MECHANISM OF BORON DERIVATIVES FOR TREATMENT OF RENAL CELL CARCINOMA

by
Sıdıka TAPŞIN

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2014

MECHANISM OF BORON DERIVATIVES FOR TREATMENT OF RENAL CELL CARCINOMA

APPROVED BY:

Prof. Dr. Fikrettin Şahin
(Thesis Supervisor) .....

Assoc. Prof. Dilek Telci .....

Assist. Prof. Ayşegül Kuşkucu .....

DATE OF APPROVAL: .. / .. / 20..

This thesis is dedicated to my family…

ACKNOWLEDGEMENTS

I would like to acknowledge to Yeditepe University for funding this study and I would like to express my deepest appreciation to my supervisor Fikrettin Şahin for his reliance on me and supports both morally and financially throughout my time at Yeditepe University.

I would like to show my greatest appreciation to my colleagues Nezaket Türkel Sesli for her encouraging supports and guidance during my experimental and typing stages of my thesis and the interview stages of my PhD application.

I would like to offer my special thanks to Sukru Gulluoglu for the stimulating discussions, for his patience, motivation, and immense knowledge in the times we were working together.

At the end, I owe my deepest gratitude to my unique family; my dear mother Sabriye Tapşın, my dear father Gürcan Tapşın and my little sister İrem Tapşın for their great effort to carry me these days and for their supports and loves which will never end in my life, I know.

ABSTRACT

MECHANISM OF BORON DERIVATIVES FOR THE TREATMENT OF RENAL CELL CARCINOMA

Renal cancers are approximately two per cent of all cancer types worldwide and renal cell carcinoma (RCC) is the most prevalent type of kidney cancer seen at a rate of 90 per cent in the world. Although there are number of different methods are used to fight with RCC including surgical, chemotherapeutic and radiotheraeutic approaches, because of its drug resistance and highly metastatic capability, these techniques are generally ineffective. In recent years, boron derivatives have been started to use as a targeted drug for cancer cells. Bortezomib is first and well known proteosome inhibitor that is used for different cancer types such as mantle cell lymphoma and multiple myeloma. Although, it is very efficient drug, it has number of side effects. Therefore, development of new boron derivatives other than bortezomib to use in targeted therapy and deciphering their effect mechanism on cancer cells is very important. In this study, we aimed to investigate the effects of boron derivatives such as sodium pentaborate pentahydrate (NaB), boric acid (BA) and bortezomib (B) in cell proliferation, cell migration and cell death in primary A-498, metastatic ACHN RCC cell lines and RPTEC normal renal cell line as an *in vitro* model were used. Our results demonstrated that NaB and BA could be a safer and efficient targeted therapeutic on renal cancer cells instead of B. Additionally, our results were the first findings and evidences about the mechanism of boron derivatives on cell proliferation, cell migration and cell death on ACHN, A498 and RPTEC cell lines.

ÖZET

RENAL HÜCRE KARSİNOMUNUN TEDAVİSİNDE BORON TÜREVLERİNİN MEKANİZMASI

Renal kanser tüm kanserlerin yüzde ikisini oluşturmaktadır ve en sık görülen renal kanser çeşidi olan renal hücre karsinomu (RHK) tüm renal kanser çeşitleri içinde yüzde 90 çoğunlukla gözlenmektedir. RHK kemoterapi ve radyoterapi gibi yöntemler ile tedavi edilmeye çalışılsa da ilaca dirençli yapısı ve metastatik özelliğinden dolayı, tedaviler etkisiz kalmaktadır. Son yıllarda, boron türevleri kanser hücreleri için hedeflenmiş ilaç şeklinde kullanılmaya başlanmıştır. Bortezomib, bu anlamda ilk kullanılan ve iyi bilinen bir proteozom inhibitörüdür ve mantle hücreli lenfoma ve multipl miyelom gibi hastalıklar için kullanılmaktadır. Etkili bir ilaç olmasına rağmen, bir çok yan etkisi vardır. Bu nedenle, hedeflenmiş tedavi olarak bortezomibin yerine yeni boron türevlerinin geliştirilmesi ve kanser hücreleri üzerindeki etki mekanizmalarının çözülmesi çok önemlidir. Bu çalışmada, boron türevi olan sodyum pentaborat pentahidrat (NaB), borik asit (BA) ve bortezomib (B)'in *in vitro* model olarak kullanılan primer A498, metastatik ACHN RHK hücre hatları ve RPTEC normal renal hücre hattı üzerindeki hücre çoğalması, hücre göçü ve hücre ölümü üzerine etkisini araştırmayı amaçladık. Sonuçlarımız, NaB ve BA'nın B'ye göre daha güvenli ve etkili bir hedeflenmiş teropotik olabileceğini gösterdi. Dahası, bu çalışmada boron türevi olan sodyum pentaborat pentahidrat (NaB), borik asit (BA) ve bortezomib (B)'in *in vitro* model olarak kulanılan primer A498, metastatik ACHN RHK hücre hatları ve RPTEC normal renal hücre hattı üzerindeki hücre çoğalması, hücre göçü ve hücre ölümü üzerine etki mekanizması hakkında ilk bulgular ve kanıtlar elde edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS / ABBREVIATIONS

AP2	activator protein-2
B	Bortezomib
BA	Boric acid
BNCT	Neutron capture therapy
c-CBL	casitas B-lineage lymphoma
cDNA	Complementary deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffer Saline
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Ets	E-twenty six
FBS	Fetal bovine serum
HER2/neu	Receptor tyrosine kinase ERBB2
HIFs	hypoxia inducible factors
g	Gram
IC50	50 per cent inhibition concentration of proliferation
IGF-1	Immunoglobulin-like factor-1
IFN-γ	Interferon-γ
IL-2	Interleukin-2
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Mikromolar
mg	Milligram
mL	Milliliter
mM	Milimolar
mmol	Millimole

mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NaB	Sodium pentaborate pentahydrate
nm	Nanometer
nM	Nanomolar
NO	Nitric oxide
NSS	Nephron-saving surgery
Pax3	Paired box 3
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-kinase
PMS	Phenazine methosulfate
PS	Membrane phospholipid phosphatidylserine
PSA	Penicillin streptomycin amphotericin B
PTPs	Protein tyrosine phosphatases
RCC	Renal cell carcinoma
RECM	Renal Epithelial Cell Growth Kit
RNA	Ribonucleic acid
rpm	Rotation per minute
RPTEC	Primary Renal Proximal Tubules Epithelial Cells
RTCC	Renal transitional cell carcinoma
SFM	Serum free medium
Tcf-4	Transcription factor 4
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

1. INTRODUCTION

1.1. GENERAL VIEW OF CANCER

Cancer is a disease which occurs due to genetic changes and multiple deficiencies of molecular, cellular and biochemical pathways [1]. According to statistics, 14,1 million new cancer cases and 8,2 million cancer deaths occurred in 2012; therefore, cancer is the most common cause of mortality after heart diseases [2] There are ten distinguishing aspects of cancer gained during the multistep development of human tumor which show the complexities of the disease. The characteristics of cancer comprise of a continuous proliferative signaling, resisting to cell death, enabling replicative immortality, evading growth suppressors, pronounced angiogenesis, activating invasion and metastasis, genome instability, reprogramming of energy metabolism and evading immune destruction [3] (Figure 1.1).

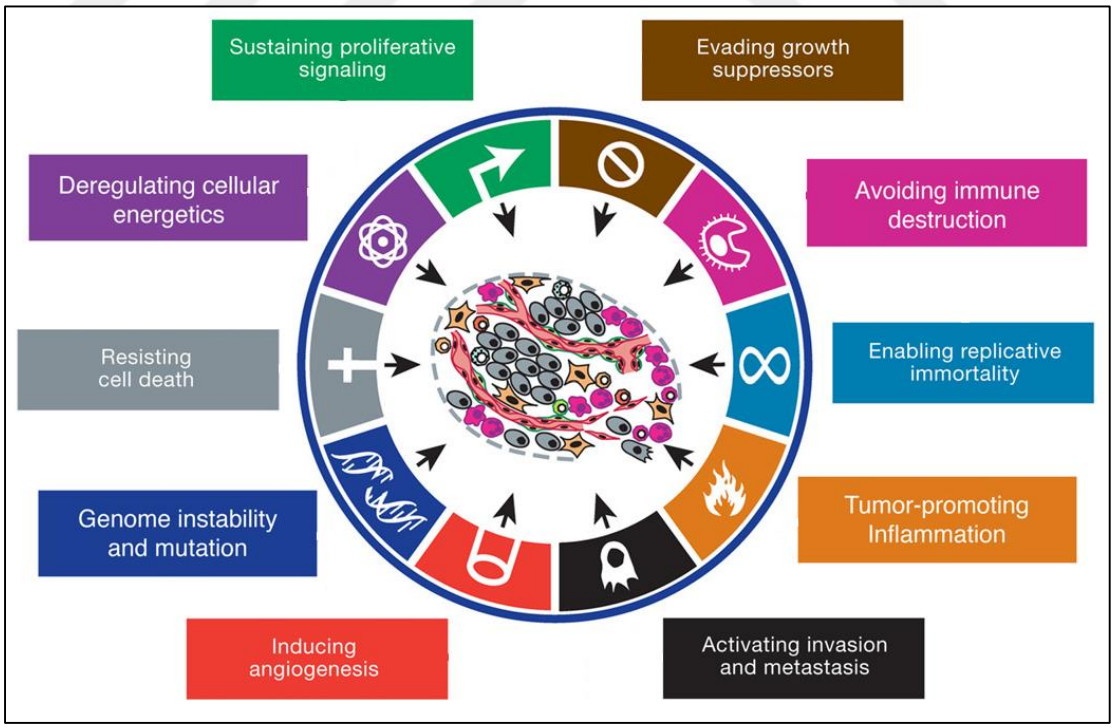

Figure 1.1. Hallmarks of cancer, new generation [3]

1.2. KIDNEY CANCER

Kidney is a bean shape organ that is located in the abdominal cavity, at the both side of spine. It is a major member of the both urinary and endocrine systems and play a role in acid-base balance, several hormones synthesis and excretion of urine [4]. Kidney has two main parts; parenchyma part consist of cortex and medulla, and collecting part comprises ureters calyses and renal pelvis as shown in Figure 1.2 [5]. Parenchyma part is formed by nephrone which is the smallest functional subunit of a kidney [6]. Nephrones are composed of glomerulus and tubules that plays role in urine formation and blood filtration. There are approximately 1 million nephron in a kidney [4].

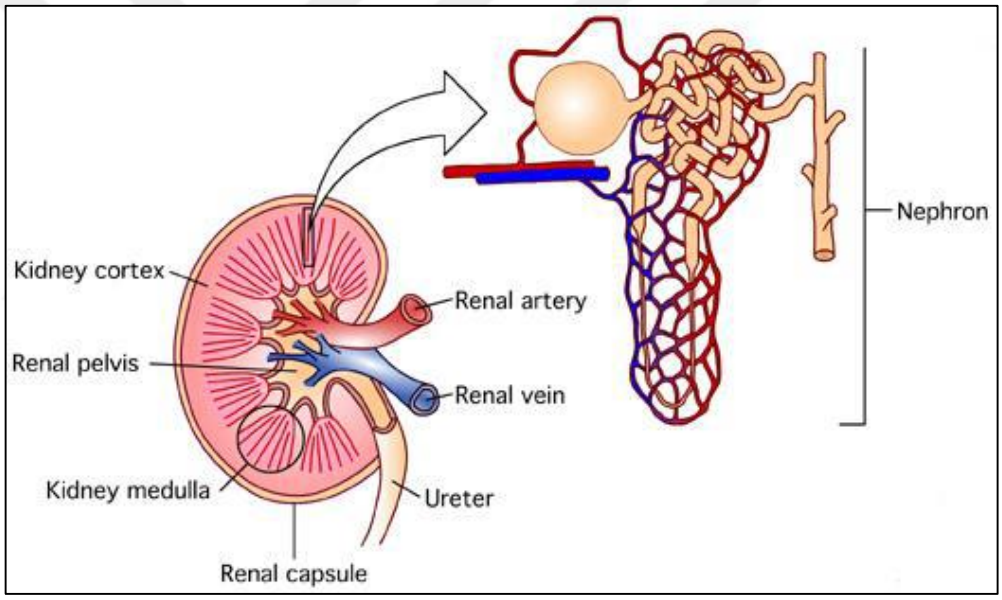

Figure 1.2. Kidney Anatomy [5]

Cancer derived from kidney is classified in two groups named renal transitional cell carcinoma (RTCC) and renal cell carcinoma (RCC). RTCC originates from renal pelvis, whereas RCC is derived from renal parenchyma part of the kidney.

Kidney cancer is twelfth most common cancer in the world with 338,000 new case diagnosed in 2012 and generates approximately 2 per cent of all cancer types worldwide [6]. Even so, its frequency is similar to breast, prostate and bladder cancer, it is the most lethal amoung these malignancies [6]. Interestingly, incidence rate and death rate are

higher in men (20 per cent; 6 per cent respectively) than women (10 per cent; 3 per cent respectively) suggesting that obesity and over smoking may induce this imparity [6]. These rates are observed similarly in Turkish population, as well.

1.3. RENAL CELL CARCINOMA

Renal cell carcinoma (RCC), also known as renal cell adenocarcinoma is the most prevalent type of kidney cancer seen at a rate of 90 per cent in the world [6]. Incidence rate and death rate of RCC is approximately 210,000 case and 100,000 death per year, respectively [6]. RCC could be characterized by its poor prognosis and high potential of metastasis [7].

Clear cell renal cell carcinoma (Figure 1.3.a) is the most common type of RCC seen at a rate of 80 per cent in RCC patients. Other common RCC histological subtypes are papillary (Figure 1.4.b) and chromophobe (Figure 1.5.c); ~10 per cent and 5 per cent respectively [7].

