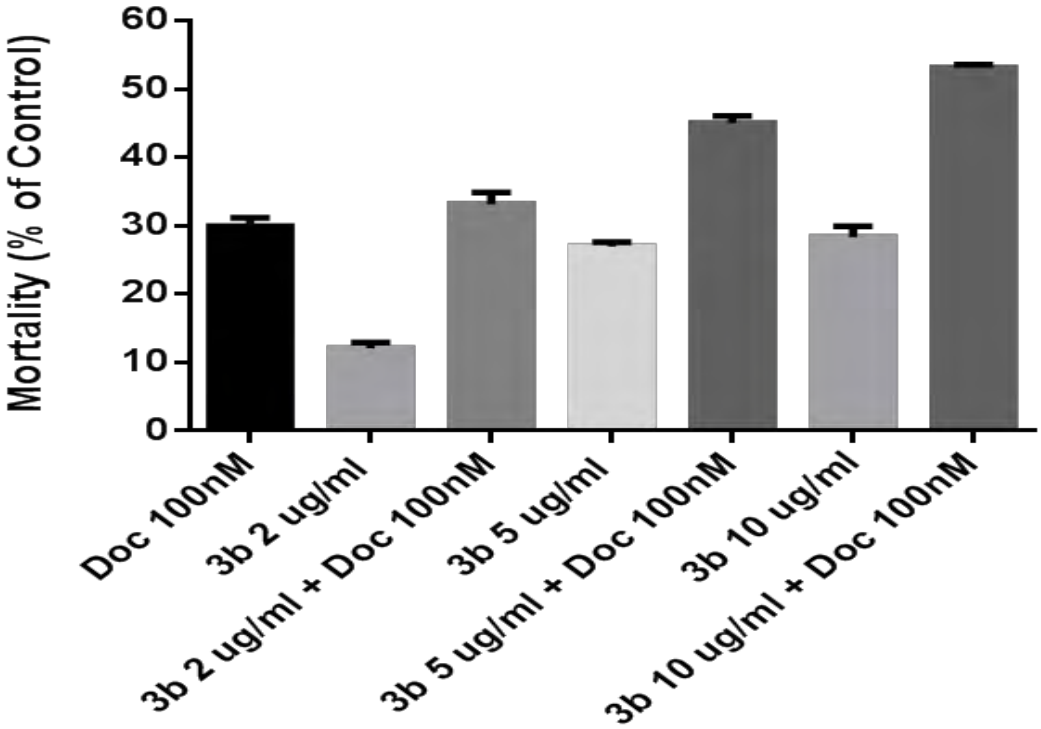


Figure 3.5 Mortality of PC-3 cells in combination therapy of the analog 3b with docetaxel (100nM). The analog **3b** (2, 5 and10µg/ml) and docetaxel (100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The percent of mortality obtained for docetaxel alone was: 30%. The percent of mortality obtained for **3b** alone (2, 5, and 10 µg/ml) were: 10%, 25% and 26%, respectively. The percent of mortality obtained for **3b** (2, 5, and 10 µg/ml) in combination with docetaxel (100nM) were: 33%, 45% and 50%. Data is based on the mean of three experiments.

Figure 3.6 Mortality of PC-3 cells in combination therapy of the analog 3b-Nanoparticle with docetaxel (100nM).

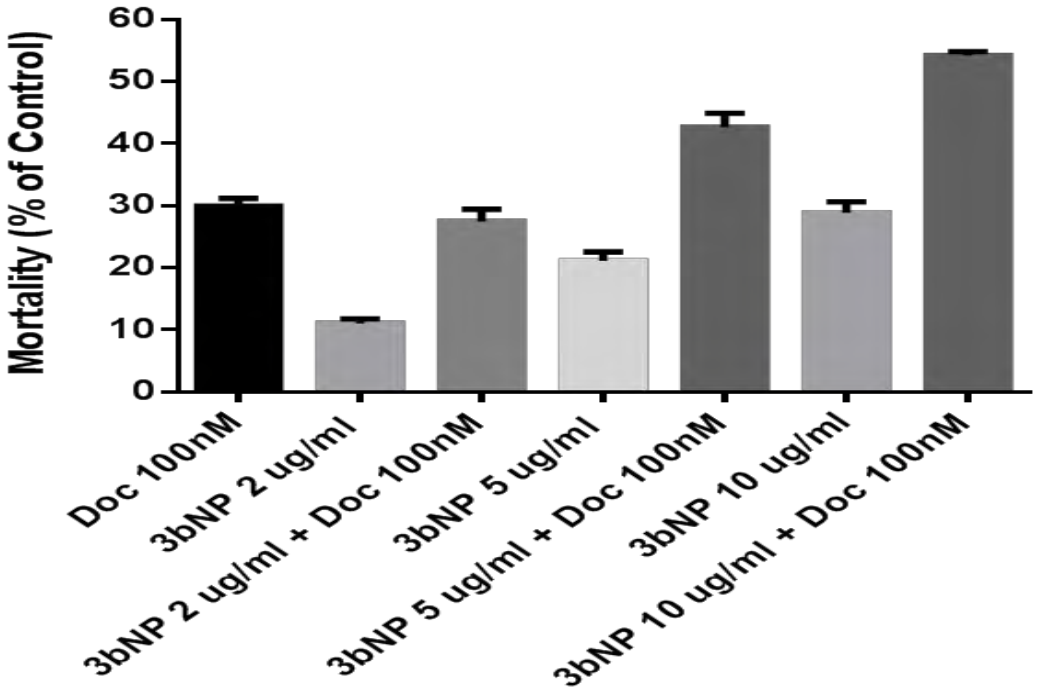


Figure 3.6 Mortality of PC-3 cells in combination therapy of the analog 3b-Nanoparticle with docetaxel (100nM). The analog 3b-Nanoparticle (2, 5 and10µg/ml) and docetaxel (100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The percent of mortality obtained for docetaxel alone was: 30%. The percent of mortality obtained for 3b-Nanoparticle alone (2, 5, and 10 µg/ml) were: 10%, 20% and 29%, respectively. The percent of mortality obtained for 3b-Nanoparticle (2, 5, and 10 µg/ml) in combination with docetaxel (100nM) were: 26%, 45% and 54%, respectively. Data is based on the mean of three experiments.

Figure 3.7 Mortality of PC-3 cells in combination therapy of the analog biocurcumin with docetaxel (100nM).

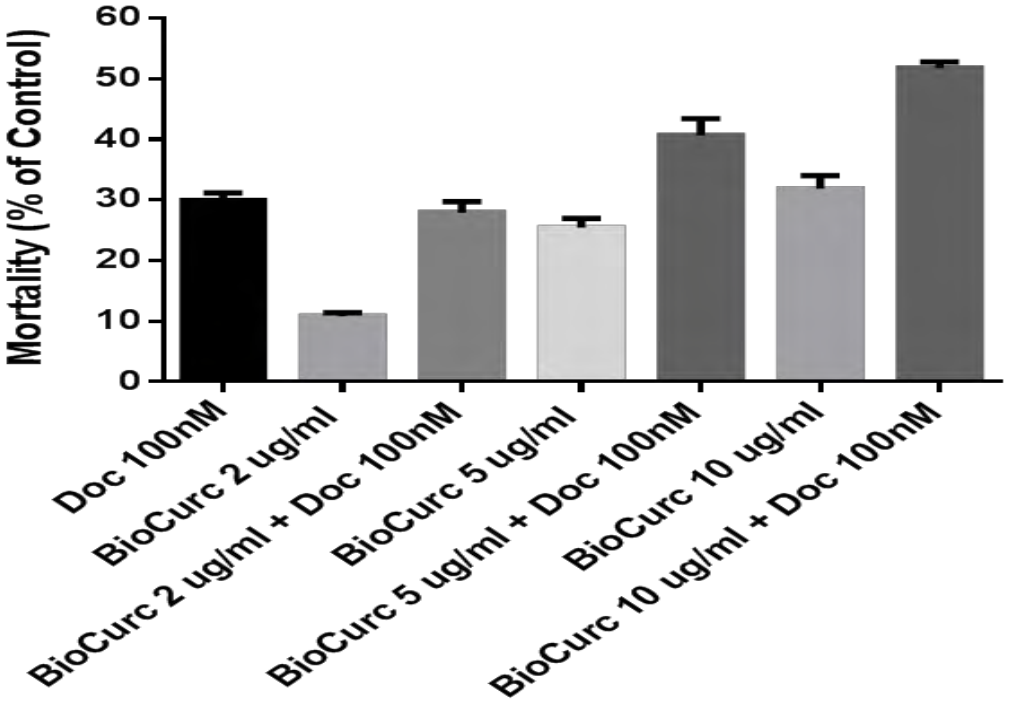


Figure 3.7 Mortality of PC-3 cells in combination therapy of the analog biocurcumin with docetaxel (100nM). The analog biocurcumin (2, 5 and10µg/ml) and docetaxel (100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The percent of mortality obtained for docetaxel alone was: 30%. The percent of mortality obtained for biocurcumin alone (2, 5, and 10 µg/ml) were: 10%, 23% and 31%, respectively. The percent of mortality obtained for biocurcumin (2, 5, and 10 µg/ml) in combination with docetaxel (100nM) were: 26%, 40% and 50%, respectively. Data is based on the mean of three experiments.

Figure 3.8 Mortality percent curve of curcumin and its analogs (10µg/ml) in combination with various concentrations of docetaxel.

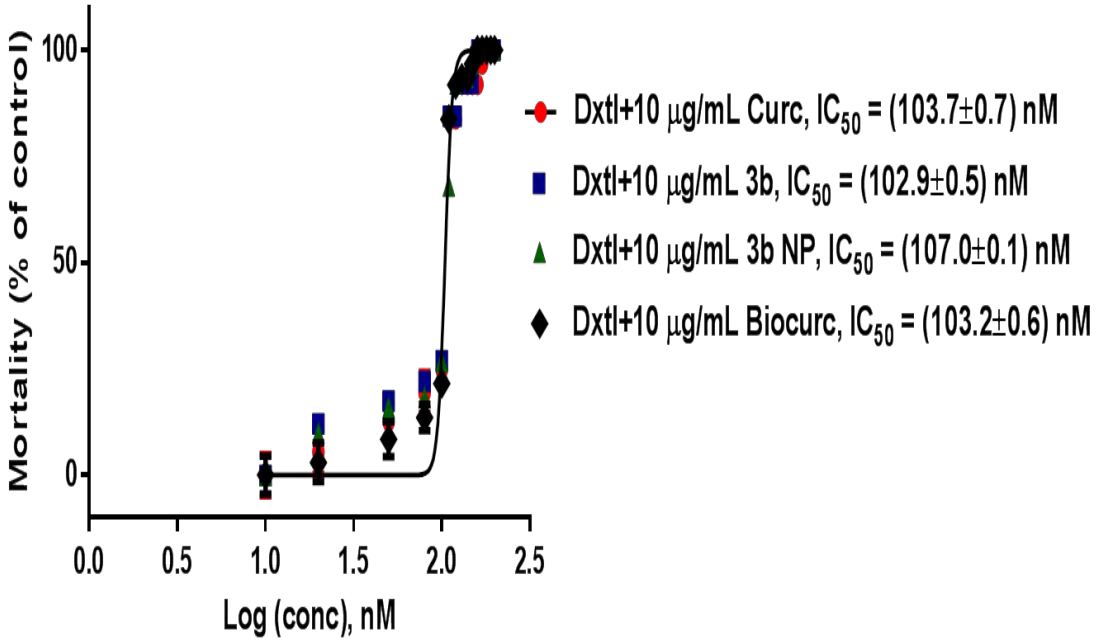


Figure 3.8 Mortality percent curve of curcumin and its analogs (10µg/ml) in combination with various concentrations of docetaxel. Curcumin or its analogs (10µg/ml) in combination with docetaxel (0-100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The IC50 obtained for curcumin, **3b**, 3b-Nanoparticle and biocurcumin were: 103.7±0.7nM, 102.9±0.5nM, 107.0±0.1nM and 103.2±0.6nM, respectively. Data is based on the mean ± SEM of three experiments.