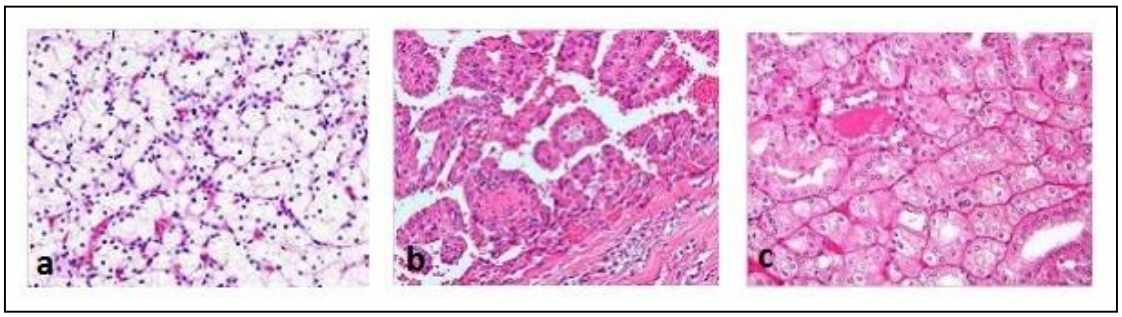

Figure 1.3. Types of renal cell carcinoma: a. Clear cell renal carcinoma, b. Papillary renal cell carcinoma, c. Chromophobe renal cell carcinoma [7]

The main curative treatment of renal cancer is surgical removal either by radical nephrectomy means removal of the entire kidney, or by nephron-saving surgery (NSS). NSS is the recommended procedure if tumor fully localized to the kidney and smaller than 4 cm [12]. However, the prognosis of patients is significantly low because of vascularization in kidney and higher metastatic capability of renal cancer cells [13,14].

Therefore, standard treatments such as chemotherapy [15] and radiotherapy [16] are generally ineffective against renal cell carcinoma [17]. RCC is one of the most immune responsive of human malignancy. Thus, various immunotherapeutic strategies, such as interferon-α and interleukin-2 have been used to increase anti-tumor immunity [18,19]. Clinical response to immunotherapy is limited because of tumor induced immune suppression. Therefore, vaccine therapy that stimulates immune system directly through the tumor cell could be an alternative, clinically safe and effective therapy although in a minority of patients [20]. Since metabolic pathways were associated with renal cell carcinoma, researches on molecular targeting therapies have been significantly increased. Targeted inhibitors for RCC are mainly separated to two groups correlated with two main pathways; the tyrosine kinase inhibitors for VEGF pathways such as bevacizumab, sunitinib, sorafenib axitinib and pazopanib; and for the mammalian target of rapamycin (mTOR) pathway such as temsirolimus and evorilimus [21].

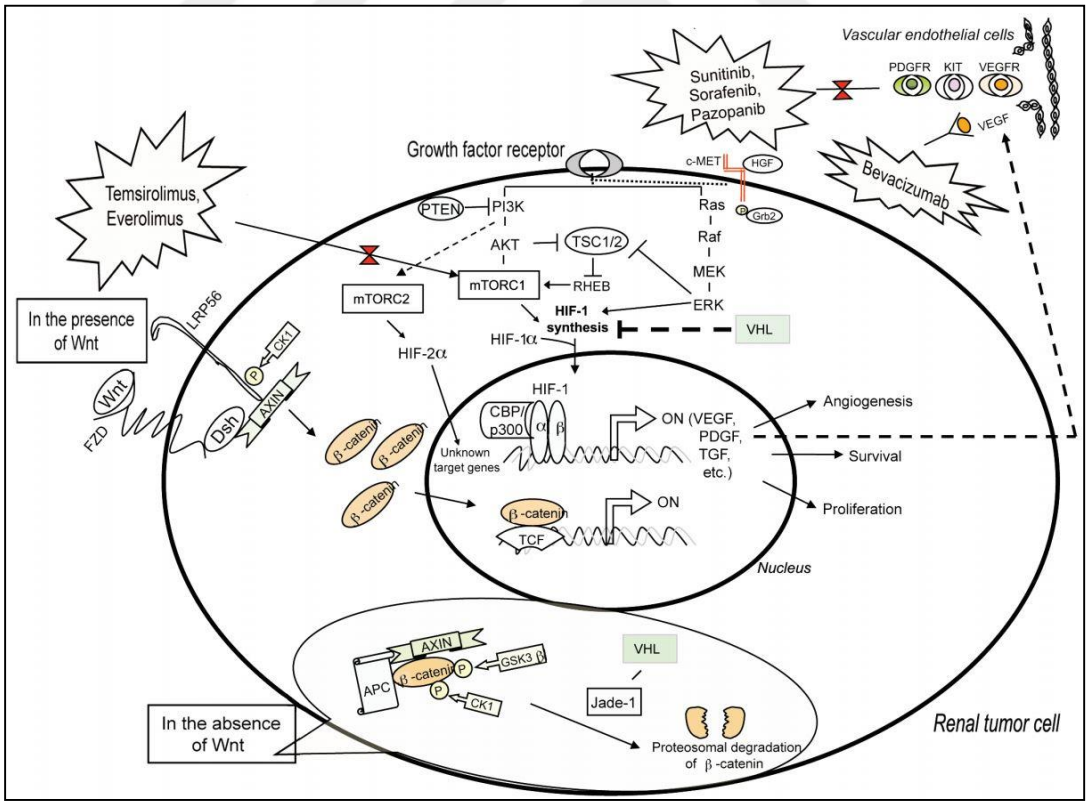

Figure 1.4. Targeted therapeutic drugs in renal cell carcinoma [21]

1.4. MOLECULAR PATHOGENESIS OF RENAL CELL CARCINOMA

Renal cell carcinoma is a metabolic disease [22] and around 4 per cent of RCC are hereditary and 96 per cent of are sporadic [23, 24]. Deficiencies in either tumor suppressor genes (*VHL, FH, TSC, BHD*) or oncogenes (*MET*) are all involved in metabolic pathways related to oxygen, iron, energy and nutrient sensing and are associated with at least six hereditary syndromes (Table1.1) [8]: von Hippel-Lindau (*VHL*) syndrome, hereditary papillary renal carcinoma, Birt-Hogg-Dube' syndrome, hereditary leiomyomatosis, familial clear cell renal cell carcinoma syndrome and renal cell tumors associated with tuberous sclerosis [9]. Additionally, several environmental risk factor such as smoking, obesity, hypertension and tuberus sclerosis are associated with prognosis of RCC [10-11] (Table 1.1).

Table 1.1. Histopathological and genetic characteristics of RCC [8]

Tumor type	**Gene**	**Pathway**	**Syndrome**
Clear cell	VHL	VEGF	von Hippel-Lindau
	FHIT	TGF-β	Familial clear cell RCC
	BHD	AMPK-mTOR	Birt-Hogg-Dube
Papillary type1 type2	MET FH	MET-HGF VEGF TGF-β	Hereditary papillary RCC Hereditary leiomatosis
	TSC1 TSC2	mTOR	Tuberous sclerosis complex
Chromophobe	BHD	AMPK-mTOR	Birt-Hogg-Dube

1.4.1. The Oxygen-Sensing Pathway

Kidney tumors are characterized by hypoxic conditions due to local imbalance between oxygen supply and consumption as many solid tumors [25]. In fact, hypoxia and compensatory hyper-activation of angiogenesis are immensely important for renal cancer prognosis compared to other tumor types because of highly vascularized nature of kidney [26].

A small group of transcription factors called hypoxia inducible factors (HIFs) mediates hypoxic signaling. These oxygen-sensitive basic helix–loop–helix transcription factors regulate biological process by taking role on oxygen delivery and cellular adaptation to oxygen deprivation [27]. HIFs are a heterodimer protein consisting of an unstable α-subunits (either HIF-1α or HIF-2α) which varies depending on oxygen availability and a stable constitutively expressed β-subunits. Under hypoxic conditions, HIF-1α translocates to the nucleus, where it binds to HIF-β and induces the expression of genes that are involved in energy metabolism, angiogenesis, erythropoiesis, iron metabolism, cell proliferation, apoptosis and other biological processes (Figure 1.5.a) [28].

In normal cells, this activation is oxygen-dependent and transient [28]. Under normoxic conditions, HIF-1α is hydroxylated by proline hydroxylase and asparagine hydroxylase, which allow it to be bound by VHL [29, 30]. *VHL* is a tumor-suppressor gene that encodes VHL protein. This protein forms a ubiquitin ligase complex associated with elongins B and C, and Cullins (Cul2) and targets proteasomal degradation of HIF-1α. In this way, it limits the transcription of HIF-dependent targets required for tumor progression (Figure 1.5.a) [31].

It has been shown that *VHL* gene is inactivated in retinal angiomas, central nervous system hemangiomas and clear cell RCC [32]. The *VHL* mutation is transmitted in an autosomal dominant manner on chromosome 3p and appears in all hereditary RCC and approximately 80 per cent of sporadic RCC [33]. In hypoxic RCC tumors, HIFα proteins remain constitutively expressed with the absence of *VHL* expression. Increased expressions of the HIFs' targets are implicated in promotion of cancer hallmarks by inducing changes both within the tumor cells (cell-intrinsic) and in the growth of adjacent endothelial cells to promote blood vessel growth (Figure 1.5.b).

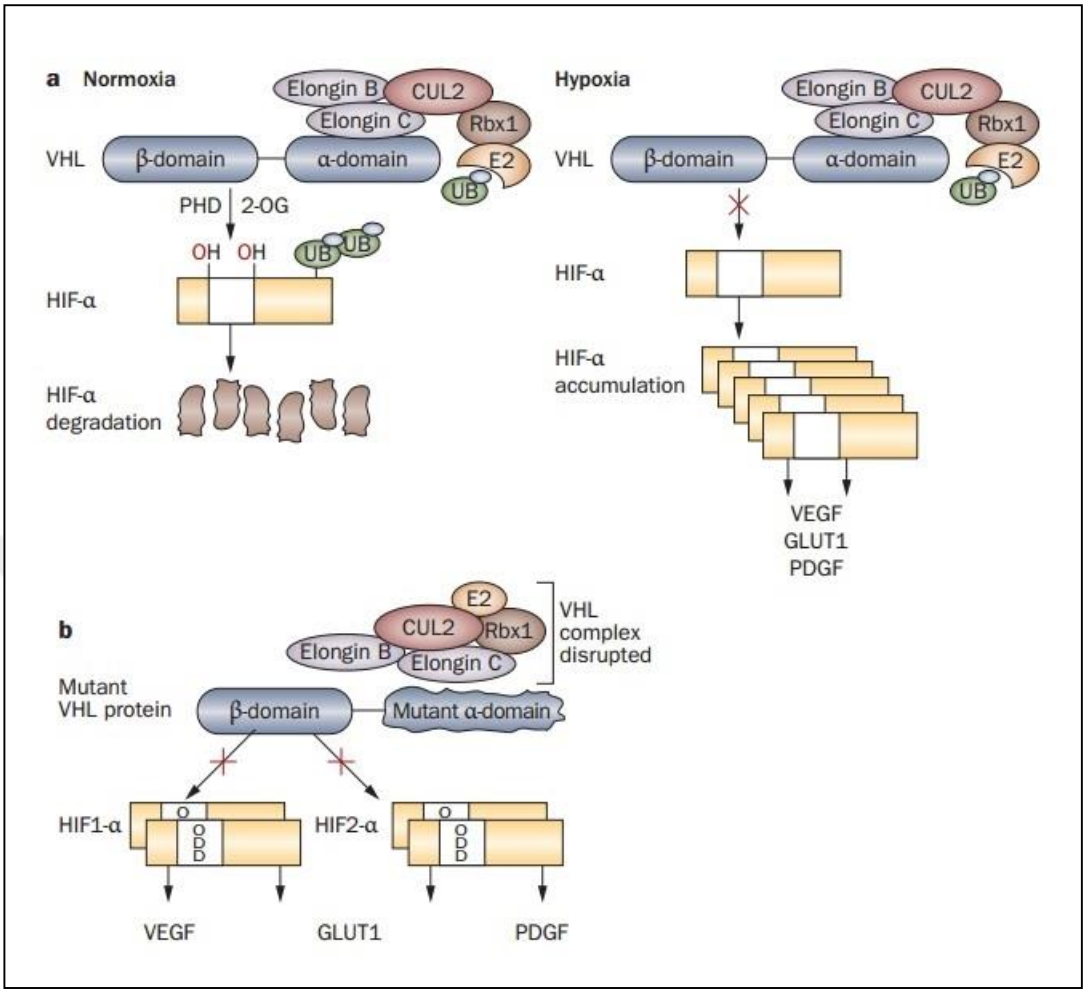

Figure 1.5 VHL activity: a. in normal cells b. in VHL gene-mutated cells [28]

Transcription of HIF-1α and thereby hypoxia-inducible genes can also be induced by physiological stimuli other than hypoxia. Signaling via the receptor tyrosine kinase ERBB2 (HER2/neu) or insulin-like growth factor-1 (IGF-1) receptor tyrosine kinase induces HIF-1 protein synthesis by an oxygen-independent mechanism [34, 35]. IGF-1 induced HIF-1 synthesis is dependent upon both the phosphatidylinositol 3-kinase (PI3K) and MAP kinase pathways [35].

In addition, some nitric oxide (NO) donors induce HIF-1 activation under normoxic conditions by suppressing the prolyl hydroxylase activity [36]. Under hypoxic conditions, NO inhibits the induction of HIF-1α, a phenomenon which one study has attributed to by showing the inhibition of mitochondrial O_2 consumption [37], whereas another study has

provided evidence that under hypoxic conditions, NO increases the concentration of intracellular free iron, thus stimulating prolyl hydroxylase activity [38].

Table 1.2. Hypoxia-Inducible Factor (HIF) Transcriptionally Induced Genes [39]

Function	**Hypoxia/HIF Target Genes**
Proliferation/Survival	IGF-BP1/2/3, IGF2, CCD1, TGF- α/β, P21, Cyclin G2, NOS2, EGFR
Apoptosis	P53, BNIP3, NIX, Bax, RTP801/REDD1, Ref-1, Bcl-2, NFκB, HSP70, Bid
Migration/ Invasion	CXCR4, MMP-2, Lox, PAI-1, c-MET, LRP1, MIC2/CD99, fibronectin, UPAR, collagen type V, AMF/GPI, CATHD, integrin-linked kinase, integrins, Muc1
Transcriptional regulation	DEC1, DEC2, ETS-1, NUR77
Cytoskeletal structure	KRT14, KRT18, KRT19, vimentin
Cell Metabolism	
Glucose	PDK, PFK, PGK, LDHA, GLUT-1/3, hexokinase-1/2, enolase-1, GAPDH, ALDA, ALDC, PKM, TPI, endoglin, GLUT1
Iron	Transferrin, transferrin-R, ceruloplasmin
pH	Carbonic anhydrase-9
Nucleotide	Adenylate kinase-3, ecto-5 ′-nucleotidase
Amino acid	Transglutaminase2

HIF-1α overexpression is associated with different biological processes (Table 1.2) [39] and correlated with an increased risk of mortality in several types of carcinoma [40]. The well-known HIF targets, vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), are potent endothelial cell mitogens. The upregulation of growth factor receptors such as VEGF in RCC is known to strongly correlate with microvessel density and the degree of angiogenesis [41].

Other HIFs target, MMP-2 is a direct transcriptional target of HIF-1α and when it is upregulated, it degrades the extracellular matrix (ECM) and mediates the migration [42].

MUC1, a heterodimeric O-glycosylated transmembrane mucin, is another target of HIFs that correlates with increased severity of disease and metastatic progression in gastric, prostate, breast, and pancreatic cancer in addition to thymic epithelial tumors (43-48). Furthermore, cellular mislocalization of MUC1 has been shown to correlate with disease progression in non–small cell lung cancer (49).

1.4.2 HGF-c-MET pathway

c-MET is a receptor tyrosine kinase which binds to a receptor, hepatocyte growth factor, and activates a wide range of different cellular signaling pathways including proliferation, motility, migration and invasion. [50-53]. Although c-MET is important for controlling tissue homeostasis under normal physiological conditions, it has also been found to be abnormally activated in human cancers via mutation, amplification or protein over-expression [50]. c-MET is a proto-oncogene located on chromosome 7q21-31 and its transcription is regulated by Ets (E-twenty six), Pax3 (paired box 3), AP2 (activator protein-2) and Tcf-4 (transcription factor 4) [54-57].

c-Met is synthesized firstly as a single-chain precursor protein [58, 59] and then is cleaved to produce a glycosylated alpha-chain subunit (50 kDa) and a transmembrane beta-chain subunit (145 kDa), which are linked by a disulfide bond to form the mature receptor [60] (Fig. 1B). The extracellular portion of c-Met containing Sema domain (homologous to semaphorins), a cysteine-rich Met-related-sequence (MRS) domain, and four immunoglobulin-like structure (IgG) domain is responsible for binding to HGF, whereas

intracellular portion of c-Met which is composed of a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal regulatory tail [61] is responsible for signal transduction. The juxtamembrane domain is important for downregulation of receptor. Because the phosphorylation of a serine residue (Ser 985) in this domain inhibits the tyrosine kinase activity of c-Met [62]. Furthermore, phosphorylation of a tyrosine residue (Tyr 1003) is responsible for polyubiquitination and degradation of the receptor [63] by recruiting c-CBL (casitas B-lineage lymphoma) [64, 65].

HGF is a multifuncional factor [66-68] expressed only by mesenchymal origin cells [69], while c-Met is expressed mainly by epithelial cells [69], also various other cell types including vascular endothelial cells [70], lymphatic endothelial cells [71], neural cells [72], hepatocytes [73], hematopoietic cells [74], and pericytes [75]. Studies show that, activated HGF and upregulated c-Met expression correlate with negative prognosis in human cancers [76-81].

c-Met mediated downstream signalling is activated by binding of ligand HGF to c-Met. C-Met-HGF binding results in receptor homodimerization and phosphorylation of two tyrosine residues (Y1234 and Y1235) located within the catalytic loop of the tyrosine kinase domain [82] and subsequently, phosphorylation of tyrosines 1349 and 1356 in the carboxy-terminal tail. When these tyrosines are phosphorylated, they recruit signalling effectors and activate multiple signal transduction pathways including the Src/focal adhesion kinase (FAK) pathway, the p120/signal transducer and activator of transcription (STAT) 3 pathway, the phosphoinositide-3 kinase (PI3K)/Akt pathway, and the Ras/MEK pathway [83,84].

Src/FAK pathway regulates cell adhesion, anchorage-independent growth and migration [85-87], while p120/STAT3 pathway stimulates branching morphogenesis of cells [85-87]. PI3K/Akt pathway activates cell motility and cell survival [85-88], Ras/MEK pathway mediates HGF-induced cell scattering, cell proliferation and cell cycle progresion [85-87].

Negative regulation of c-Met mediated-pathways is crucial for its tightly controlled activity. This is conducted by either dephosphorylation of the tyrosines in the c-MET kinase domain by various protein tyrosine phosphatases (PTPs) or PLCγ-mediated-PKC

activation. Independently, an increase in intracellular calcium levels can also lead to negative c-MET regulation [89].

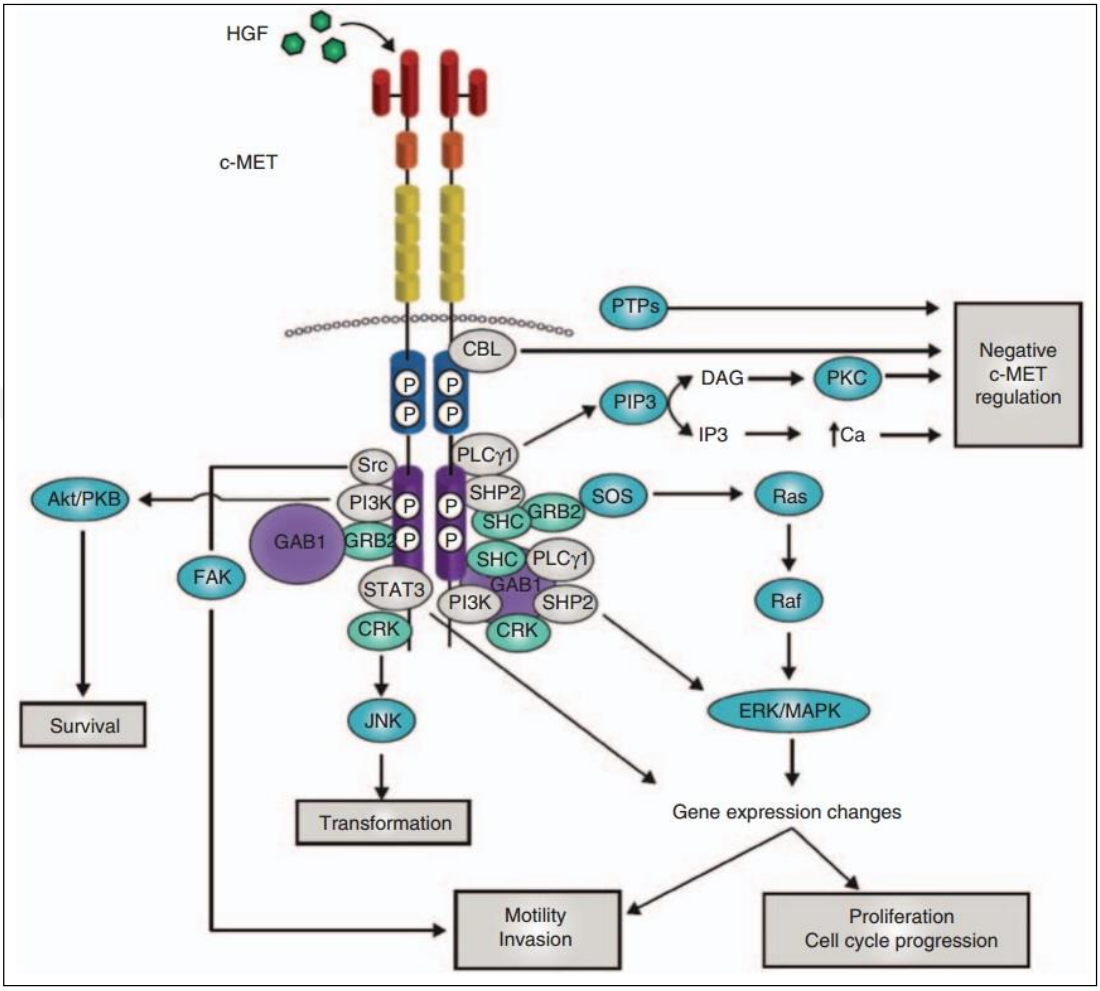

Figure 1.6 HGF-cMET pathway [50]

1.4.3. Programmed cell death (PCD) pathway: apoptosis, autophagy and programmed necrosis

1.4.3.1. Apoptosis pathway

Apoptosis is a type of PCD, which was characterized by specific morphological changes of dying cells, including 1) cell shrinkage, 2) nuclear condensation and fragmentation, 3)

dynamic membrane blebbing, 4) loss of adhesion to neighbors or to extracellular matrix and additional specific biochemical changes including 1) chromosomal DNA cleavage into inter-nucleosomal fragments, 2) phosphatidylserine externalization and 3) a number of intracellular substrate cleavages by specific proteolysis [90, 91].

Apoptosis can be induced by either extrinsic (or death receptor) pathway or intrinsic (or mitochondrial) pathway [92]. The extrinsic pathway is activated by binding of Fas plasma membrane death receptor (or tumor necrosis receptor-1 and its relatives) with Fas-L extracellular ligand. When the Fas/Fas-L complex occurs, it recruits death domain proteins containing FADD and pro-caspase-8 to produce death-inducing signaling complex (DISC). DISC activates pro-caspase-8 which proceeds to activate pro-caspase-3, the penultimate enzyme for execution of the apoptotic procedure [93].

On the other hand, the intrinsic pathway is under the control of mitochondrial pro-enzymes and Bcl-2 family members including pro-apoptotic members such as Bax, Bak, Bad, Bcl-XS, Bid, Bik, Bim and Hrk, plus further anti-apoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1 [94]. The reduced-expression of Bcl-2 is associated with apoptotic response against anticancer drugs while over-expression of Bcl-2 leads to resistance to chemotherapeutics and radiation therapy. Following a death signal, pro-apoptotic proteins undergo posttranslational modifications and translocate to mitochondria [95]. By the contribution of Bax and Bak, outer mitochondrial membranes become permeable to internal cytochrome-c and release it into cytosol. Cytochrome-c interact with Apaf-1 and pro-caspase-9 to produce apoptosome, leading to activation of caspase-9 and caspase-3 cascade, subsequently generating apoptosis [96].

NF- κB is a class of protein activated by degradation of IκB and plays a role in transcriptional regulation of stress responses, cell proliferation, differentiation, extrinsic apoptosis and tumorigenesis [97-98]. The nuclear transcription factor, p53 is an important pro-apoptotic factor and tumor-suppressor promoting extrinsic apoptotic cell death by activating a number of positive regulators of apoptosis such as DR-5 and Bax [100,101].

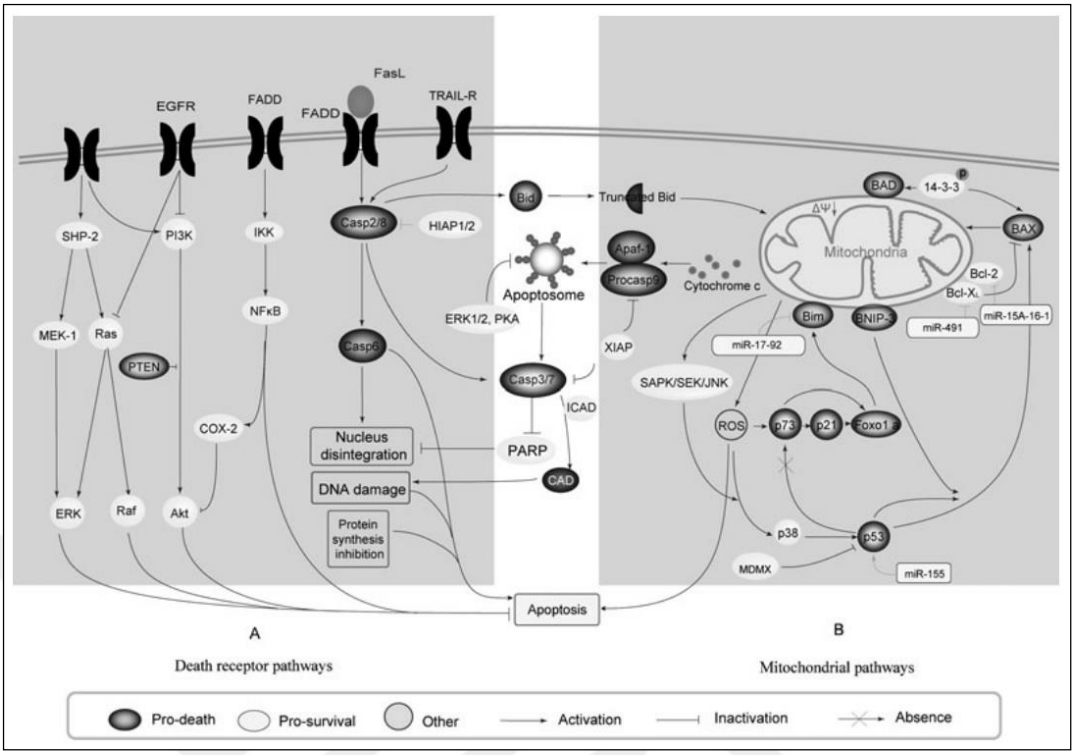

Figure 1.7 Apoptosis pathway: a. Extrinsic (Death receptor) pathway, b. Intrinsic (Mitochondrial) pathway [99]

1.4.3.2 Autophagy pathway

Autophagy is a second type of PCD that is a catabolic control process including starvation, cell differentiation, cell survival and death [102]. This evolutionarily conserved process begins with the formation of autophagosomes, subsequently double membrane-bound structures surrounds cytoplasmic macromolecules and organelles and destines recycling [103-105]. The number of researches indicate that autophagic activation can act as activator for tumor progression by regulating a number of pathways involving Beclin-1, Bcl-2, Class III and I PI3K, mTORC1/C2 and p53 [105] on the other hand, as a tumour suppressor by activating pro-autophagic genes and blocking anti-autophagic genes in oncogenesis [106].

PI3K/Akt pathway plays a role in mTORC1-inhibited autophagy in malignant cells. PI3KCI provoke autophagy by activation of Akt and subsequently mTORC1 [107].

Furthermore, Ras-Raf-MAPK pathway also plays role in inhibition of autophagy by pX-mediated activation of acceleration entry of cells into S phase of the cell cycle [108, 109], moreover, induction of apoptosis by specific mediators such as JNK, SAPK, 14-3-3 and NF-κB (110) (Figure 1.8).