Figure 3.9 IC 50 of curcumin or its analogs (10µg/ml) in combination therapy with docetaxel against PC-3 cell line.

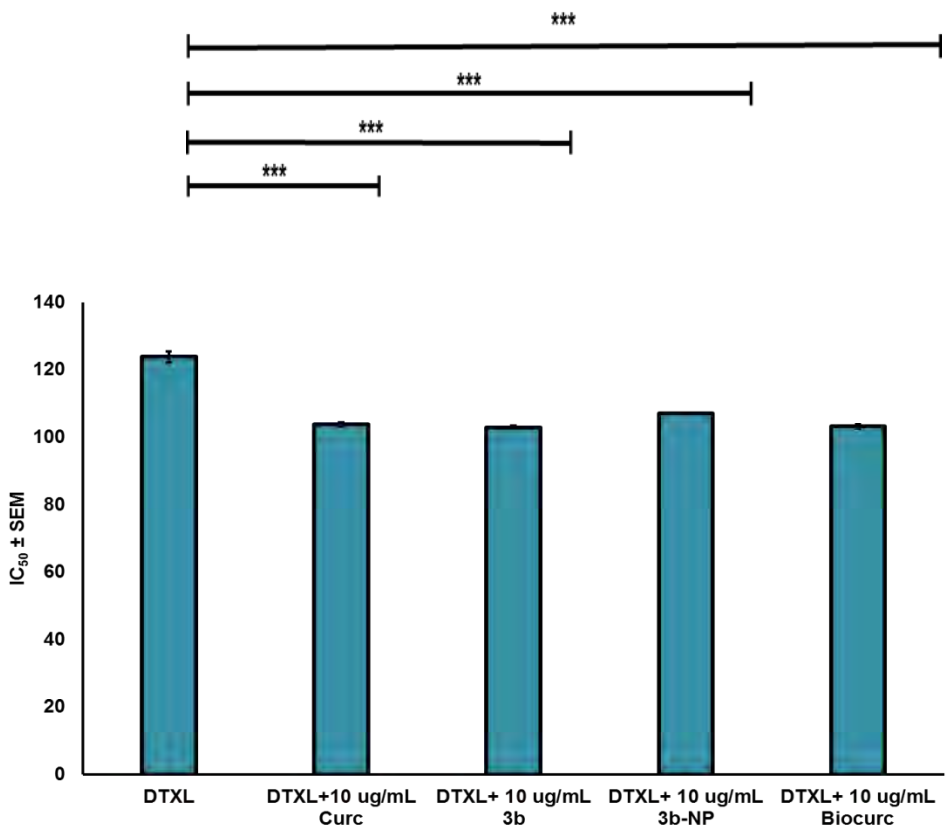


Figure 3.9 IC 50 of curcumin or its analogs (10µg/ml) in combination therapy with docetaxel against PC-3 cell line. Curcumin or its analogs (10µg/ml) in combination with docetaxel (100nM) were added to PC-3 cells per three days. Cytotoxicity was measured by MTT assay. The IC50 obtained for docetaxel alone was: 120nM. The IC50 obtained for curcumin, **3b**, 3b-Nanoparticle and biocurcumin were: 100 nM, 99 nM, 103nM and 99nM, respectively. Data is based on the mean ± SEM of three experiments, ***P<0.001.

Discussion

Docetaxel (100 nM) plus curcumin combination can lower their IC50. . The IC50 of Curcumin and its analogs decreased with the addition of Docetaxel (100nM). Docetaxel can cause drug resistance in cancer cells, and here we demonstrate that lower doses of Docetaxel in combination with curcumin are effective against prostate cancer cell line PC-3. Docetaxel could be administered in combination with other anticancer agents, such as curcumin, to diminish its toxicity and the resistance of prostate cancer cells.

Chapter 4: Expression of proinflammatory cytokines and angiogenesis related proteins

Introduction

Inflammation promotes tumor initiation, angiogenesis and metastasis (De Marzo et al., 2007). Biopsies of patients with high levels of PSA, abnormal digital-rectal examination, radical prostatectomy and tissue from patients with benign prostatic hyperplasia demonstrated inflammation (Bora et al., 2014). In the tumor microenvironment, prostate cancer has the ability to evade the immune system by causing a dormant-like state to CD8+ Tcells and natural killer cells (Figure 4.1.) (Ferrone and Dranoff , 2010). Activation of immunosuppressive Tregs, increase of inflammatory cytokines (e.g. IL- 6, IL-1, TNF-a), increase expression of PD-L1 receptor, IL-10 and TGF-b are immune evasion mechanisms of cancer.

Figure 4.1 Dual roles for immunity in gastrointestinal cancer.

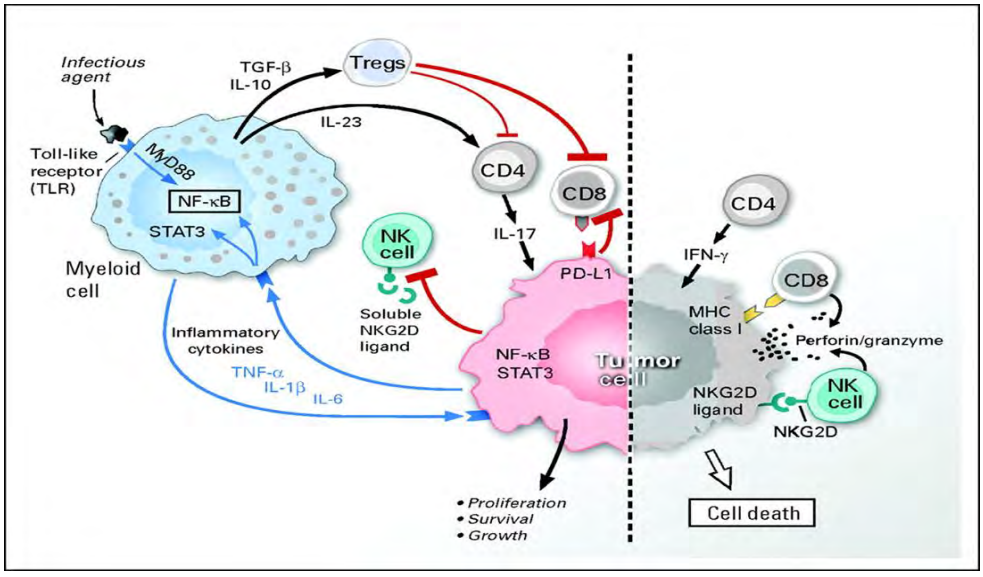


Figure 4.1 Dual roles for immunity in gastrointestinal cancer. Tumors can cause the dormancy and editing of immune cells by increasing IL-6, IL-10 and TGF-beta.

Cancer cells need stromal cells to survive, promoting a relationship with non-cancerous cells in the tumor microenvironment. Cancer cells secrete lactate to the tumor microenvironment to inhibit cytokines and the cytotoxic activity of Tcells. The inactivation of Tcells in some cancers as prostate and melanoma, could lead to the inhibition of the expression of PD1 and CTLA-4 which are immune inhibitory molecules (Hadrup,et al.,2012). In addition, cancer cells can also cause the inhibition of natural killer cells (NK) which correlates with an increased risk of cancer (Waldhauer and Steinle, 2008). This increase in risk of cancer could be possible because NK cells would not be able to destroy abnormal self cells (Morice, 2007).

Both, NK cells and CD8+Tcells are involved in the process of tumor destruction. NK cells and CD8+Tcells contain perforin and granzyme B in cytotoxic granules. Perforin and granzyme B are proteins that induce apoptosis together. The function of perforin is to make a pore whereas granzyme b is a caspase-like serine protease that enters the target cells after the pore formation. During the immunological synapse, perforin and granzyme b are released from cytotoxic granules after the fusion of those granules with the tumor cell membrane, causing apoptosis (Fang et al., 2012).

Besides the mechanisms of tumor immune surveillance, tumor cells can escape the process in different manners. Mechanisms of tumor immune evasion are listed as follow (Yefenof et al., 2008):

1. Induction of antitumor immune response: Paucity of co-stimulatory signals due to decrease of co-stimulatory molecules on tumor or antigen presenting cells surface.

2. Compromised function/survival of effector T lymphocytes: Suppression of Tcells responses by Tregs, suppression by MDSC, apoptosis of effector Tcells (in tumor and in periphery) and secretion of exosomes.

3. Loss of tumor cell recognition by immune cells: Down regulation of surface molecules by tumors and suppression of NK cells in the tumor.

4. Resistant to tumor cells to immune intervention: Lack of susceptibility to immune effector cells, immunoselection of resistant variants, tumor stem cells.

Another mechanism of tumor immune evasion is the production of pro inflammatory cytokines which are mediators of cancer. High serum levels of pro-inflammatory cytokines like IL-1α, IL-6 and Tumor Necrosis Factor alpha (TNFα) are found in cancer patients (Mantovani et al., 2006). IL-1 and TNF attract neotrophils promoting carcinogenesis. Pro-inflammatory cytokines also induce metaloproteinases and adhesion molecules which promotes invasion. This can suggest that the blockage of pro-inflammatory cytokines could stop tumor growth (Dinarello, 2006).

Previous studies have shown that bacterial infections can cause inflammation leading to initiation of prostate cancer in mouse (Oh-Joon et al., 2014). In order for inflammation to occur it needs cytokines like IL-1α, IL-1ß, IL-6, and TNF-α that can promote PCa development (Hua et al., 2015).