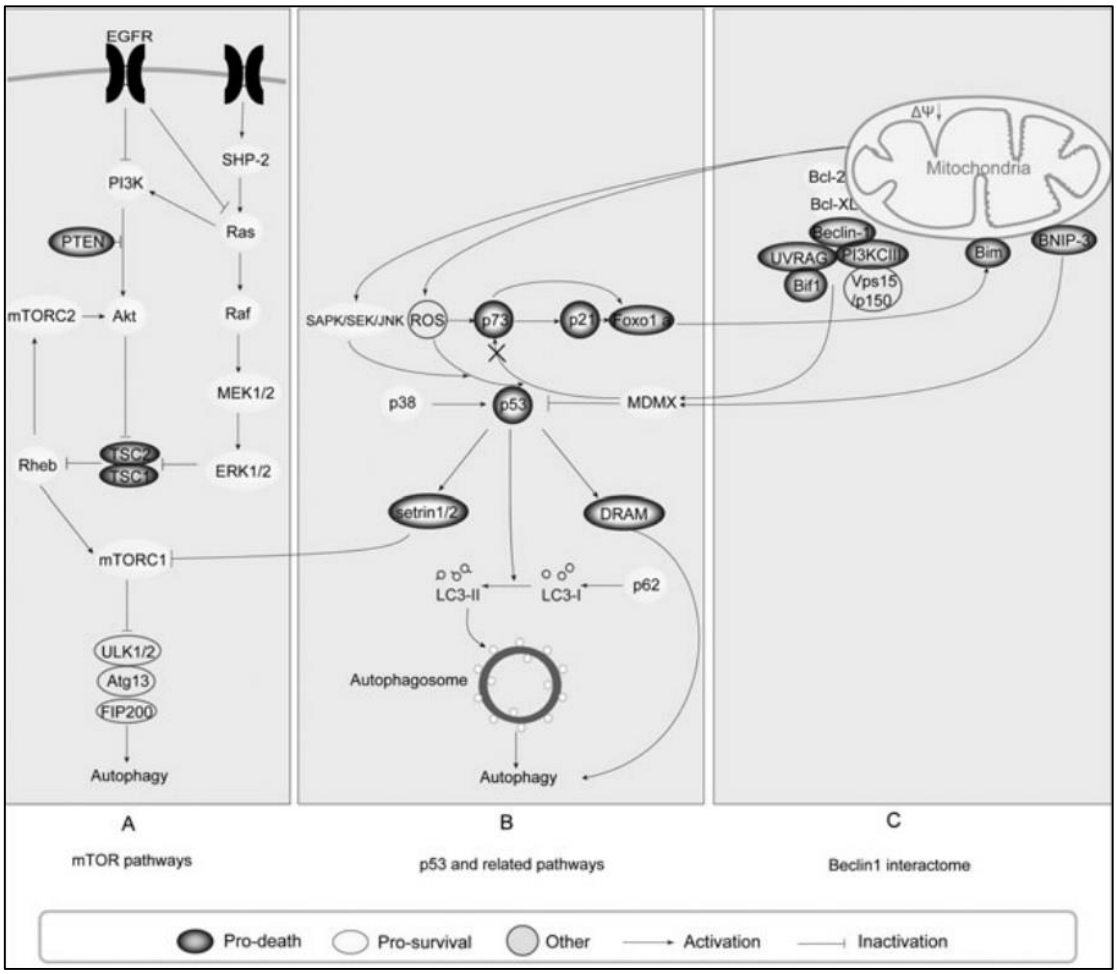

Figure 1.8 Autophagy pathway [99]

1.4.3.3 Programmed necrosis pathway

Necrosis has always been considered to be almost ‘accidental’ cell death, a random, uncontrolled process. Thanks to the discovery of key mediators of necrotic death such as receptor interacting protein (RIP) kinases, poly(ADP-ribose) polymerase-1(PARP1),

NADPH oxidases and calpains, the concept of programmed necrosis has recently been gaining ground [111-112]. Programmed necrosis is characterized by cell swelling, organelle dysfunction and cell lysis [113-115] and plays an important role on tissue homeostasis and elimination of damaged cells [116]. When cells undergo cell death, integrity of the cell membrane is disrupted, so that intracellular materials are released into the extracellular milieu, leading to inflammatory responses by immune cells and consequently, promoting tumor growth.

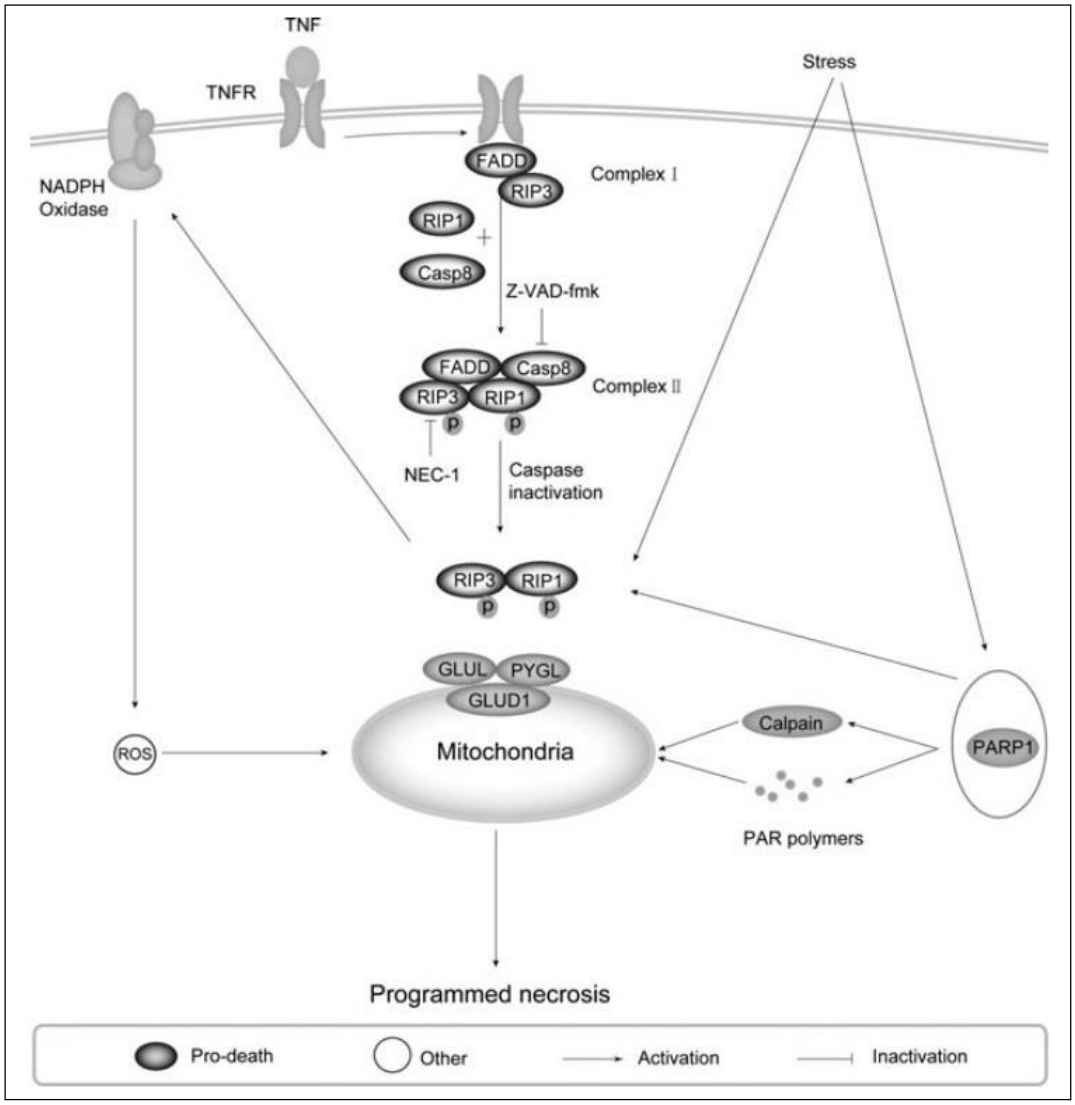

Figure 1.9 Programmed necrosis pathway [99]

1.5. BORON

Element boron has atomic number 5 and is classified as a metalloid [117]. It was first recognized as an essential mineral in vascular plants in 1923 and thanks to this investigation multiple researches were conducted to understand the functions of boron in plant life [118]. Plants need boron for pollination [119], cell wall stability [120], crop size modulation [118] and regulation of multiple enzyme activity [121]. Since the abundance of boron derivatives in food such as in fruits and seeds, boron deficiency is seen in population very rare [117]. Therefore, importance of boron is recognized in animals much later [122].

The first cellular boron transporter called *bor1-1* was identified in *Arabidopsis thaliana* by observation of *bor1-1* mutated plants under normal boron concentration [123]. The experiments revealed that boron has high affinity to *bor1-1* transporter and a mutation on this protein causing low boron transport into the cells, and thereby, slow growth, reduced expansion of rosette leaves, reduced fertility and loss of apical dominance [124]. Subsequent cloning and identification studies indicated that a homolog boron transporter protein exists in animal cells named BTR1 which is a member of SLC4 $Na^{+}HCO^{-}$ co-transporter family. Recent studies revealed that, BTR1 has a role on borate transport and with this way cell growth and proliferation. Therefore, this protein is defined as a unique Na^{+-} coupled-borate transporter and renamed as NaBC1 [125]. It acts as a transporter for Na^{+} and H^{+} in the absence of borate. However, in the presence of borate, it acts as a selective electrogenic Na^{+}-$B(OH)_4^{-}$ co-transporter regulated by voltage and conducts bilateral borate flux [126] (Figure 1.9)

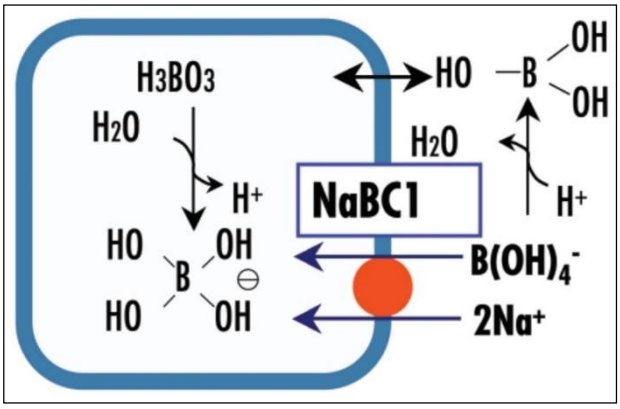

Figure 1.10 Possible role of NaBC1 in cellular borate transport and homeostasis [126]

Boric acid has a unique property as a strong Levis acid. Most phenylboronic acids have a pKa in the range of 4.5–8.8 [127] in another words they are electrophiles depending upon the phenyl substitution [128,129]. Under appropriate physiological conditions, trigonal boron atom accepts a pair of electrons from a nucleophile and converts its neutral-trigonal planar sp2 boron structure to an anionic tetrahedral sp3 boron structure. The process of enzyme inhibition by boric acid requires this easy conversion from trigonal to a tetrahedral form [117].

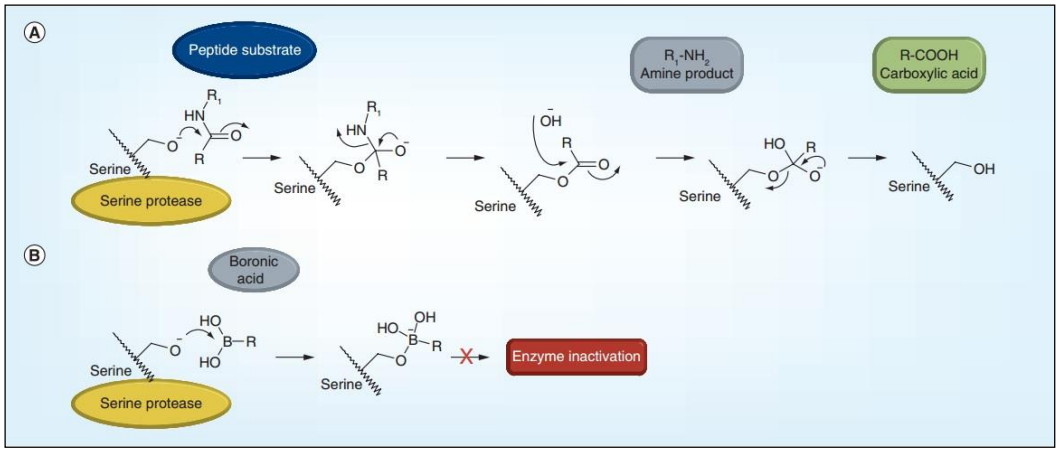

Figure 1.11. a. Possible mechanism of action of boron-based compounds for enzymatic inhibition b. Boronic acid forms a transition state analog thereby causing inactivation of enzyme [117]

Boron is present in tissue and bodily fluids as boric acid or borate anion and vary from tissue to tissue according to their function. Boron content in human body is estimated between 3-20mg and the highest concentration is in bones, nail and hairs with 4.3-17.9 ppm, whereas 0.06 ppm in blood, 0.02 ppm in plasma, 0.75 ppm in urine. Furthermore, differences in boron content were also found depending on the health of the individual; for example boron was 3 ppm in arthritic bones relative to 56 ppm in healthy bones [130].

There are more evidence indicating that boron intake effects cancer progression [131, 132].

Prostate cancer is one of the most common cancer type and its incidence is decreased by regulation of steroid hormones, anti-cancer metabolites and cell proliferation via increased

boron intake. Studies revealed that boric acid reduces levels of immunoglobuline F (IgF) in tissue and prostate specific antigen (PSA) in plasma playing a key role in expression of steroid hormones via progression of prostate cancer [133].

Furthermore, boric acid inhibit the growth and metastasis of prostate cancer through decreased expression of A, B1, C, D1 and E cyclin proteins and increased Ca(II) storage by NAD+ cADPR system [134, 135]. Low dietary of boron is also associated with lung cancer [136]. Studies showed that the nutrition of boron compounds have antioxidant and anti-inflammatory effects correlated with low estrogen level [137]. Herewith, boron intake decreases the lung cancer risk via mimicing the 17β-estradiol-based hormone therapy and increasing estrogen expression even in smokers [138]

Boric acid is one of the most studied boron derivative that plays role as an inhibitor of peptidase, proteases, proteasomes, arginase, nitric oxide synthase and transpeptidase by binding to OH groups from NAD and serine [130, 136, 139, 140].

The prostatic serum antigen (PSA) is a serine protease and a putative target for BA effect on proliferation androgen-sensitive-cancer cells such as (LNCaP). The results affirmed that boric acid leads to 25-38 per cent decrease in tumor growth via 88 per cent reduction of PSA levels [133].

Furthermore, boric acid has also been found to have inhibitory effect on androgen-independent cell lines such as DU-145 and PC-31, suggesting that another serine protease-independent mechanisms exist [139]. It is shown that boric acid controls the cell cycle and proliferation of DU-145 via inhibition of agonist stimulated release of Ca(II) from ryanodine receptor sensitive cell stores [141] Additionally, high dose of boric acid induces apoptosis in both melanoma and MDA231 breast cancer cells by slowing down the cell replication [142]

Boronic acid is another types of boric acid and plays a role on selectively inhibition of the migration and cell viability [143]. The well-known cancerogenic drug, Bortezomib (Velcade®, PS-341) is a dipeptide boronic acid derivative containing pyrazinoic acid, phenylalanine and leucine with boronic acid [117]. This drug is known to plays a role as a proteasome inhibitor (a novel target in cancer therapy), disrupts the regulation of cell cycle and induces apoptosis [144-145]. Bortezomib has been reported to inhibit nuclear factor-kB, to induce cell cycle blockade and apoptosis *in vitro*, as well as tumor growth inhibition

in vivo [146]. Moreover, intracellular calcium metabolism deregulation, which causes caspase activation and apoptosis, is also responsible for the anticancer activity of bortezomib [147].

It is shown that bortezomib has strongly cytotoxic effect in both hematologic and solid tumor malignancies, including prostate cancer cells, MCF-7 and EMT-6 breast carcinoma cells [148], myeloma [149], mantle cell lymphoma [150], cell lung cancer [151], ovarian cancer [152], pancreatic cancer [153, 154], prostate cancer [152, 155], and head and neck cancers [156].

However, limitations of bortezomib, including limited activity in solid tumors [157], side effects such as the emergence of reversible peripheral neuropathy [158] and the invasive intravenous route of administration, have prompted researchers to develop various second generation proteasome inhibitors for anticancer activity [159].

Neutron capture therapy (BNCT) is another field that boron-based compounds are used for anti-carcinogenic therapy. Basically, boron is irradiated with low energy (0.0025V) thermal neutrons and turn into high linear energy transfer alpha particles (He) and recoiling Li nuclei. This high linear energy transfer particles have ability to move just in limited distances in tissue (5–9 μm), with this way the destructive effects of these high-energy particles is limited to boron-containing cells, providing a way to selectively destroy malignant cells except adjacent normal cells. This therapy is mainly used for high grade gliomas, recurrent head and neck tumors, melanomas and hepatic metastasis (117).

In the light of these accumulating evidence, this study is designed to investigate the effect of boron derivatives in cell proliferation, cell migration and cell death in renal cell carcinoma (RCC). In order to demonstrate the effect of NaB, BA and B on cell proliferation, cell migration and cell death in RCC, *in vitro* model primary A-498 and metastatic ACHN RCC cell lines were used. By testing this, it is expected that NaB and BA might be used instead of B as a therapeutic agent in the treatment of cancer disease.

2. MATERIALS

2.1. INSTRUMENTS

Inverted Phase Contrast Microscope (Carl Zeiss Primo Vert)

Laminar Flow Cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A)

CO_2 Mammalian Incubator (New Brunswick Galaxy 170S)

Centrifuge (Hettich Mikro 22R, Germany and SIGMA 2-5 centrifuge)

pH meter (Hanna Instruments PH211)

Vortex (Stuart SA8)

-80 ^oC freezer (Thermo Forma -86C ULT Frezer)

Real Time PCR (Bio-Rad CFX96 Real Time System)

Flow Cytometer (BD Bioscience, BD FACSCalibur)

Water Bath (Stuart, Shaking Water Bath SBS40)

96well Plate Reader (BIOTEK ELx800)

NanoDrop (ThermoScientific NanoDrop 2000 Spectrophotometer)

Hemocytometer (Neubauer Chamber)

ELISA plate reader (BioTek Instruments, Inc., VT, USA).

iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA).

2.2. EQUIPMENTS

Cell Culture Flask T175-T75-T25 (Corning)

Cell Culture Plates 6-well, 24-well (TPP)

Cryovials (TPP)

Centrifuge Tubes 50-15 ml (Isolab)

Centrifuge Eppendorfs 2-1,5 ml (Isolab)

Micro pipettes 1000-200-10µl (Eppendorf Research Plus)

Serological Pipettes 50-25-10-5ml (Biofil)

Automatic Pipette (Accupette)

Tips (ExpellPlus)

Sterile Disposable Filter units 0.02µm (TRP Spritzen)

Sterile Disposable Syringe 10mL (Hayat Syringe, Istanbul/Turkey)

Isopropanol freezing container (Nalgene Cryo 1°C Freezing Container, 5100-0001)

Flow cytometry tubes (BD Falcon 5ml Plystrene Round Bottom Tube, ref. 352054)

2.3. CHEMICALS

2.3.1. Cell Culture Media

Dulbecco's Modified Eagle's Medium, High glucose (DMEM) (Gibco 41965-062)

Renal Epithelial Basal Medium (ATCC PCS-400-030)

2.3.2. Growth Supplements and Chemicals

Fetal Bovine Serum (FBS) (Gibco, 10270-098)

Renal Epithelial Cell Growth Kit (ATCC, PCS-400-040)

2- Propanol (AppliChem, A3928)

Absolute Ethanol (AppliChem, A3928)

Dulbecco's Phosphate Buffer Saline (DPBS) (PAN Biotech, P04-36500)

Penicillin- Streptomycin (Pen/Strep) (Gibco, 15070063)

Triton-X100 (Biomatik Corporation)

Trypsin-EDTA: 0.025 per cent Trypsin and 0.01 per cent EDTA in Phosphate Buffered Saline (PBS) (Gibco, R-001-100)

Sodium pentaborate pentahydrate (National Boron Research Institute-BOREN, Ankara, Turkey)

Boric acid (Biobasic, 10043-35-3)

Bortezomib (Velcade, Millenium, USA)

Propidium Iodide (Sigma Aldrich, P4170)

2.4. KITS AND SOLUTIONS

CellTiter96 AQueousOne Solution (Promega, Southampton, UK)

High Pure RNA Isolation Kit (Roche, Germany)

High Fidelity cDNA Synthesis Kit (Roche, Germany)

Maxima SYBR Green/ROX qPCR Master Mix (2X)

Annexin V Apoptosis Detection Kit (Santacruz Biotechnology, Inc., sc-4252AK)

2.5. CELL LINES

A498, Primary Kidney Epithelial Carcinoma, Human, Adherent (ATCC Number: HTB-44)

ACHN, Metastatic Renal Cell Adenocarcinoma, Human, Adherent (ATCC Number: CRL-1611)

RPTEC, Primary Renal Proximal Tubules Epithelial Cells, Normal, Human, Adherent (ATCC Number: PCS-400-010)

3. METHODS

3.1. CELL CULTURING METHODS

3.1.1. Cells Characteristics and Culturing Conditions

A498 is an epithelial originated human kidney carcinoma derived from 52 years old female patient. The cell line contains a single VHL allele with a frame-shift mutation at codon 142. [160]

ACHN is human renal cell adenocarcinoma isolated from 22 year old male patient. It has highly metastatic characteristics due to derive from the fluid that accumulates in the pleural cavity.

RPTEC is human primary epithelial cell derived from renal proximal tubules. It has a cubodial structure with a characteristic patter of swirled cells. These primary cells do not cause a disease in healthy adult humans.

All cell lines were purchased from ATCC. Cells passages of ACHN and A498 were performed every 2-3 days as the confluency reaches to 85 per cent and attended to the experiments until passage 30. However, cell passage of RPTEC was performed every 4-5 days as the confluency reaches to 85 per cent and attended to the experiments until passage

8. ACHN and A498 cell were cultured in DMEM completed with 10 per cent FBS and 1 per cent PSA whereas RPTEC was cultured in Renal Epithelial Cell Basal Medium completed with Renal Epithelial Cell Growth Kit and 1 per centPSA (RECM). All cells were cultured periodically at 37^{o}C, 5 per cent (v/v) CO_2 and 95 per cent (v/v) air.

3.1.2. Cell Passaging

Medium was discarded and cell monolayer was firstly washed gently with Dulbecco's Phosphate Buffer Saline (DPBS), pH=7.4 and then treated with trypsin-EDTA solution pH=7.4 for 2-3 min in 37^{o}C mammalian incubator. In order to block trypsin activity, cells were collected in completed medium equal to the volume of trypsin added and centrifuged 300xg for 5min. Supernatant was discarded and pellet was re-suspended with complete media in order to get the intended dilution.

3.1.3. Determination of Cell Number

Before the centrifugation (Section 3.1.2) 10µl aliquots of the cell suspension were placed in hemocytometer. Cells in the four corners squares of area were counted by using inverted microscope, then average number of the cells was calculated. Cell number per milliliter is calculated according to the formula "(average of counted number of cells x dilution factor) / (area of 1 square x chamber depth).

3.1.4. Cell Freezing

Freezing medium is prepared by mixing 5 per cent (v/v) Dimethyl sulfoxide (DMSO) and 95 per cent completed medium according to the ATCC's freezing protocol. Following the centrifugation (Section 3.1.2) cell pellets were re-suspended in freezing mix 1 x 10^6 cells/ml per vial to be frozen. Vials were then aliquoted and placed on isopropanol freezing container and taken immediately to -80^{o}C. The isopropanol freezing container helps to

decrease temperature 1 ^{o}C per min. Therefore, at least 4h to overnight incubation at -80^{o}C is required before the vials are transferred into N_2 tank for longer storage.

3.1.5. Cell Thawing

Cryovial containing the frozen cells is taken out from the liquid nitrogen storage or – 80^{o}C. Cells were immediately thawed by gently swirling the vial at 37°C. 5 ml of pre-warmed complete growth medium was placed into 15 ml Falcon and cell suspension was then transferred onto the suspension slowly. Cell suspension was centrifuged at 300xg for 5 min. Supernatant was decanted without disturbing the cell pellet and cells were re-suspended with complete growth medium and transferred into the appropriate culture vessel. In the following day, media was changed for the removal of remaining DMSO. Cells were passaged when they reached to 85 per cent confluency.

3.1.6. Preparations of Medias Including Boron Derivatives

Sodium pentaborate pentahydrate (NaB) or boric acid (BA) stock was prepared as 10mg/ml in appropriate complete media for each cell, freshly. The stock then diluted with complete media in order to get the intended dilution.

Commercial Velcade (Bortezomib) (B) was aliquoted from Yeditepe University Hospital, Genetic Diagnostic Laboratory as 800μM dissolved in DPBS. The stock diluted with DPBS in order to get 100μM and then diluted with appropriate complete media for each cell in order to get intended dilution.

3.2. MEASUREMENT OF CYTOTOXICITY OF BORON DERIVATIVES ON CELL LINES

Toxicity of NaB, BA and B were tested for each cell type by The CellTiter 96 AQueous One Solution Cell Proliferation Assay according to the manufacturer's instructions. This

assay is a colorimetric method which determines the number of viable cells in proliferation or cytotoxicity assay. The assay solution is prepared in laboratory by mixing a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] with an electron coupling reagent (phenazine methosulfate; PMS) according to the manufacturer's instructions. Basically, MTS tetrazolium compound is bioreduced by dehydrogenase enzymes in metabolically active cells into a colored formazan product which is soluble in tissue culture medium. In this manner, PMS plays a role to enhance the chemical stability of MTS solution.

ACHN cells and RPTECs were seeded at a density of 10 000 cells/well, whereas A498 were seeded at a density of 7500 cells/well on a 96 well plate. The following day, cells were treated with different concentration of chemicals.

For A498 and ACHN cells, NaB and BA solutions were prepared with seven different concentrations such as 500 mg/ml, 1000 mg/ml, 1500 mg/ml, 2000 mg/ml, 2500 mg/ml, 3000 mg/ml and 3500 mg/ml and B solutions were prepared with seven separate concentrations such as 30 nM, 50 nM, 80 nM, 100 nM, 130 nM, 150 nM and 180 nM by dilution in DMEM.

However, for RPTEC, six different concentrations of NaB and BA solutions were prepared such as 500 mg/ml, 1000 mg/ml, 2500 mg/ml, 5000 mg/ml, 7500 mg/ml and 10 000 mg/ml, additionally, six different concentration of B were prepared such as 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1000 nM by dilution within RECM.

After incubating cells with defined range concentrations of NaB, BA, B for 24, 48 and 72 hours, medium in the wells was discarded, then 10 µl MTS solution and 100 µl DMEM mix was applied to cells for two hours. Absorbance at the end of two hours was recorded at 490nm with a 96-well plate reader.

3.3. ANNEXIN-V APOPTOSIS ASSAY

Apoptosis is a normal physiologic process of programmed cell death to remove unwanted cells. When the process is triggered, apoptotic cells undergo rapid morphological changes

that indicate the progression of cell death such as condensation of the cytoplasm and nucleus and inter-nucleosomal cleavage of DNA. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine (PS) from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by utilizing the preferentially binding properties of AnnexinV, a calcium dependent phospholipid binding protein, to negatively charged phospholipids including PS. Cells progressing through apoptosis can be monitored according to their AnnexinV and propidium iodide staining pattern. Early apoptotic cells bind to AnnexinV but are not sensitive to intracellular staining with propidium iodide (PI). As cells progress through apoptosis the integrity of the plasma membrane is lost, allowing PI to penetrate and label the cells with a strong yellow-red fluorescence (Figure3.1).

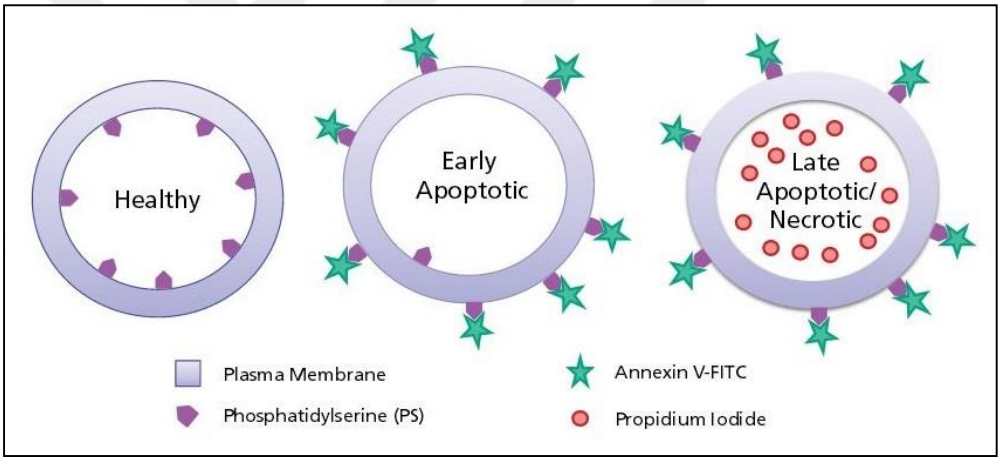

Figure 3.1. Diagram showing healthy and apoptotic cells with markers for detection of apoptosis

ACHN, A498 cells and RPTECs were seeded at a density of 250 000, 140 000 and 200 000 cells/T25 flask, respectively. The following day, cells were treated with defined IC50 and EC50 concentrations of chemicals for 3 days which were indicated in Table 3.1.