Inflammation can increase nucleotides alteration and reactive oxygen species (Endo et al., 2007) .

Curcumin is a scavenger of free radicals and it is also an inhibitor of inflammation, for that reason it has been studied in different types of cancer as an anti-proliferative agent (Dorrah et al., 2004). Curcumin can inhibit the phosphorylation of IkBα resulting in inhibition of the nuclear factor -kB, which is involved in the regulation of inflammation (Dorrah et al., 2004). Deeb, et al, 2004 demonstrated the inhibition of NF-kB by curcumin (12.5 to 50 μmol/L) in LNCaP cells. Curcumin can act as a kinase inhibitor in the NF-kB pathway in metastatic prostate cancer cells (Killian et al.2012). In studies by (Duarte et al., 2010) liposomal curcumin demonstrated to down-regulate IkBα by inhibition of Nf-kB as shown in Figure 4.2. Curcumin can cause the inhibition of Nf-kB, which downregulates IkBα, COX-2, and IL-8. The inhibitory effect of curcumin in head and neck squamous cell carcinoma (HNSCC), is due to the inhibition of Nf-kB by the interaction of curcumin with IKKB. Immunofluorescence studies with HNSCC cells (CAL27, oral cancer) treated with liposomal curcumin, showed that phospho-IkBα expression was seen in the cytoplasm but the treatment with curcumin decreased the expression of IkBα in cytoplasm. This suggests that curcumin can cause the inhibition of IKK kinase which leads to the reduction in the phosphorylation of inhibitor-kB-α. In the same study, mass spectrometry analysis with cell lysates of CAL27 cells treated with curcumin was performed to demonstrate the binding of curcumin to IKKB protein and a five-fold increased binding of curcumin to IKKB was measured in comparison with controls.

Figure 4.2 Mechanism of action curcumin and cisplatin.

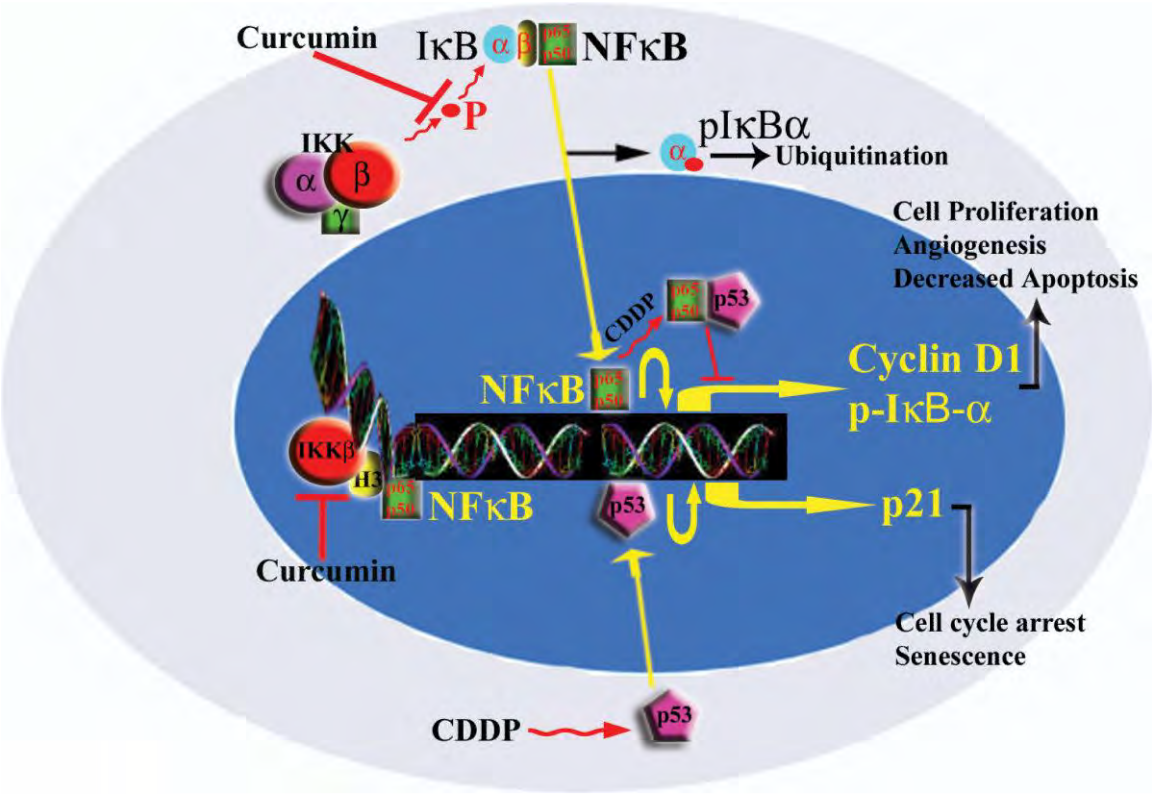


Figure 4.2 Mechanism of action curcumin and cisplatin. Curcumin downregulates IKK kinase in cytoplasm and nucleus, interacting with IKKB. The downregulation of IKKB mediated by curcumin causes the inhibition of Nf-kB. This study suggests that the chromatin remodeling of Nf-kB binding sites is done by IKKB and histone H3.

Cells can respond to stimuli by signaling cascades that promote the activation of mitogen-activated protein kinases (MAPKs). Five groups of MAPKs have been found in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, γ, and δ , ERKs 3 and 4, and ERK5 (Chen et al., 2001 and Kyriakis et al. 2001). In a study by (Uzgare et al., 2003), activation of p38 MAP kinase activation was seen in in well differentiated tumors in TRAMP mice SAPK/JNK are translocate to the nucleus after their activation and phosphorylation ,from the

cytosol to the nucleus (Gupta et al., 1995). Once in the nucleus it can cause the phosphorylation of c-Jun and ATF-2 which are transcription factors (Gupta et al., 1995). Phospho IKBα can be inhibited by curcumin, resulting in the reduction of pro-inflammatory cytokines produced by NF-kB (Killian et al. 2012).

IL-8 is involved in inflammation and it can increase the activity of androgen receptor (Seaton et al., 2008). This chemokine promotes angiogenesis in prostate cancer by increasing vascular endothelial growth factor (Maxwell et al., 2013). IL-8 (CXCL8) in cancer, could triggers many cellular pathways such as: serine/threonine kinases, tyrosine kinases, Rho GTPases through Gprotein coupled receptor (Brysse et al., 2012). In prostate cancer, IL-8 can be linked to pathologic state (Uehara et al., 2005). Previous studies have shown that higher IL-8 levels are found in patients with metastatic prostate cancer (Veltri et al.,1999). Figure 4.3. demonstrates the chemokines at the tumor microenvironment.

Figure 4.3 Crosstalk between chemokines in the tumor microenvironment.

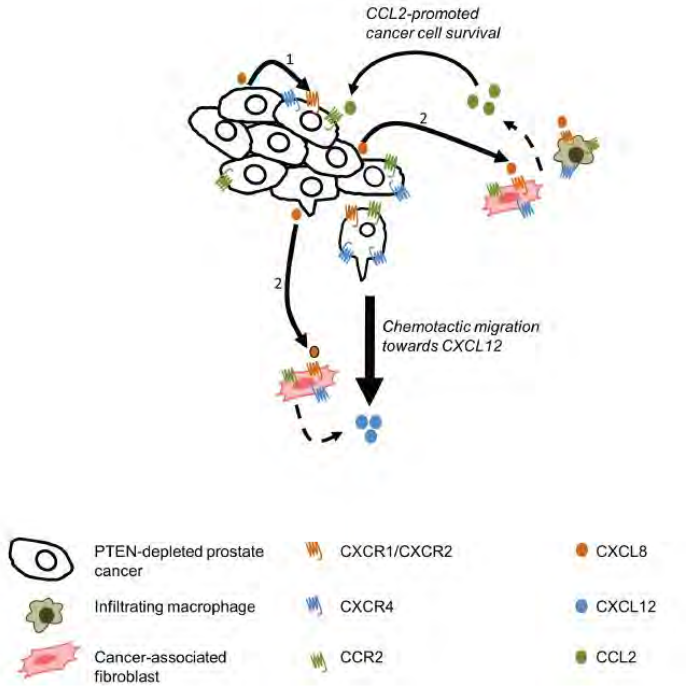


Figure 4.3 Crosstalk between chemokines in the tumor microenvironment. 1. Up- regulation of CXCR1,CXCR2, CXCR4, and CCL2 by CXCL8 on tumor cells. 2. Paracrine CXCL8 signaling results in the production of CXCR1, CXCR2, CXCR4 and CCR2 by tumor-associated stromal cells. Paracrine CXCL8 promotes secretion of CCL2 by macrophages and fibroblasts (Maxwell et al., 2014).

In a study with PC-3 cells and DU-145 cell lines, IL-8 (CXCL8) induced the expression of cyclin D1 expression causing tumor cell proliferation (MacManus et al., 2007). (Nakamura et al, 2002), demonstrated that curcumin down-regulates AP-1 and also NF-kB activation in prostate cancer cell lines resulting in lower IL-8 gene expression.

Tissue inhibitors of metalloproteinases (TIMPS) are regulators of matrix metalloproteinases and are constituted by four members (TIMP1-4; 6-8) (Ross et al., 2003). TIMP1 inhibits MMP9 and TIMP2 inhibits MMP2 (Baker et al. 2000). TIMP2 is related to advanced tumor stage (Still et al., 2000). TIMP 2 has showed

matrix degradation with the enzyme MT1-MMP. MMP2 and TIMP2 can be significant in the prognostic of prostate cancer patients (Ross et al., 2003).

In this study we aimed to investigate if curcumin, or its analogs in combination therapy with docetaxel could decrease the expression of human chemokines associated to tumor progression such as (Eotaxin, GROa, I-309, IL-8, IP-10, MCP-1, MCP-2, RANTES, TARC) and pro-inflammatory proteins such as pNF-kB, p38 , SAPK/JNK and pIkBα .

Materials and methods

Experimental design.

Human cancer cell line PC-3 (prostate cancer) was purchased from ATCC. The cell line was cultured in 19ml of F-12K medium in culture flasks. Trypsin EDTA (2ml) (Hyclone) was used to detach cells from cultutred flasks. Cancer cells were seeded $2X10^6$ cells/well in six wells culture plates. The PC-3 cells were incubated with the different concentrations of curcumin and its analogs as described in the table below, at 37°C for three days in humidified 5% CO2 incubator. The ELISA test was performed and the absorbance (450nm) was measured with a spectrophotometer (Tecan). Experiments were performed in four biological replicates and three technical replicates. Experimental design is describe in Table 4.1.

Treatments	Docetaxel (100nM)	Curcumin (10µg/mL)	3b Analog (10µg/mL)
1	-	-	-
2	+	-	-
3	-	+	-
4	-	-	+
5	+	+	-
6	+	-	+

Table 4.1 Experimental design used in PC-3 cells before measurement of pro-inflammatory proteins.