Table 3.1. IC50 concentrations of chemicals for ACHN cells, A498 cells and RPTECs; and EC50 concentrations of chemicals for RPTECs at day3

	ACHN (IC50)	A498 (IC50)	RPTEC (EC50)
NaB	1550mg/ml	1400mg/ml	1550mg/ml
BA	1550mg/ml	1400mg/ml	1550mg/ml
Bortezomib	100nM	20nM	100nM

Three days later, mediums were collected to falcon tubes, cells trypsinized (Section 3.1.2) and collected into the same tubes then centrifuged at 300g for 5 min. Supernatants were discarded, pellet was washed ones with cold DPBS and then re-suspended in 1X assay buffer at a concentration of $1x10^6$ cells/ml. 100 µl of cells transferred to four 1,5 ml eppendorf tubes and added 2,5 µl (0,5 µg) of Annexin V FITC and 10µl of propidium iodide per 100 µl of cell sample in such manner as no - Annexin V FITC/no - PI; Annexin V FITC alone; PI alone and Annexin V FITC/ PI. Cells were pipetted gently and incubated for 15 min at room temperature. After 15 min, 400 µl of 1X assay buffer added into each sample tube and analyzed immediately by flow cytometer using a double laser emitting light at 488nm with fluorescence channel FL1 for FITC and at 535 nm with fluorescence channel FL2 for PI.

3.4. CELL CYCLE ASSAY

Replication states of a cell population can be detected by utilizing the preferentially binding properties of propidium iodide (PI), an intercalating agent between bases and fluorescent molecule, to nucleic acids including DNA. Analysis of replication state is performed depending on fluorescence intensity, for example, cells in quiescence or G1 phase of the cell cycles which have one copy of DNA and therefore have 1X fluorescence intensity however cells in G2/M phase of the cell cycles which have two copies of DNA and therefore have 2X fluorescence intensity.

In this experiment, cells were cultured same as in Section 3.4. After cells were pelleted with 300g for 5 min, cells were fixed with 70 per cent ethanol at -20^{o}C for 1 hours,

permeabilized with 0.1 per cent triton-X-100 at room temperature for 20 min, and then incubated with RNase (0.3mg/ml) at 37ºC for 30min. Cells were washed ones with cold DPBS and centrifuged 300g for 5 min in every step interval. At the end, cells were stained with PI (5µg/ml) at room temperature for 5 min and then immediately analyzed in 15 min by flow cytometer using a single laser emitting light at 488nm with fluorescence channel FL2.

3.5. TOTAL RNA ISOLATION AND RELATIVE REAL-TIME PCR ANALYSES

Primers were designed by using Perlprimer software in our laboratory.

Table 3.2. Primers used in this study

Primer	**Sense (5'-3') *forward***	**Antisense (5'-3') *reverse***
Bcl2	AACGGAGGCTGGGATGCCTTTGTG	ACCAGGGCCAAACTGAGCAGAGT
NF-κB	GCCACCCGGCTTCAGAATGGC	TATGGGCCATCTGCTGTTGGCAG
c-MET	GGCTGGTGCCACGACAAATG	CATGGCAGGACCAACTGTGC
TORC1	GCTGGAGAAGGACCAATTCTG	TCTGCCTCTGATGTGGTCTC
DKK2	AGTGATAAGGAGTGTGAAGTTGG	GGATACAGATGCCATTATTGCAG
MMP2	CTTTGCAGGGAATGAATACTGG	GTCTCCAGCAAAGATGTATGTC
SLC4A11	CTCTACATCCAGGTGATTCGT	TCTTGAAGAGACTCATGACCA
STAT3	GAAGAATCCAACAACGGCAG	CAAGGAGTGGGTCTCTAGGT

STAT5a	GGAACATGTCACTGAAGAGGA	ACAACCACAGGTAGGGACAG
Caspase3	GAGGCGGTTGTAGAAGAGTTTCGTG	TGGGGGAAGAGGCAGGTGCA
TP53	ATTTGCGTGTGGAGTATTTGG	CCAGTAGATTACCACTGGAGTC
Bax	TGCAGAGGATGATTGCCGCCG	ACCCAACCACCCTGGTCTTGG
Rps13	TCGGCTTTACCCTATCGACGCAG	ACGTACTTGTGCAACACCATGTGA

Cells were cultured same as in Section 3.4. Total RNA isolation was performed after three days incubation of each cell line with three different chemicals individually used in this thesis using High Pure RNA Isolation Kit according to the manufacturer's instructions then immediately cDNA synthesis were done using High Fidelity cDNA Synthesis Kit. To determine the expression levels of genes (Table 3.2), cDNAs from the each groups were used as template and were mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2X) (Table 3.3). Real time PCR experiments were conducted using iCycler RT-PCR detection system (Table 3.4).

Table 3.3. Reagents used for RT-PCR

Reagents	**Volume**
SYBRGreen	5 µl
Primer Forward (10 µM)	0.3 µl
Primer Reverse (10 µM)	0.3 µl

Distilled water	2.9 µl
Template (500 ng/µl)	1.5 µl

Table 3.4. RT-PCR Protocol

Cycle 1	Step 1	50ºC	2 min
	Step 2	95ºC	15 min
Cycle 2 x40	Step 1	95ºC	15 sec
	Step 2	60ºC	1 min
	Step 3	72ºC	30 sec
Cycle 3	Step 1	95ºC	1 min
Cycle 4	Step 1	55 ºC	1 min
Cycle 5- Melt Curve	Step 1	55ºC -95ºC	10 sec/0.5ºC up

3.6. CELL MIGRATION ASSAY

A-498 cells and ACHN cells were seeded 150.000 and 400.000 cells/well respectively in 12-well plates in complete growth medium and incubated overnight at 37ºC. Following day, complete medium was replaced with serum free medium (SFM) and incubated for 16h in order to achieve quiescent state, and then scratched using a 100µl tip along one direction. This time point assumed as T=0h and cells were incubated in serum reduced medium (SFM: 2 per cent FBS (v/v)) (in order to limit the proliferation of cells) containing NaB, BA or B until closure of NC. ACHN cells were incubated for 36h and every 18h images were taken, on the other hand, A498 cells were incubated for 24h and images were taken for 8h and 24h. Three non-overlapping digital images covering the central area of each well were captured at 4X magnification using ECLIPSE TE200 fluorescence microscope.

3.7. STATISTICAL ANALYSIS

All data are shown as the means ± standard error (S.E.M.). GraphPad Prism 5 software was used to draw graphs and to perform the statistical analyses with student t-test. Statistical significance was determined at $p < 0.05$. All data's were arranged using Adobe Photoshop CS6 and Adobe Illustrator CS6.

4. RESULTS

4.1. CYTOTOXICITY of NaB, BA and B

Toxicity of NaB, BA and B were measured for ACHN, A498 and RPTEC cells using MTS assay at different concentrations for three days separately as mentioned before *(Method Section 3.2)* (Figure 4.1, Figure 4.3 and Figure 4.5).

The results showed that inhibition concentration of 50 per cent of population (IC50) of NaB and BA at 3th days were almost the same for A498 (1400µg/ml, Figure 4.2.a and Figure 4.2.b) and ACHN (1550µg/ml, Figure 4.4.a and Figure 4.4.b), however, were significantly higher for RPTEC (5000µg/ml, Figure 4.6.a and Figure 4.6.b).

Similar results were observed for B of which IC50 was nearly same for A498 (20nM, Figure 4.2.c) and ACHN (100nM, Figure 4.4.c), however, was notably higher for RPTEC (750nM, Figure 4.6.c).

For the subsequent experiments, their own IC50 concentrations of NaB, BA, and B used for ACHN and A498, however on RPTEC healthy cells, maximum IC50 concentration for cancer cells were used to analyze the effect of effective dose of NaB, BA and B (Table 4.1).

Table 4.1. Defined IC50 of NaB, BA and for further experiments on ACHN, A498 and RPTEC

	NaB	**BA**	**B**
ACHN	1550 µg/ml	1550 µg/ml	100 nM
A498	1400 µg/ml	1400 µg/ml	20 nM
RPTEC	1550 µg/ml	1550 µg/ml	100 nM

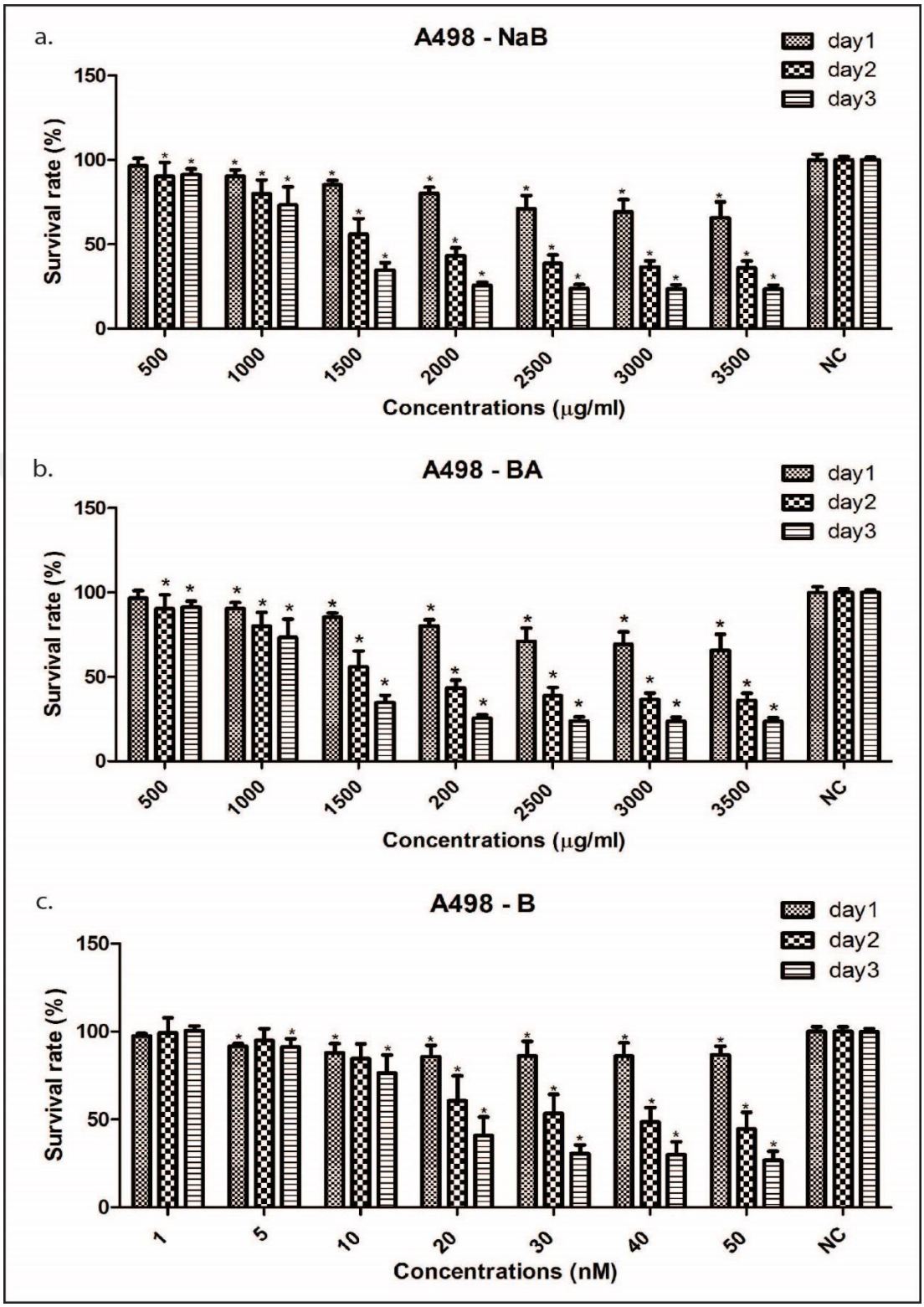

Figure 4.1. Survival rate (per cent) in NaB, BA, and B treated A498 cells by MTS assay at 24h, 48h and 72h, *$p<0.05$, Data compared with their corresponding controls