This table describes the different treatments applied to PC-3 cell cultures. After three days supernatants were collected to detect pro-inflammatory proteins.

Quantification of total protein.

Cell lysates were prepared after cell treatments. To assure that all the samples had the same amount of total protein, a Bradford assay was performed. After the Bradford assay 100µg of total protein was added to each well of the 96 well plate of PathScan Sandwich ELISA array.

Measurement of inflammation associated intracellular molecules.

PC-3 cells were treated as descripted in the table above. The PathScan Sandwich ELISA array was purchased from Cell Signaling Technology and protocol was followed as directed by manufacturer: The plate was incubated overnight (4^{o}C). Then it was washed and100µL of detection antibody were added. It was incubated 1hr, washed, then 100µl of secondary antibody were added and incubated for 30 minutes at 37°C. TMB substrate was added (100µl) and incubated for 10 minutes. Stop solution was added (100µl) for 30 minutes and the absorbance was read at 450nm.

Measurement of molecules associated to tumor growth and metastasis.

Experimental design.

Human cancer cell line PC-3 (prostate cancer) was purchased from ATCC. The cell line was cultured in 19ml of F-12K medium in culture flasks. Trypsin EDTA (2ml) (Hyclone) was used to detach cells from cultured flasks. Cancer cells were seeded 2X10^{6} cells/well in six wells culture plates. The PC-3 cells were incubated with the different concentrations of curcumin and its analogs as described in the table below, at 37°C for three days in humidified 5% CO2

incubator. Supernatants were collected and the ELISA test was performed. The absorbance (450nm) was measured with a spectrophotometer (Tecan). Experiments were performed in four biological replicates and three technical replicates. Experimental design is describe in Table 4.2.

Treatments	Docetaxel (100nM)	Curcumin (10µg/mL)	3b Analog (10µg/mL)
1	-	-	-
2	+	-	-
3	-	+	-
4	-	-	+
5	+	+	-
6	+	-	+

Table 4.2 Experimental design used in PC-3 cells before measurement of molecules associated to tumor growth and metastasis.

This table describes the different treatments applied to PC-3 cell cultures. After three days, supernatants were collected to detect molecules associated to tumor growth and metastasis, (angiogenesis chemokines).

Quantification of total protein.

Supernatant was collected from cell cultures. To assure that all the samples had the same amount of total protein, a Bradford assay was performed. After the Bradford assay 50µg of total protein were added to each well of the 96 well plate of the Q-plex Human Angiogenesis/Chemokine Array.

Measurement of inflammation associated intracellular molecules.

PC-3 cells were treated as descripted in the table above. The Q-plex Human Angiogenesis/Chemokine Array was purchased from QUANSYS BIOSCIENCES and protocol was followed as directed by manufacturer: The supernatants were added to the plate (50µg protein/well) . The plate was in shaker at 500rpm, 1 hour, RT. Then it was washed and 50µL of detection antibody were added. It was incubated 1hr, shaker at 500rpm, RT. Then the plate was washed and 50µl of secondary antibody were added and incubated for 15 minutes, shaker at 500rpm, RT. After that, the substrate (50µl) was added. Then, Chemiluminescence was measured by Q- View Imager.

Statistics

Values were analyzed using the program GraphPad Prism 6. Values were transformed using Y transformation.

Figure 4.4 Expression levels of NF-kB following drug treatment in PC-3 cells.

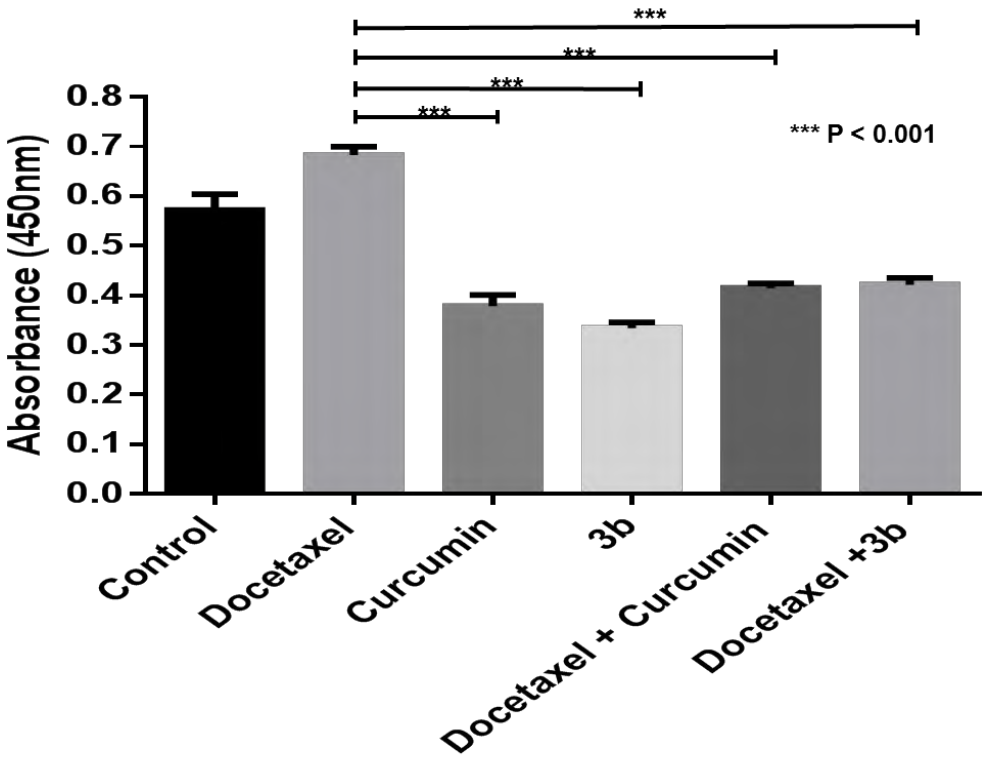


Figure 4.4 Expression levels of NF-kB following drug treatment in PC-3 cells. Curcumin and **3b** alone decreased the expression levels of NF-kB by 34% and 41%, respectively in comparison with the treatment with docetaxel alone. Combination therapy of docetaxel with curcumin and combination therapy of docetaxel with **3b** showed a decreased in the expression levels of NF-kB by 27% and 26% respectively in comparison with the treatment with docetaxel alone.

Figure 4.5 Expression levels of SAPK//JNK following drug treatment in PC-3 cells.

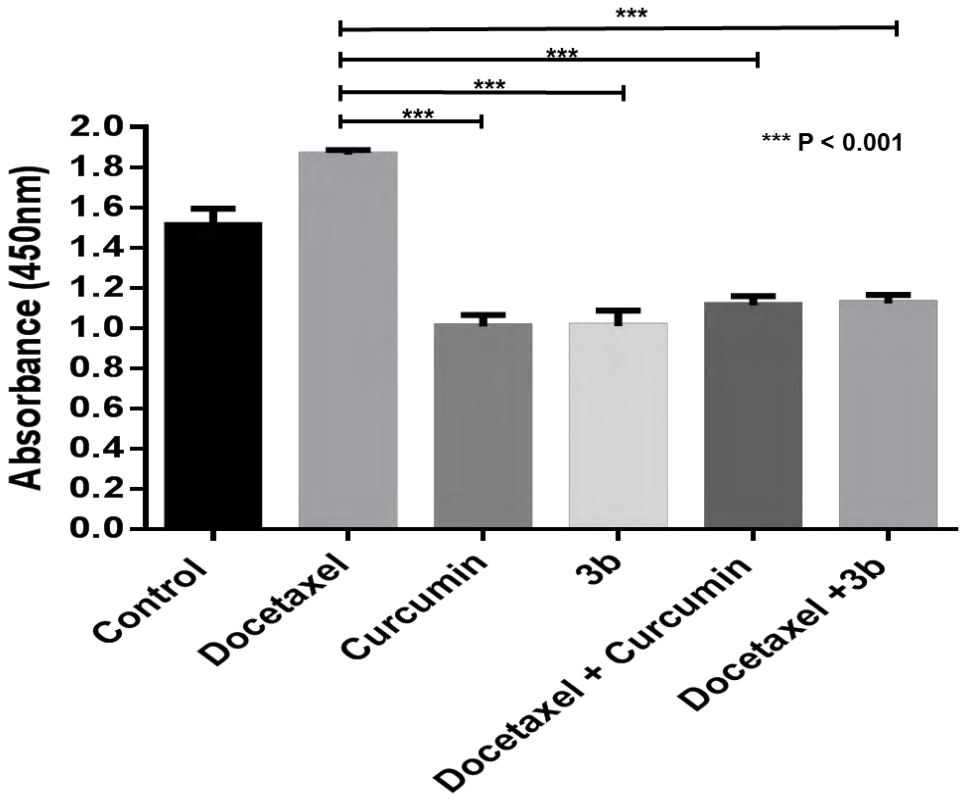


Figure 4.5 Expression levels of SAPK//JNK following drug treatment in PC-3 cells. Curcumin and **3b** alone decreased the expression levels of SAPK/JNK both by 33%, in comparison with the treatment with docetaxel alone. Combination therapy of docetaxel with curcumin and combination therapy of docetaxel with **3b** showed a decreased in the expression levels of SAPK/JNK by 27% and 26% respectively in comparison with the treatment with docetaxel alone.

Figure 4.6 Expression levels of phospho-p38 following drug treatment in PC-3 cells.

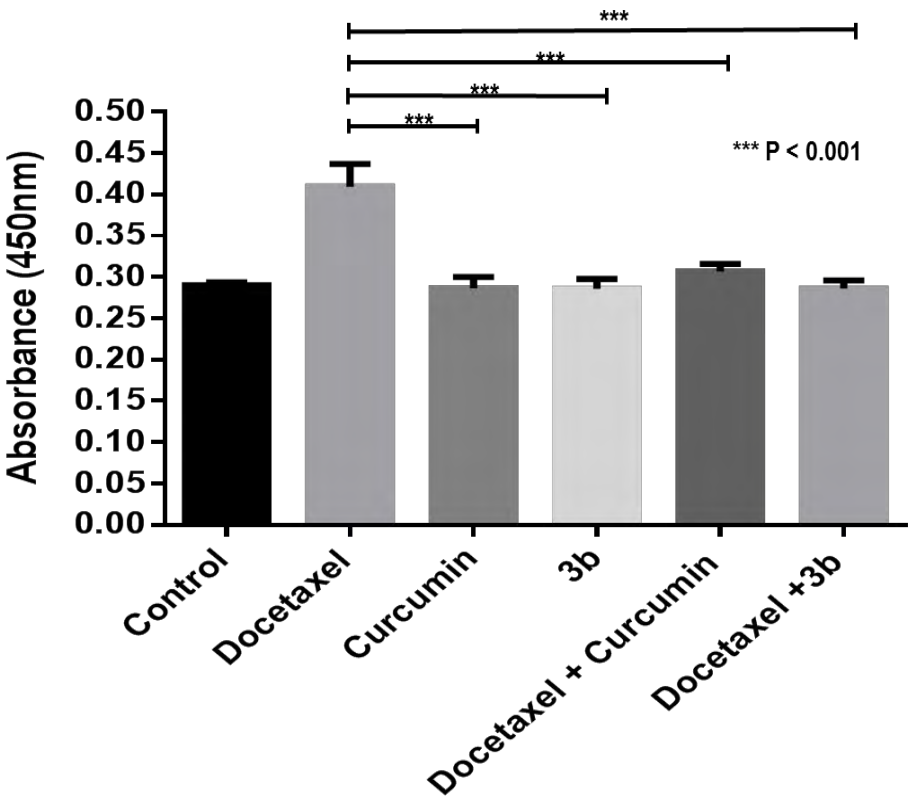


Figure 4.1 Dual roles for immunity in gastrointestinal cancer. Curcumin and **3b** alone decreased the expression levels of phospho-p38 both by 30%, in comparison with the treatment with docetaxel alone. Combination therapy of docetaxel with curcumin and combination therapy of docetaxel with **3b** showed a decreased in the expression levels of phospho-p38 by 25% and 30% respectively in comparison with the treatment with docetaxel alone.

Figure 4.7 Expression levels of phospho-IkBα following drug treatment in PC-3 cells.

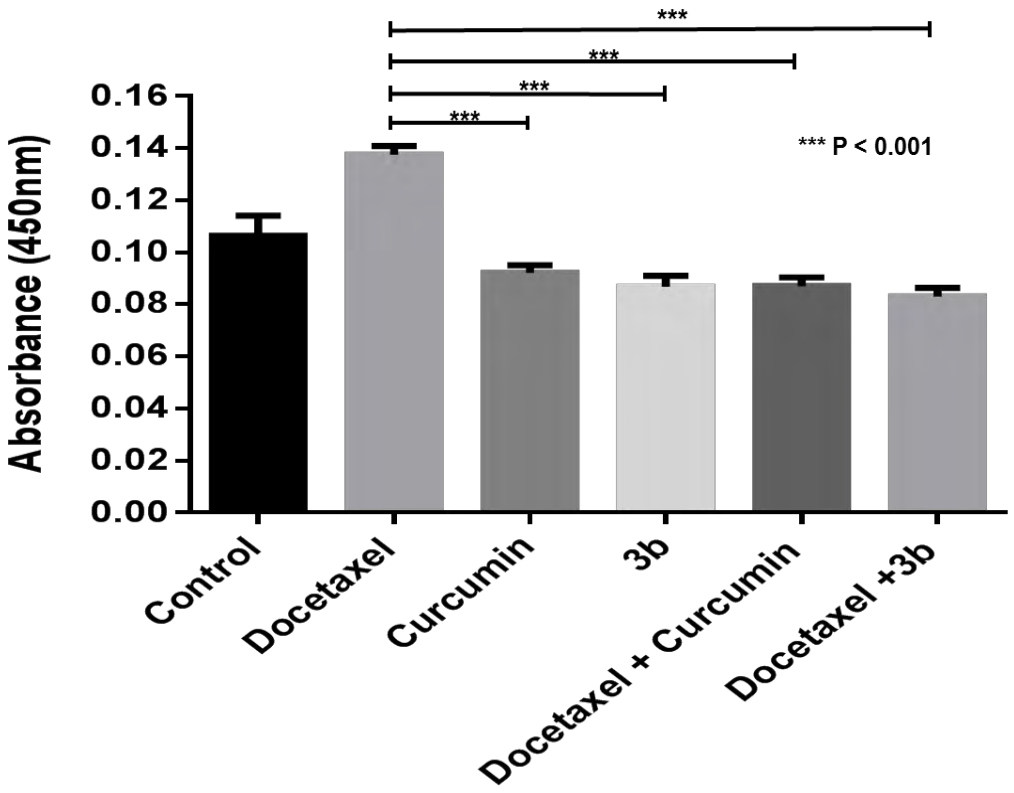


Figure 4.7 Expression levels of phospho-IkBα following drug treatment in PC-3 cells. Curcumin and **3b** alone decreased the expression levels of phospho-IkBα by 33% and 37% in comparison with the treatment with docetaxel alone. Combination therapy of docetaxel with curcumin and combination therapy of docetaxel with **3b** showed a decreased in the expression levels of phospho-IkBα by 37% and 39%, respectively in comparison with the treatment with docetaxel alone.

Figure 4.8 Concentration of hTIMP-2 following drug treatment in PC-3 cells.

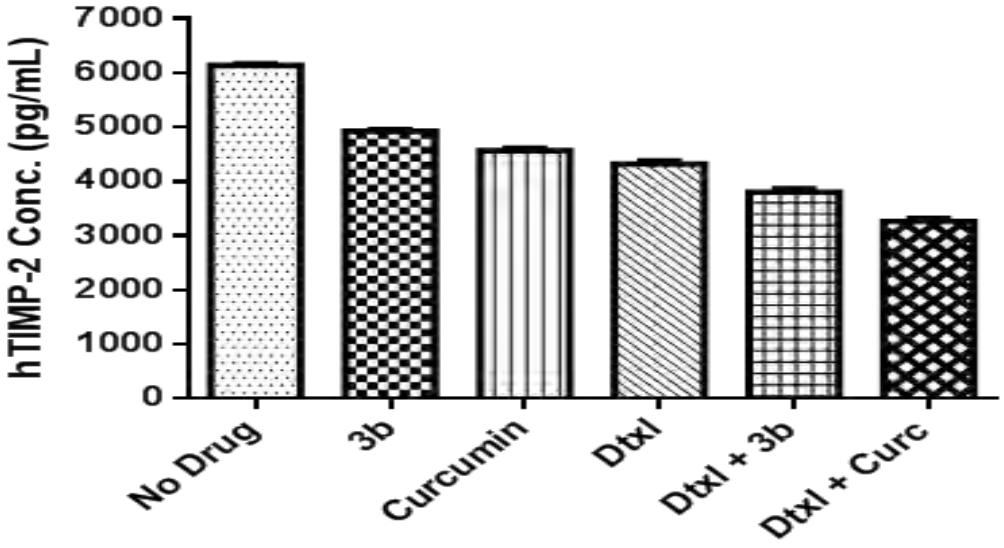


Figure 4.8 Concentration of hTIMP-2 following drug treatment in PC-3 cells. The concentration of hTIMP-2 in the treatment with **3b** was 5000pg/ml (20% of inhibition in comparison with control). The concentration of hTIMP-2 in the treatment with curcumin was 4,900pg/ml (25% of inhibition in comparison with control). The concentration of hTIMP-2 in the treatment with docetaxel was 4,800pg/ml (26% of inhibition in comparison with control). The concentration of hTIMP-2 in the treatment with docetaxel plus **3b** was 3,900pg/ml (38% of inhibition in comparison with control). The concentration of hTIMP-2 in the treatment with docetaxel plus curcumin was 3,000pg/ml (48% of inhibition in comparison with control).

Figure 4.9 Percent of Inhibition of hTIMP2 following drug treatment in PC-3 cells.

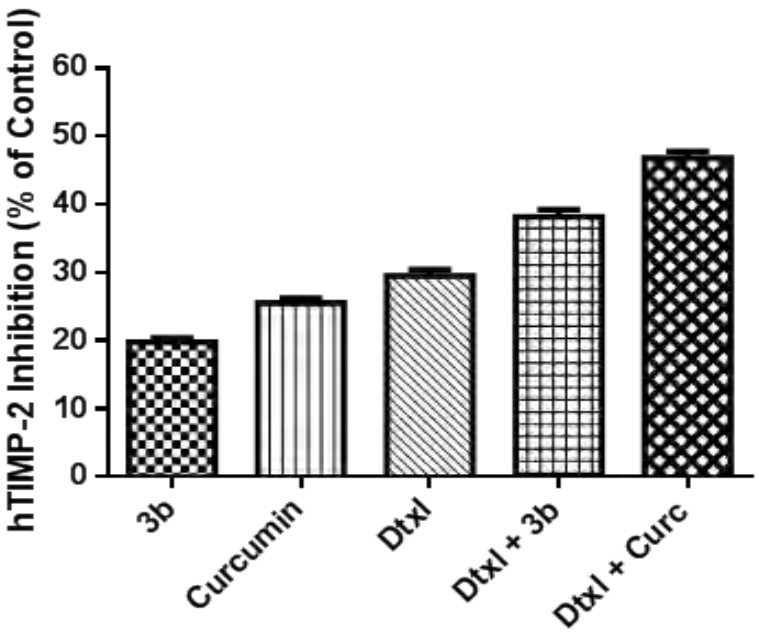


Figure 4.9 Percent of Inhibition of hTIMP2 following drug treatment in PC-3 cells. The curcumin analog **3b** demonstrated a 20% of inhibition in comparison with control. Curcumin showed a 25% of inhibition in comparison with control. Docetaxel caused a 26% of inhibition in comparison with control. Combination therapy with docetaxel plus **3b** demonstrated a 38% of inhibition in comparison with control. Combination therapy with docetaxel and curcumin showed a 48% of inhibition in comparison with control.

Figure 4.10 Concentration of hIL-8 following drug treatment in PC-3 cells.

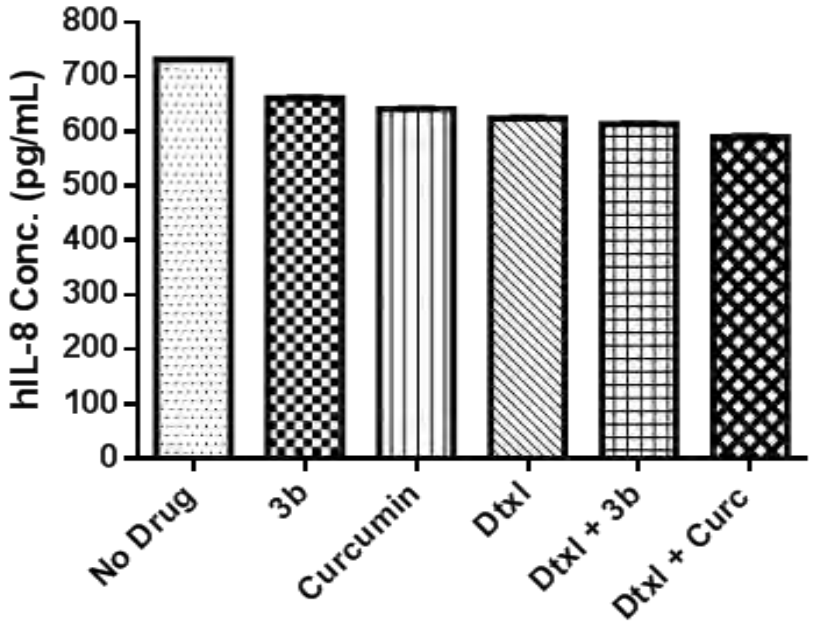


Figure 4.10 Concentration of hIL-8 following drug treatment in PC-3 cells. The concentration of hIL-8 in the treatment with **3b** was 650pg/ml (10% of inhibition in comparison with control). The concentration of hIL-8 in the treatment with curcumin was 630pg/ml (13% of inhibition in comparison with control). The concentration of hIL-8 in the treatment with docetaxel was 620pg/ml (15% of inhibition in comparison with control). The concentration of hIL-8 in the treatment with docetaxel plus **3b** was 610pg/ml (16% of inhibition in comparison with control). The concentration of hIL-8 in the treatment with docetaxel plus curcumin was 550pg/ml (19% of inhibition in comparison with control).