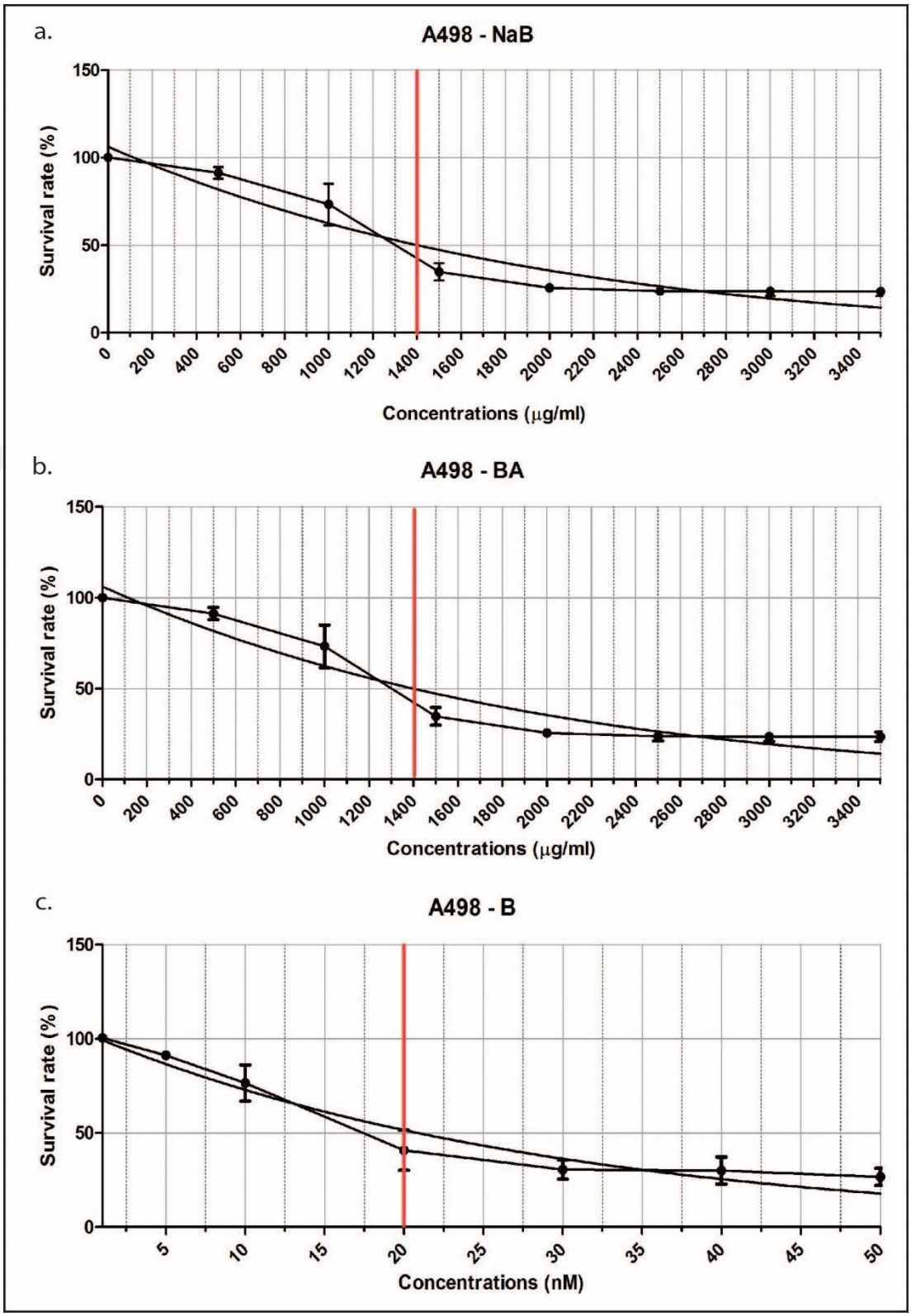

Figure 4.2. 50 per cent inhibition concentration of proliferation (IC50) in NaB, BA, and B treated A498 cells by MTS assay at 72h

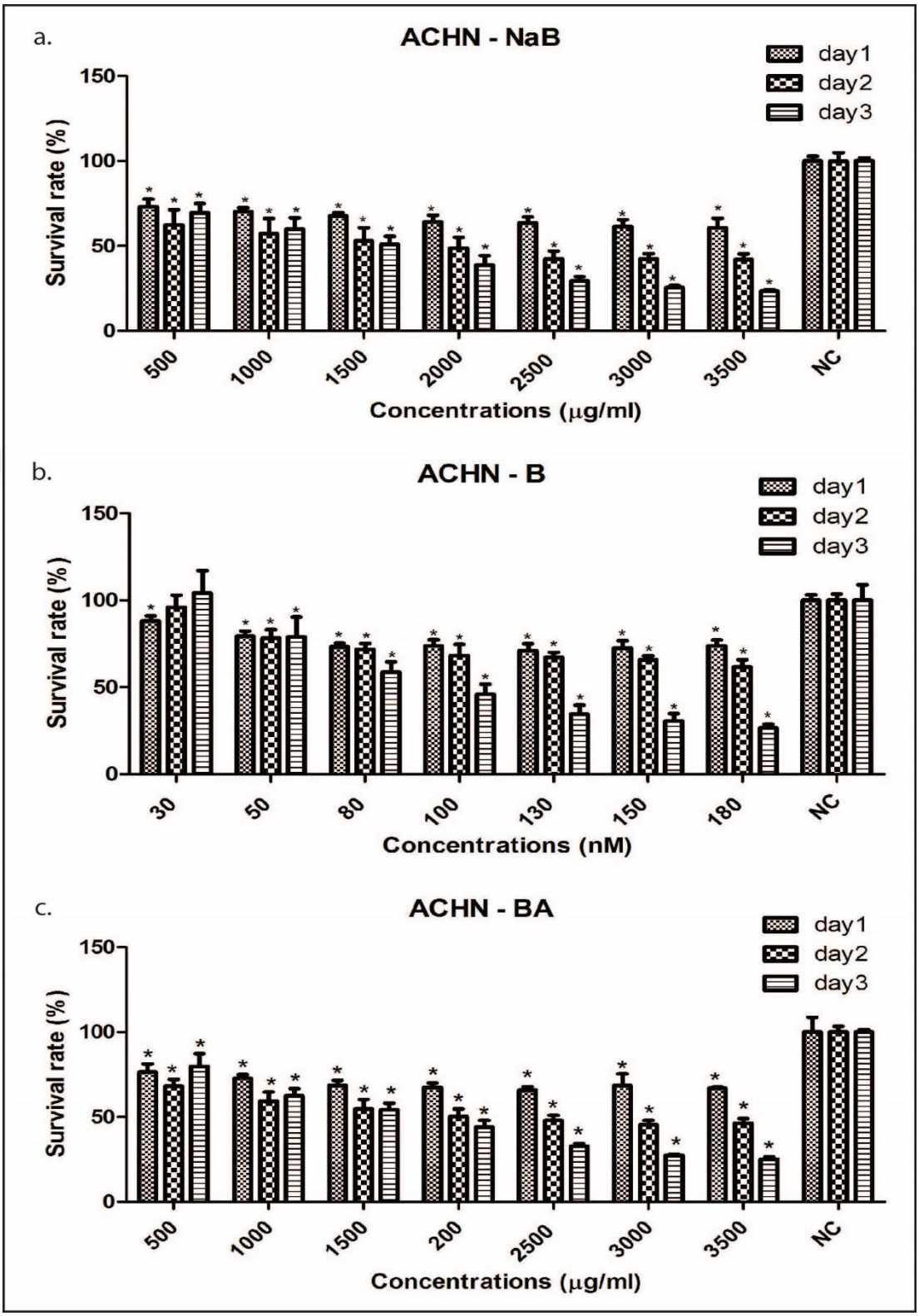

Figure 4.3. Survival rate (per cent) in NaB, BA, and B treated ACHN cells by MTS assay at 24h, 48h and 72h, *$p<0.05$, Data compared with their corresponding control

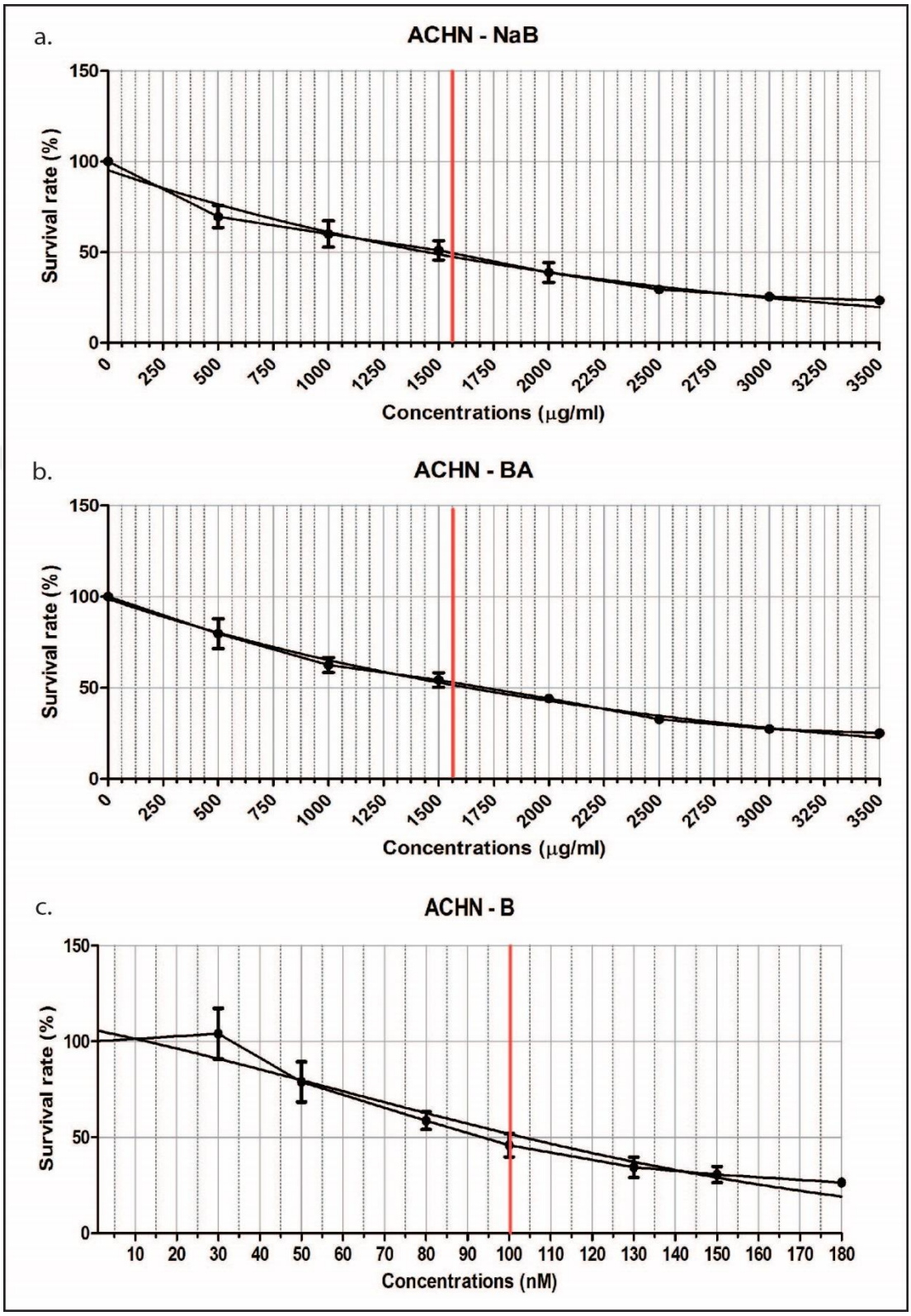

Figure 4.4. 50 per cent inhibition concentration of proliferation (IC50) in NaB, BA, and B treated ACHN cells by MTS assay at 72h

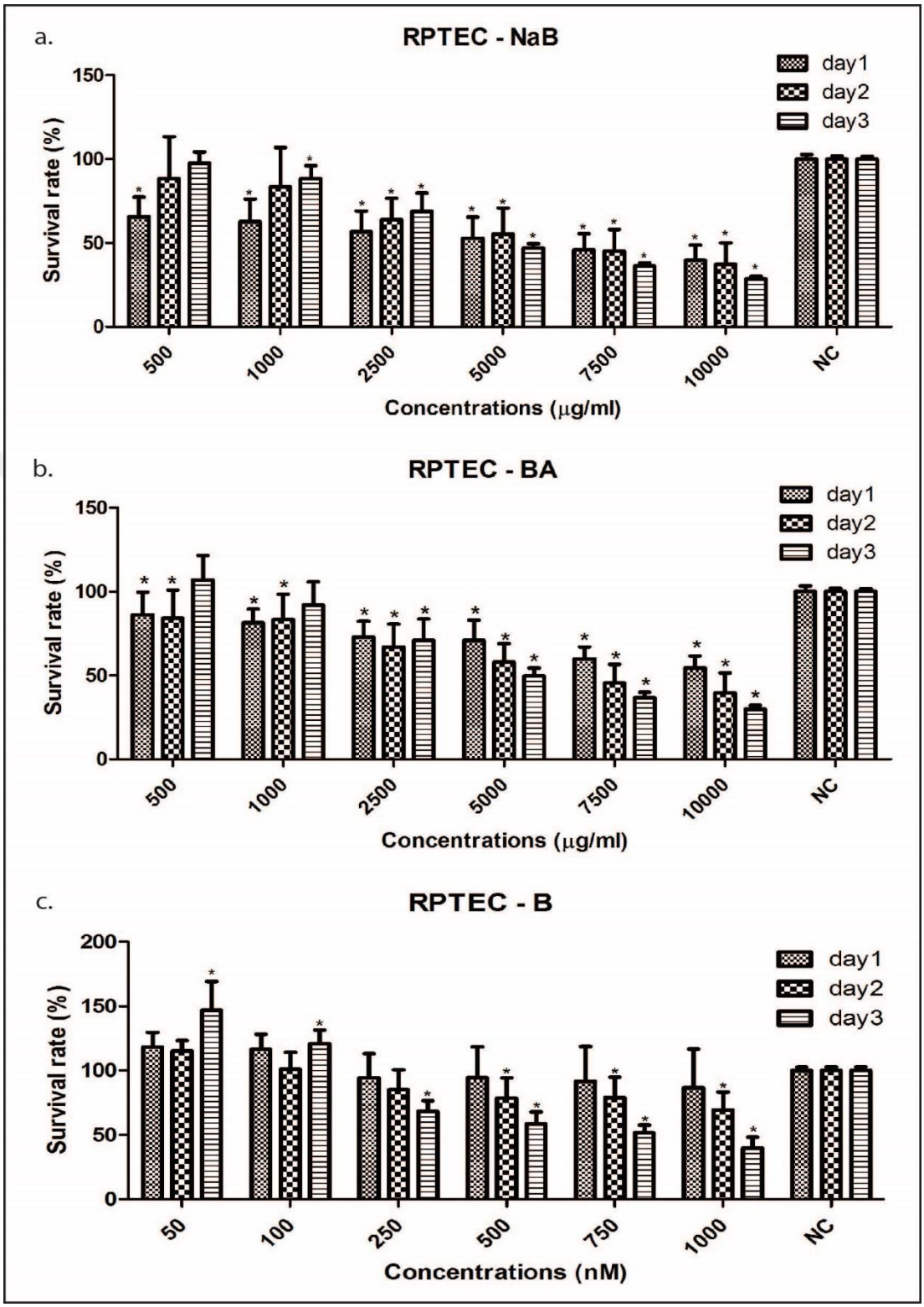

Figure 4.5. Survival rate (per cent) in NaB, BA, and B treated RPTEC cells by MTS assay at 24h, 48h and 72h, *$p<0.05$, Data compared with their corresponding controls