Figure 4.11 Percent inhibition hIL-8 following drug treatment in PC-3 cells.

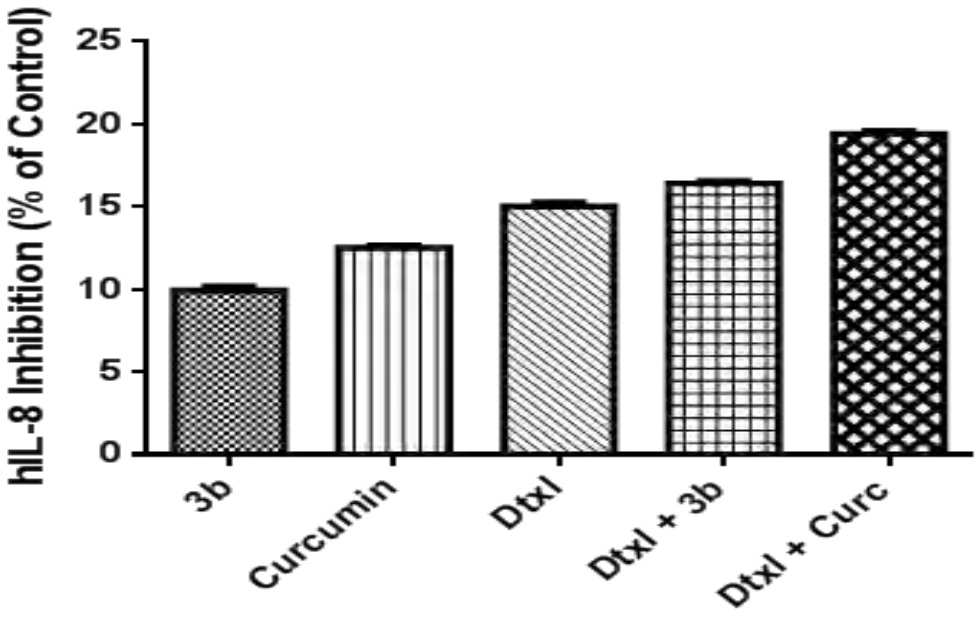


Figure 4.11 Percent inhibition hIL-8 following drug treatment in PC-3 cells. The curcumin analog **3b** demonstrated a 10% of inhibition in comparison with control. Curcumin showed a 13% of inhibition in comparison with control. Docetaxel caused a 15% of inhibition in comparison with control. Combination therapy with docetaxel plus **3b** demonstrated a 16% of inhibition in comparison with control. Combination therapy with docetaxel and curcumin showed a 19% of inhibition in comparison with control.

Discussion

Docetaxel demonstrated to enhance NF-kB in our experiments. The chemotherapeutic agent docetaxel has increased the expression of NF-kB by 16% in comparison with control. It also increased the expression levels of SAPK/JNK, phospho-p38 and IkBα by 19%, 29%, and 23%, respectively in comparison with control. This suggests that this could be a reason for drug resistance and toxicity seen in clinic. Combination therapy with docetaxel, curcumin and **3b** was not effective due to the increased in expression of NF-kB that could be caused by docetaxel. Curcumin and **3b** were more effective working alone than in combination to inhibit NF-kB, SAPK/JNK, phospho-p38 and phospho-IkBα. Treatment with curcumin or its analog **3b** alone or in combination with docetaxel were effective decreasing the concentration of IL-8 and TIMP-2.

Chapter 5: Conclusions

A previous Phase I dose escalation trial of docetaxel plus curcumin, (Bayet-Robert, et al., 2010) recommended a curcumin dose of 6,000mg/d for seven days every three weeks in combination with docetaxel as administered in standard dose. In that same study, hematological toxicity did not increased, neither febrile aplasia. Neutropenia due to docetaxel was transient and reversible. No progressive disease was seen in the fourteen patients that participated in the study.

In our study, the analog **3b** was effective as curcumin. The therapeutic index of **3b** was better than the therapeutic index of curcumin. Docetaxel (100nM) plus curcumin combination can lower their EC50. In future studies a combinatorial index must be calculated to determine whether exist a synergistic effect when curcumin is combined with Docetaxel, one of them working inside the cell and the other outside of the cell. Similar synergistic studies need to be performed when PLGA Nanoparticle encapsulating **3b** is combined with Docetaxel. It is important to take into account that Docetaxel can cause drug resistance in cancer cells by the activation of the NF-kB protein (Hong et al., 2014). In this study we have demonstrated that lower doses of Docetaxel in combination with curcumin enhanced the cytotoxic activity of Docetaxel against prostate cancer cell PC-3 and decreased the expression of NF-kB. Thus Docetaxel can be administered in combination with other anticancer agents, such as **3b** PLGA Nanoparticle, to decrease its toxicity and the inherency of prostate cancer cells towards Docetaxel. Curcumin reduces Docetaxel's IC50 and vice

versa. Curcumin works at protein level (pNf-kB, SAPK/JNK, phosphor-Ikbα, IL-8) docetaxel works in the cell cyle (microtubules stabilization, G2M phase).

Our proposed mechanism of curcumin based on our experimental results is that curcumin can cause the downregulation of extracellular IL-8 and TIMP-2 and also the downregulation of intracellular phospho-Ikß-α, Nf-kB, p38 and SAPK/JNK leading to the suppression of survival, proliferation, and invasion. This effect could also be accompanied by the inhibition of inflammatory cytokines.

We have demonstrated that all, docetaxel, curcumin and its analogs can exert their cytotoxic effect at low concentrations (μM, nM). Our results suggest that low concentrations of curcumin analogs can be increased to obtain a stronger cytotoxic effect. Recommendations for future studies include: 1) In vitro studies with other prostate cancer cell lines such as DU-145 and LNCaP , 2) In vivo studies with prostate cancer mouse model, (non-immunocompetent, immunocompetent) to investigate the potential of curcumin analogs as anticancer agents, 3) Further experiments aimed at determining the inhibitory effect on the activity of molecules such as Rac-1 and Vav3, which are involved in biological process such as: proliferation, apoptosis, invasion (Myant et al., 2013) and cell transformation(Uen et al., 2015) , respectively. In conclusion, combination therapy of curcumin and its analogs with docetaxel are a promising treatment for highly metastatic prostate cancer.

References

1. Aggarwal, BB, Takada Y, Oommen, OV. (2004). From chemoprevention to chemotherapy: Common targets and common goals. *Expert Opin. Investig. Drugs*. *13*: 1327–1338.

2. American Cancer Society. (2012). Datos y Estadísticas sobre el cáncer entre los Hispanos/Latinos 2012-2014. Atlanta: Sociedad Americana Contra El Cáncer, 2012. Retrieved July 31, 2015, from http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-036792.pdf

3. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. (2007).Bioavailability of curcumin: problems and promises. *Mol Pharm*. 4(6):807–18.

4. Antony B, Merina B, Iyer VS, Judy N, Lennertz K, Joyal S. (2008). A Pilot Cross-Over Study to Evaluate Human Oral Bioavailability of BCM-95®CG (BiocurcumaxTM), A Novel Bioenhanced Preparation of Curcumin. *Indian Journal of Pharmaceutical Sciences.* 70(4):445-449.

5. Baker AH, AhonenM, Kahari V-M. (2000). Potential applications of tissue inhibitor metalloproteinase (TIMP) overexpression for cancer gene therapy*ADV. Exp Med Biol*.465-469-83.

6. Barve A, Oo Khor T, Hao X., Keum YS, Yang CS, Reddy B, Kong AT. (2008). Murine Prostate Cancer Inhibition by Dietary Phytochemicals-Curcumin and Phenyethylisothiocyanate. *Pharm. Res*.25(9):2181-2189.

7. Bayet-Robert M, Kwiatkowski F, Leheurteur M, Gachon F, Planchat E, Abrial C, Mouret-Reynier M, Durando X, Barthomeuf C, Chollet P. (2010). Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer. Cancer Biol & Ther.9:1, 8-14.

8. Bhattacharyya S, Sakib D, Mohanty S, Sen G, Chattopadhyay S, Banerjee S, Chakraborty J, Das K, Sarkar D, Das Tand Sa G. (2010). Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. *Cell & Molecular Immunol*. 7*,* 306-315.

9. Bisht S, Feldmann G, Soni S, Ravi R, Karikar C, Maitra A, & Maitra A. (2007). Polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"): a novel strategy for human cancer therapy. *Journal of Nanobiotechnology*, *5*, 3.

10. Botchkina GI, Zuniga ES, Rowehl RH, Park R, Bhalla R, Bialkowska AB, Johnson F, Golub LM, Zhang Y, Ojima I, Shroyer KR. (2013) .Prostate Cancer Stem Cell-Targeted Efficacy of a New-Generation Taxoid, SBT-1214 and Novel Polyenolic Zinc-Binding Curcuminoid, CMC2.24, DOI: 10.1371/journal.pone.0069884 PLOSone.

11. Brysse A , Mestdagt M , Polette M , Luczka E , Hunziker W, No A, Birembaut P, Foidart JM , Gilles C. (2012).Regulation of CXCL8/IL-8 Expression by Zonula Occludens-1 in Human Breast Cancer Cells. *Mol Cancer Res*. 10(1) 121-132.

12. Cancer Statistics 2013 A presentation from the American Cancer Society. (2013). American Cancer Society Inc. Retrieved July 31, 2015, from http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2013/cancer-statistics-2013-slide-presentation.pdf

13. Cancer Treatment, Side Effects. (2015). National Cancer Institute. Retrieved July 31, 2015, from http://www.cancer.gov/about-cancer/treatment/side-effects

14. Cancer Treatment, Types of Treatment. (2015). National Cancer Institute. Retrieved July 31, 2015, from http://www.cancer.gov/about-cancer/treatment/types

15. Carter HB, Partin AW, Luderer AA et al (1997) Percentage of free prostate-specific antigen in sera predicts aggressiveness of prostate cancer a decade before diagnosis. Urology 49:379.

16. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH.(2001) . MAP kinases. *Chem. Rev*.101:2449-2476.

17. Cheng AL., Hsu, CH., Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*.21:2895-2900.

18. Choudhary N, and Singh, B. (2012). Potential Therapeutic Effect of curcumin- an update. *J Pharm Educ Res.* 3: 64-71.

19. Citalingam, K., Abas, F., Lajis N, Othman I, Naidu R. (2015).Anti-Proliferative Effect and Induction of Apoptosis in Androgen-Independent Human Prostate Cancer Cells by 1,5-Bis(2-hydroxyphenyl)-1,4-pentadiene-3-one. *Molecules*. 20, 3406-3430.

20. Clarke SJ., Rivory, LP. (1999). Clinical pharmacokinetics of docetaxel. *Clinical pharmacokinetics*. 36:99-114.

21. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. (2007).Inflammation in prostate carcinogenesis. Nat Rev Cancer. 7:256–69.

22. Dinarello, C.A. (2006). The paradox of pro-inflammatory cytokines in cancer. *Cancer Metastasis Rev.*25 (3):307-313.

23. Dorai T., Gehani, N., Katz A. (2000).Therapeutic potential of curcumin in human prostate cáncer-I. Curcumin induces apoptosis in both androgen-dependent and androgen-independent prostate cancer cells. *Prostate Cancer Prostatic Dis* 3:84 -93.

24. Dorai, T., Gehani, N., Katz A. (2000). Therapeutic potential of curcumin in human prostate cáncer – II. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. *Mol Urol*.4:1-6.

25. Dorrah D, Hao J, Xiaohua G,1 Hafner MS, Wong H, Divine G, Chapman RA, Dulchavsky SA, Gautam SC. (2004). Curcumin sensitizes prostate cancer cells to tumor necrosis factor–related apoptosis-inducing ligand/Apo2L by inhibiting nuclear factor-KB through suppression of IKBA phosphorylation. *Mol Cancer Ther.* 3(7). 803-812.

26. Drugs and supplements Docetaxel (Intravenous Route). (2015). Mayo Foundation for Medical Education and Research. Thompson Healthcare Inc. Retrieved July 31,2015, from http://www.mayoclinic.org/drugs-supplements/docetaxel-intravenous-route/side-effects/drg-20068305

27. Duarte V, Han E, Veena M, Salvado A, SuhJ, Liang LG, Faull K, Srivatsan ES, Wang MB. (2010). Curcumin enhances the effect of cisplatin in suppression of head and neck squamous cell carcinoma via inhibition of IKKβ protein of the nuclear factor kB pathway. *Mol Cancer Ther.* 9:2665-2675.

28. Endo Y, Busslinger M, Chiba T, Sabyasachi D, Anja E, Mustapha F, Bodo G, Masayuki H, Emilie M, Hiroyuki M, Chelsea Mc, Jasna M, Bertrand N, Nikolas N, Sandrine R, Gilles S, Stéphanie S, Hiromi T, Atsushi T, Rea T,Thaventhiran J.E.D, Yong P.F.K. (2007). Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene* 26(38):5587–5595.

29. Epstein JI. (2010). An update of the Gleason grading system. *J Urol*. 183:433-440.

30. Fajardo AM, MacKenzie DA, Ji M, Deck LM, Vander DL, Thompson TA, Bisoffi M. (2012). The Curcumin Analog ca27 Down-Regulates Androgen Receptor Through an Oxidative Stress Mediated Mechanism in Human Prostate Cancer Prostate.72(6): 612–625.

31. Fang, Y., Herrick, E., Nicholl MB. (2012). A possible Role for Perforin and Granzyme B in Resveratrol-Enhanced Radiosensitivity of Prostate Cancer. *J Androl*. 33:752-760.

32. Ferrone C and Dranoff G. (2010). Dual Roles for Immunity in Gastrointestinal Cancer. *Journal of Clinical Oncology*. 28: 4045-4051.

33. Freedland SJ, Moul JW. (2007) Prostate-specific antigen recurrence after definitive therapy. *J Urol*. 177(6):1985-1991.

34. Fuchs JR, Pandit B, Bhasin D, Etter JP, Regan N, Abdelhamid D, Li C, Lin J, Li PK. (2009). Structure-activity relationship studies of curcumin analogues. *Bioorg Med Chem Lett*. 19:2065-2069.

35. Gota VS, Maru GB, Soni TG, Gandhi TR, Kochar N, Agarwal MG. (2010). Safety and pharmacokinetics of a solid lipid curcumin particle formulation in osteosarcoma patients and healthy volunteers. *J Agric Food Chem*. 58(4):2095–9.

36. Gupta S, Campbell D, Derijard B and Davis R (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science.* 267: 389–393.

37. Hadrup, S., Donia, M., Straten P.t. (2013).Effector CD4 and CD8 T Cells and their Role in the Tumor Microenvironment.*Cancer Microenv.* (6):123-133.

38. Hong JM, Park CS, Nam-Goong IS, Kim YS, Lee JC, Han MW, Choi J, Kim Y, Kim ES. (2014). Curcumin Enhances Docetaxel-Induced Apoptosis of 8505C Anaplastic Thyroid Carcinoma Cells. *Endocrinology and Metabolism*. 29(1), 54–61.

39. Hua X, Meng-bo H, Pei-de B, Wen-hui Z, Sheng-hua L, Jun-yao H, Zu-quan Xi, Qiang D, Hao- wen J. (2015) Proinflammatory cytokines in Prostate Cancer Development and Progression Promoted by High-Fat Diet. . *Biomed. Research Int.* 249741. doi:10.1155/2015/249741.

40. Ide H, Tokiwa S, Sakamaki K, Nishio K, Isotani S, Muto S, Hama T, Masuda H, Horie S. (2010).Combined inhibitory effects of soy

isoflavones and curcumin on the production of prostate-specific antigen. *Prostate*.70(10):1127–33.

41. Jayaprakasam B, Vanisree M, Zhang Y, Dewitt DL, Nair MG. (2006). Impact of alkyl esters of caffeic and ferulic acids on tumor cell proliferation, cyclooxygenase enzyme, and lipid peroxidation. J Agric Food Chem;54:5375–81.

42. Jones, SE., Erban, J., Overmoyer B, Budd GT, Hutchins L, Lower E, Laufman L, Sundaram S, Urba WJ, Pritchard KI, Mennel R, Richards D, Olsen S, Meyers ML, Ravdin PM. (2005). Randomized phase III study of docetaxel compared with paclitaxel in metastatic breast cancer. *Jour of Clin Oncol: official journal of the American Society of Clinical Oncology*. 23:5542-5551.

43. Kato T, Mizutani K, Kameyama K, Kawakami K, Fujita Y, Nakane K, Kanimoto Y, Ehara H, Ito H, Seishima M, Deguchi T. (2015).Serum exosomal P-glycoprotein is a potential marker to diagnose docetaxel resistance and select a taxoid for patients with prostate cancer. *Urol. Oncol.*

44. Killian PH,Kronski E, Michalik KM, Barbieri O, Astigiano S, Sommerhoff CP, Pfeffer U, Nerlich AG, Bachmeier BE. (2012). Curcumin inhibits prostate cancer metastasis *in vivo* by targeting the inflammatory cytokines CXCL1 and -2. *Carcinogenesi*s. 33: pp.2507–2519.

45. Kyriakis, J. M., and J. Avruch. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev*. 81:807-869.

46. Lilja, H, Ulmert, D, Vickers A. (2008). Prostate-specific antigen and prostate cancer: Prediction, detection and monitoring. Nat Rev Cancer. 8: 268-278.

47. Lin, L., Shi, Q., Nyarko AK, Bastow KF, Wu CC, Su CY, Shih CC, Lee KH, (2006). Antitumor Agents 250. Design and Synthesis of New Curcumin Analogs as Potential Anti-Prostate Cancer Agents. *J Med Chem.* 49(13):3963-3972.

48. Luo F, Song X, Zhang Y, Chu Y. (2011). Low-dose curcumin leads to the inhibition of tumor growth via enhancing CTL-mediated antitumor immunity. *Elsvier International Immunopharmacology. Vol. 11: 1234-1240.*

49. MacManus CF, Pettigrew J, Seaton A, Wilson C, Maxwell PJ, Berlingeri S, Purcell C, McGurk M, Johnston PG, Waugh DJJ. (2007). Interleukin-

8 signaling promotes translational regulation of cyclin D in androgen-independent prostate cancer cells. *Mol. Cancer Res. 5*: 737–748.

50. Mantovani G., Anker S.D., Inui A., Morley J.E., Rossi Fanelli F., Scevola D., Schuster M.W., Yeh S.-S. (2006). Cachexia and Wasting: A Modern Approach. Springer. ISBN-10 88-470-0471-3 Springer Milan Berlin Heidelberg New York. ISBN -13 978-88-470-0471-9 Springer Milan Berlin Heidelberg New York 1- 758.

51. Maxwell PJ, Coulter J, Walker SM, Mckenchnie M, Neisen J, McCabe N, Kennedy R.D., Salto M, Albanese C., Waugh D.J.J. (2013). Potentiation of inflammatory CXCL8 signalling sustains cell survival in PTEN-deficient prostate carcinoma*. Eur Urol*.64:177–88.

52. Maxwell PJ, Neisen J, Messenger J, Waugh DJJ. (2014). Tumor-derived CXCL8 signaling augments stroma-derived CCL2- promoted proliferation and CXCL12-mediated invasion of PTEN-deficient prostate cancer cells. *Oncotarget*, 5: 4895-4908.

53. Mimeault, M. and Batra, S. (2011). Potential applications of Curcumin and its novel synthetic analogs and nanotechnology-based formulations in cancer prevention and therapy. *Chinese Medicine*. 6:31.

54. Mohammad I, Som S , Yasumasa O, Masayoshi F, and Shigeru Okada (2003) Dietary Supplementation of Curcumin Enhances Antioxidant and Phase II Metabolizing Enzymes in ddY Male Mice: Possible Role in Protection against Chemical Carcinogenesis and Toxicity. *Pharmacol. & Toxicol.* 92: 33-38.
55. Morice WG. (2007) .The Immunophenotypic Attributes of NK cells and NK-Cell Lineage Lymphoproliferative Disorders . *Am J Clin Pathol .* 127 (6):881-886.

56. Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS. (2007). Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. *Cancer Res.* 67(10):5033-41.

57. Murakami Y, Hirata A, Ito S, Shoji M, Tanaka S, Yasui T, Machino M, Fujisawa S. (2007). Re-evaluation of cyclooxygenase-2- inhibiting activity of vanillin and guaiacol in macrophages stimulated with lipopolysaccharide. Anticancer Res;27:801–7.

58. Myant, K. B., Cammareri, P., McGhee, E. J., Ridgway, R. A., Huels, D. J., Cordero, J. B., Schwitalla, S., Kalna, G., Ogg, E.L., Athineos, D.,

Timpson,P., Vidal, M., Murray, G., Greten, F., Anderson, K., Sansom, O. J. (2013). ROS Production and NF-κB Activation Triggered by RAC1 Facilitate WNT-Driven Intestinal Stem Cell Proliferation and Colorectal Cancer Initiation. *Cell Stem Cell*. 12(6), 761–773.

59. Nakamura K, Yasunaga Y, Segawa T, Ko D, Moul JW, Srivastava S, Rhim JS. (2002). Curcumin down-regulates AR gene expression and activation in prostate cancer cell lines. *Int J Oncol* 21:825–30.

60. Oh-Joon, K, Li Z., Ittmann MM, Xin LI. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin.(2014). *PNAS*.111:E592-E600.

61. Ohori M, Wheeler TM, Scardino PT. (1994). The New American Joint Committee on Cancer and International Union Against Cancer TNM classification of prostate cancer. *Cancer*. 74: 104-114.

62. Pan MH, Huang TM, Lin JK. (1999). Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab Dispos*.27(4):486-94.

63. Plummer SM, Hill KA, Festing MF, Steward WP, Gescher AJ,Sharma RA. (2001). Clinical development of leukocyte cyclooxygenase 2 activity as a systemic biomarker for cancer chemopreventive agents. Cancer Epidemiol Biomarkers Prev. 10:1295–9.

64. Pramanik D, Campbell N, Das S, Gupta S, Chenna V, Bisht S, Sysa-Shah P, Bedja D, Karikari C, Steenbergen C, Gabrielson K, Maitra A, Maitra A.(2012). A composite polymer nanoparticle overcomes multidrug resistance and ameliorates doxorubicin-associated cardiomyopathy. *Oncotarget*. 3: 640-650.

65. Prostate Cancer, Prostate Cancer Treatment. (2015). National Cancer Institute. Retrieved July 31, 2015, from http://www.cancer.gov/types/prostate/patient/prostate-treatment-pdq#section/_120

66. Riemenschneider MJ, Betensky RA, Pasedag SM, Louis DN. (2006). AKT activation in human glioblastomas enhances proliferation via TSC2 and S6 kinase signaling. Cancer Res. 66:5618–23.

67. Rocha S, Generalov R, do Pereira CM, Peres I, Juzenas P, Coelho MA. (2011) Epigallocatechin gallateloaded polysaccharide nanoparticles for prostate cancer chemoprevention. Nanomedicine (Lond). 6:79–87.

68. Ross JS, Kaur P, Sheehan CE, Fisher HAG, Kaufman RA Jr, Kallakury BVS. (2003).Prognostic Significance of Matrix Metalloproteinase 2 and

Tissue Inhibitor of Metalloproteinase 2 Expression in Prostate Cancer. *Mod Pathol*.16:198–205.

69. Roy S, Yu Y, Padhye S, Sarkar F, Majumdar A. (2013). Difluorinated-Curcumin (CDF) Restores PTEN Expression in Colon Cancer Cells by Down-Regulating miR-21. *PLos One*. 8(7):e68543. doi:10.1371/journal.pone.0068543.

70. Russell PJ, Jackson P, Kingsley E. Human Prostate Cancer Cell Lines. Prostate Cancer Method s and Protocols. (2003), XII, 403 p. ISBN: 978-0-89603-978-0. Humana Press.

71. Sahoo SK, Ma W, Labhasetwar V. (2004). Efficacy of transferrin-conjugated paclitaxel-loaded nanoparticles in a murine model of prostate cancer. *Int J Cance*r. 112:335–340.

72. Sasaki H, Sunagawa Y, Takahashi K, Imaizumi A, Fukuda H, Hashimoto T, Wada H, Katanasaka Y,Kakeya H, Fujita M,Hasegawa K, Morimoto T. (2011). Innovative preparation of curcumin for improved oral bioavailability. Biol Pharm Bull.34 (5):660–5.

73. Seaton A, Scullin P, Maxwell PJ, Wilson C., Pettigrew J., Gallagher R., O' Sullivan JM, Johnston PJ, Waugh DJ. (2008). Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells via induction of androgen receptor expression and activation.*Carcinogenesis*.29:1148–56.

74. Sharifi N, Dahut WL, Steinberg SM, Figg WD, Tarassoff C, Arlen P, Gulley JL. (2005). A retrospective study of the time to clinical endpoints for advanced prostate cancer. *BJU Int.* 96 (7): 985-9.

75. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP. (2004). Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res*. 10:6847–54.

76. Shoji M, Sun A, Kisiel W, Lu YJ, Shim H, McCarey BE, Nichols C, Parker ET, Pohl J, Mosley CA, Alizadeh AR, Liotta DC, Snyder JP. (2008). Targeting tissue factor-expressing tumor angiogenesis and tumors with EF24 conjugated factor VIIa. *J Drug Target*. 16:185-97.

77. Siddiqui IA, Adhami VM, Bharali DJ, Hafeez BB, Asim M, Khwaja SI, Ahmad N, Cui H, Mousa SA, Mukhtar H. (2009) Introducing nanochemoprevention as a novel approach for cancer control: proof of

principle with green tea polyphenol epigallocatechin-3-gallate. Cancer Res.; 69:1712– 1716.

78. Soto-Salgado M, Suarez E, Torres-Cintron M, Pettaway C, Colon V, Ortiz AP. Prostate Cancer Incidence and Mortality among Puerto Ricans: An Updated Analysis Comparing Men in Puerto Rico with US Racial / Ethnic Groups. (2012). PR Health Sci J. 3: 107-113.

79. Still K, Robson CN, Autzen P, Robinson MC, Hamdy FC. (2000). Localization and quantification of mRNA for matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in human benign and malignant prostatic tissue. *Prostate*; 42:18-25.

80. Su WP, Cheng FY, Shieh DB, Yeh CS, Su WC. (2012). PLGA nanoparticles codeliver paclitaxel and Stat3 siRNA to overcome cellular resistance in lung cancer cells. *International Journal of Nanomedicine*, *7*, 4269–4283. doi:10.2147/IJN.S33666.

81. Sundram V, Chauhan SC, Ebeling M, Jaggi, M. (2012). Curcumin Attenuates β-catenin Signaling in Prostate Cancer Cells through Activation of Protein Kinase D1. *PLoS ONE*. *7*(4), e35368. doi:10.1371/journal.pone.0035368

82. Tamvakopoulos C, Dimas K, Sofianos ZD, Hatziantoniou S, Han Z, Liu ZL,Wyche JH, Pantazis P. (2007).Metabolism and anticancer activity of the curcumin analogue, dimethoxycurcumin. *Clin Cancer Res*.13:1269-1277.

83. Thomas, S., Zhong, D., Zhou W, Malik S, Liotta D, Snyder JP, Hamel E., Giannakakou P. (2008). EF24, a novel curcumin analog, disrupts the microtubule cytoskeleton and inhibits HIF-1. *Cell Cycle.* 7(15): 2409-2417.

84. Tortolero G, Zavala D, Pérez N, Torres CR, Ortiz KJ, Traverso M, Román Y, Veguilla I, Vázquez N, Merced MF, Ojeda G, Hayes FJ, Ramos M, López A, Pérez N (2013). Cancer in Puerto Rico, 2006-2010. Puerto Rico Central Cancer Registry. San Juan, PR.

85. Tripathi A, Gupta R , Saraf SA. (2010). PLGA Nanoparticles of Anti Tubercular Drug: Drug Loading and Release Studies of a Water In-Soluble Drug. *Int Jour of Pharm Tech Res*. 2: 2116-2123.

86. Ueda K, Cardarelli C, Gottesman MM , Pastan I. (1987). Expression of a full-length cDNA for the human “MDRI” gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci* . 84:3004-3008.

87. Uehara H, Troncoso P, Johnston D, Bucana CD, Dinney C, Dong Z, Fidler IJ, Pettaway CA. (2005). Expression of interleukin-8 gene in radical prostatectomy specimens is associated with advanced pathologic stage. *The Prostate*. 64(1):40-9.

88. Uen YH, Fang CL, Hseu YC, Shen PC, Yang HL, Wen KS, Hung ST, WangLH, Lin KY. (2015).VAV3 Oncogene Expression in Colorectal Cancer: Clinical Aspects and Functional Characterization. *Sci. Rep*. 5, 9360.

89. Uzgare AR, Kaplan PJ, Greenberg NM. (2003). Differential expression and/or activation of P38MAPK, erk1/2, and jnk during the initiation and progression of prostate cancer. *Prostate*.55(2):128-39.

90. Veltri RW, Miller MC, Zhao G, Ng A, Marley GM, Wright GL, Vessella RL, Ralph D.(1999). Interleukin-8 serum levels in patients with benign prostatic hyperplasia and prostate cancer. *Urology*.*53*:139–147.

91. Verweij, J., Clavel, M., Chevalier B. (1994). Paclitaxel (Taxol) and docetaxel (Taxotere): not simply two of a kind. *Annals of oncology: official journal of the European Society for Medical Oncology/ESMO*.5:495-505.

92. Waldhauer, I., Steinle, A. (2008).NK cells and cancer immunosurveillance. *Oncogene.* 27(45):5932-5943.

93. Wang YJ, Pan MH, Cheng AL, Lin LI, Ho YS, Hsieh CY, Lin JK. (1997). Stability of curcumin in buffer solutions and characterization of its degradation products. J Pharm Biomed Anal;15:1867–76.

94. Xiaochen N, Aili Z, Zhihong Z, Yongqing S, Shijie W. (2012). Demethoxycurcumin inhibits cell proliferation, migration and invasion in prostate cancer cells. *Oncology Reports* .28: 85-90.

95. Yallapu MM, Dobberpuhl MR, Maher DM, Jaggi M, Chauhan SC.(2012).Desing of curcumin loaded cellulose nanoparticles for prostate cancer. *Curr Drug Metab*. 13 (1): 120-128.

96. Yefenof, E. (2008). The tumor microenvironment. *Innate and Adaptive Immunity in the Tumor Microenvironment.* Vol. 1. Springer. ISBN print: 978-1-4020-6749-5. ISBN online: 978-1-4020-6750-1.

97. Zhu Y, Liu C, Nadiminty N, Lou W, Tummala R, Evans CP, Gao AC. (2013). Inhibition of ABCB1 expression overcomes acquired docetaxel resistance in prostate cancer. *Molecular Cancer Therapeutics*. *12*(9):1829–1836.