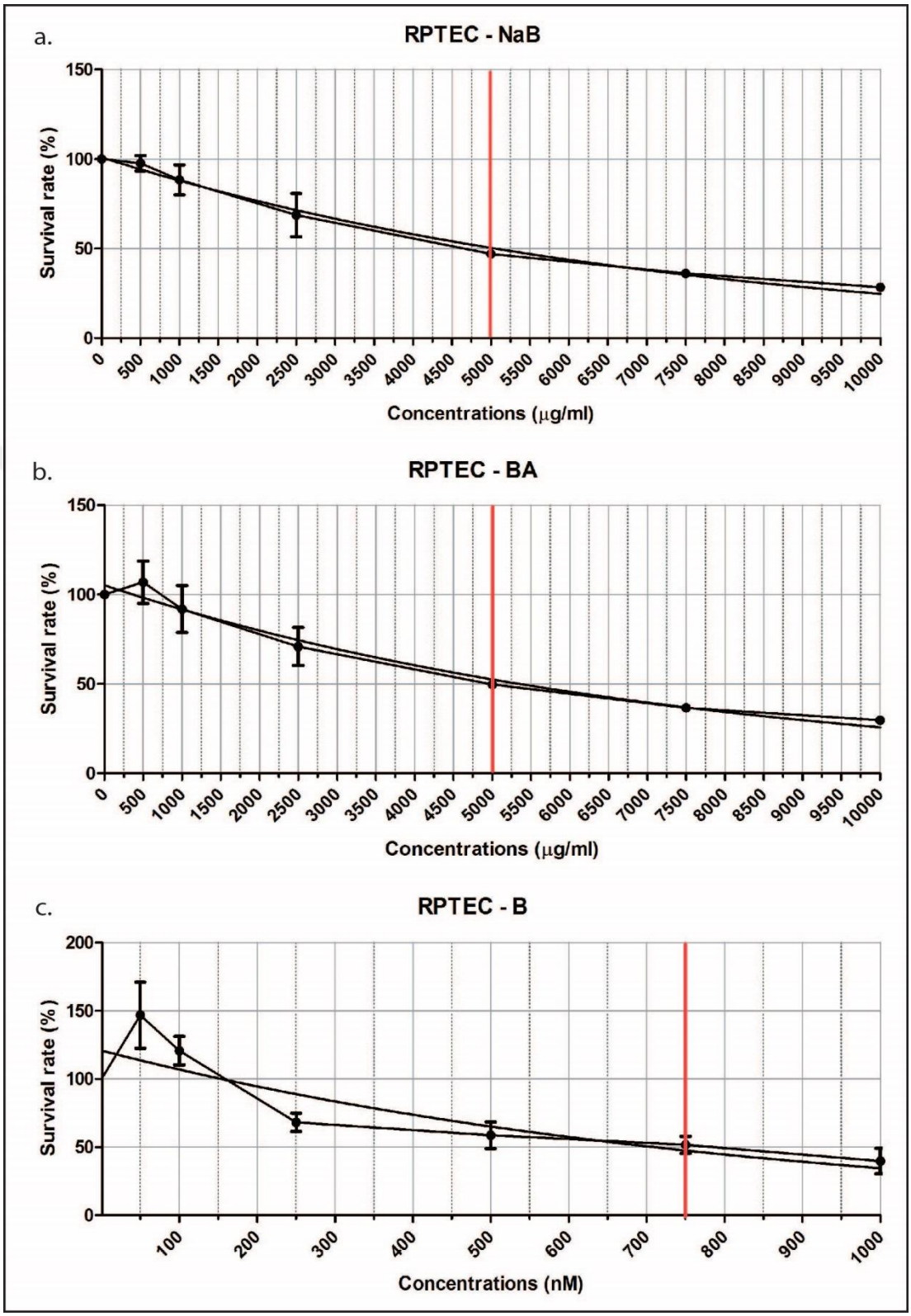

Figure 4.6. Fifty per cent inhibition concentration of proliferation (IC50) in NaB, BA, and B treated RPTECs by MTS assay at 72h

4.2. EFFECTS of NaB, BA and B on SLC4A11 BORON TRANSPORTER EXPRESSION

Solute carrier family 4, sodium borate transporter, member 11 (SLC4A11) is a member of sodium bicarbonate co-transporter family. This gene encodes a voltage-regulated, electrogenic sodium-coupled borate co-transporter which is essential for borate homeostasis, cell growth and cell proliferation.

To analyze the effect of boron chemicals on both cancer and healthy cell lines, we conducted real-time PCR analysis. All experiments were performed with three biological replicates each including three technical replicates.

Our results showed that both NaB and BA treatment increased the expression of *SLC4A11* on ACHN cells (159 per cent, per cent183 per cent, respectively), A498 cells (101 per cent, 77 per cent, respectively) and RPTEC cells (73 per cent, 72 per cent, respectively) (Figure 4.7). Interestingly, B treatment decreased *SLC4A11* expression on all cells to 72 per cent for ACHN cells, 84 per cent for A498 cells and 63 per cent for RPTEC cells. These results may indicate that NaB and BA operate through similar transport system to penetrate cells, however B use another path to enter the cells.

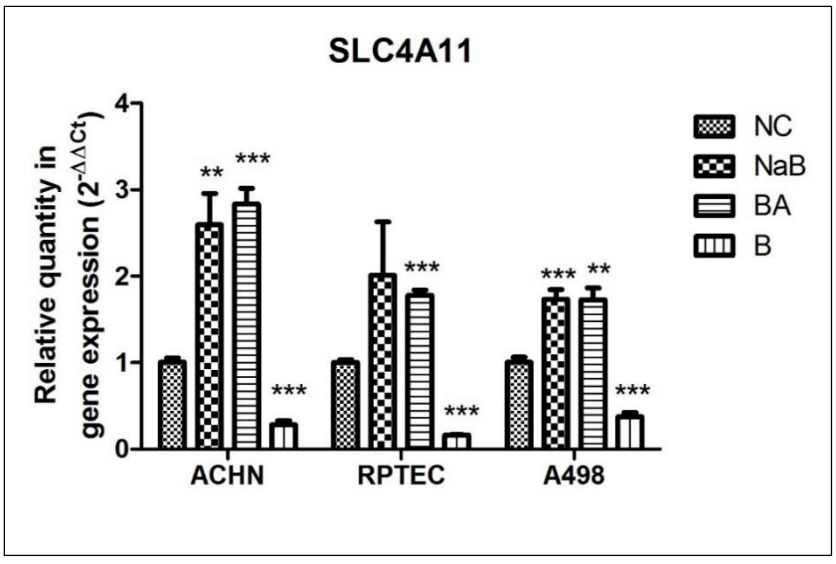

Figure 4.7. Real-time PCR results for *SLC4A11* * p<0.05 , ** p<0.01, *** p<0.001

4.3. EFFECTS of NaB, BA and B on HGF-cMET PATHWAY

As I mentioned in *introduction section 1.4.2*, it is known that c-MET is abnormally activated in human cancers. According to the literature, binding of c-MET to HGF receptor induces downstream pathways regulating proliferation, motility, migration and invasion. In this thesis, the effect of boron compounds was analysed on the regulation of c-MET-HGF pathway and its downstream pathway, metastatis through monitoring the expression of c-MET, STAT3 and STAT5a by using Real-time PCR. The assay was conducted with three biological replicates each including three technical replicates.

Results revealed that NaB, BA and B treatment induced expression of c-MET as 176 per cent, 223 per cent and 71 per cent and Stat3 in a ratio of 21 per cent, 29 per cent and 35 per cent in ACHN cell line (Figure 4.8). However, in ACHN cells, BA, and NAB treatment up-regulated Stat5a expression as 19 per cent and 56 per cent, respectively, whereas B treatment down-regulated its expression as per cent64 (Figure 4.8).

On the other hand, A498 cells responded to NaB treatment with up-regulation of c-MET as 48 per cent while expression of Stat3 and Stat5a did not change (Figure 4.8). Beside this, BA treatment did not alter the expression level of c-MET and Stat3, but significantly decreased the expression level of Stat5a to 46 per cent (Figure 4.8). Moreover, B only down-regulated the expression levels of c-MET and Stat5a to 31 per cent and 35 per cent, respectively. Though, Stat3 expression level was not altered by B treatment (Figure 4.8).

For RPTEC cell, any significant change was not observed on c-MET expression level by NaB, BA or B treatment (Figure 4.8). Although, Stat3 expression was significantly downregulated by NaB and BA treatment to 50 per cent and 64 per cent, respectively while Stat5a expression was reduced by B treatment to 88 per cent (Figure 4.8).

4.4. EFFECTS of NaB, BA and B on METASTASIS

Analyses of the effects of NaB, BA and B on cell migration were investigated by scratch assay as mentioned in the *Method Section 3.6*. For this experiment, defined IC50s of compounds were used for cell lines as mentioned in Results Section 4.1. This assay was

performed with two sample/biological replicates including two technical replicates for each sample per replicate.

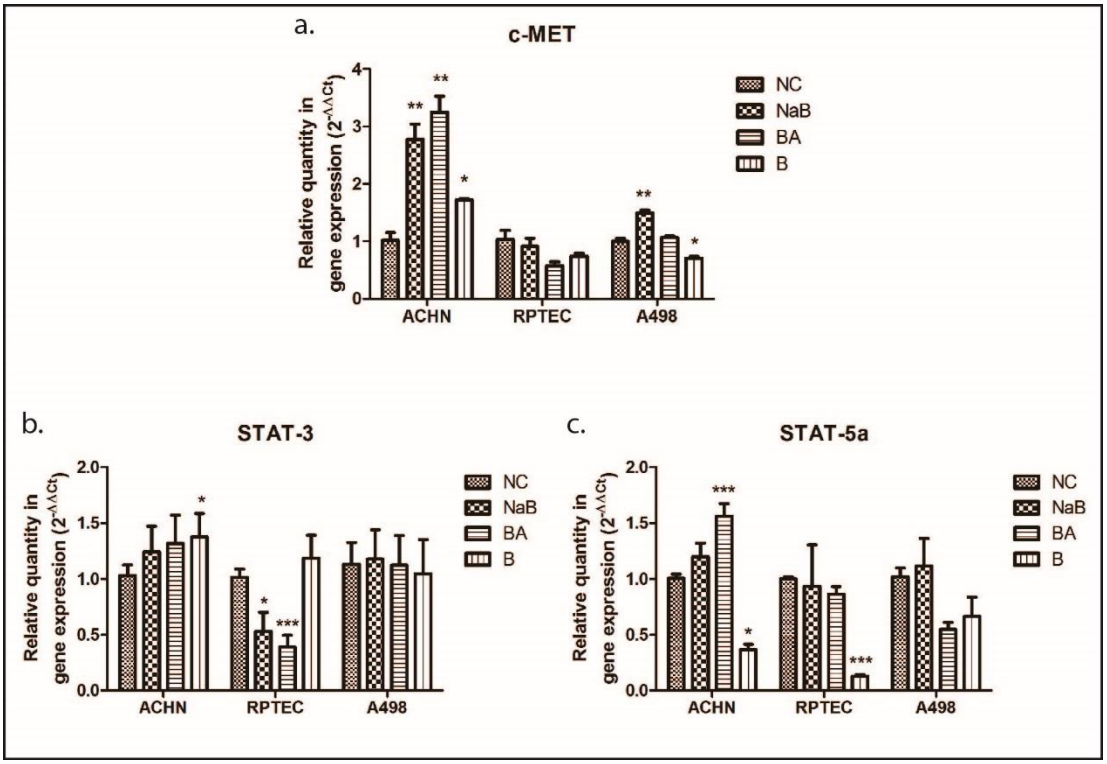

Figure 4.8. Real-time PCR results of HGF-c-MET pathway

* p<0.05, ** p<0.01, *** p<0.001

ACHN cell line appeared to be more sensitive to boron treatment compared to A498 cell line (Figure 4.10.a). Results showed that the cell migration were significantly arrested for both in 18h incubation period of NaB (26,74 per cent), BA (22,21 per cent) and B (14,5 per cent) versus NC (53.29 per cent) and in 36h incubation period of NaB (61,84 per cent), BA (66,72 per cent) and B (21,85 per cent) versus NC (100 per cent) (Figure 4.10.b). As it was observed before, NaB and BA effected the cell migration of ACHN cell line similarly, however, there were highly significant difference between B versus NaB and B versus BA.

On the contrary, A498 cells showed less sensitivity to boron derivatives. Analysis revealed that boron treated cells were significantly ceased the cell migration but not as much as ACHN cells, even though ACHN cell line is known for more migratory phenotype

compared to A498 cell line (Figure 4.9.a). Based on the results, A498 cells responded to the treatment of NaB, BA and B compared to NC as 32,66 per cent, 38,72 per cent, and 37,73 per cent compared to 57 per cent migration ratio at 8h, respectively, and 75,2 per cent, 74,42 per cent and 75 per cent85 compared to 100 per cent migration ratio at 24h, respectively (Figure 4.9.b).

4.5. EFFECTS of NaB, BA and B on CELL CYCLE

Cell cycle analysis of ACHN, A498 and RPTEC treated with NaB, BA and B were conducted by flow cytometer as mentioned in Method section 3.4. For this experiment, defined concentrations were used for corresponding cell lines as mentioned in *Results section 4.1*. This analysis was performed via three sample/biological replicates at different times.

For ACHN cell line, NaB and BA slightly arrested the cell cycle in S phase, while B treatment arrested ACHN cells in G2/M phase (Figure 11.a). ACHN is a rapidly dividing cells, therefore the cell population was distributed to the cycles as G0/G1, S, and G2/M in a ratio of 67,58 per cent, 15,46 per cent, and 17,53 per cent, respectively (Figure 11.b). NaB and BA treatment were decreased the cell number in G0/G1 phase (51,82 per cent and 51,18 per cent, respectively) significantly and increased the cell population in S phase (25,69 per cent and 25,67 per cent) and G2 phase (22,87 per cent and 23,47 per cent), slightly, compared to the control (Figure 4.11.b) . Based on the results it can be stated that NaB and BA play a similar role on cell cycle arrest for ACHN cells. Conversely, B treatment significantly arrested ACHN cells in G2/M phase (61,065 per cent) and decreased the cell population in G0/G1 phase (32.110 per cent) and S phase (7.015 per cent) (Figure 11.b).

Regarding to RPTEC cell line, none of the chemicals used in this project showed any effect on cycle cycle (Figure 4.13.a). RPTEC has a very low cell growth profile compared to cancer cells. That’s why, the cell population dispersed to the cycles as G0/G1, S, and G2/M in a ratio of 86,25 per cent, 3,38 per cent, 10,51 per cent, respectively (Figure 4.13.b). Treatments of NaB, BA and B were hardly effective on the cell population in G0/G1 phase (75,1 per cent, 74,33 per cent and 93.37 per cent, respectively), S phase (5,73

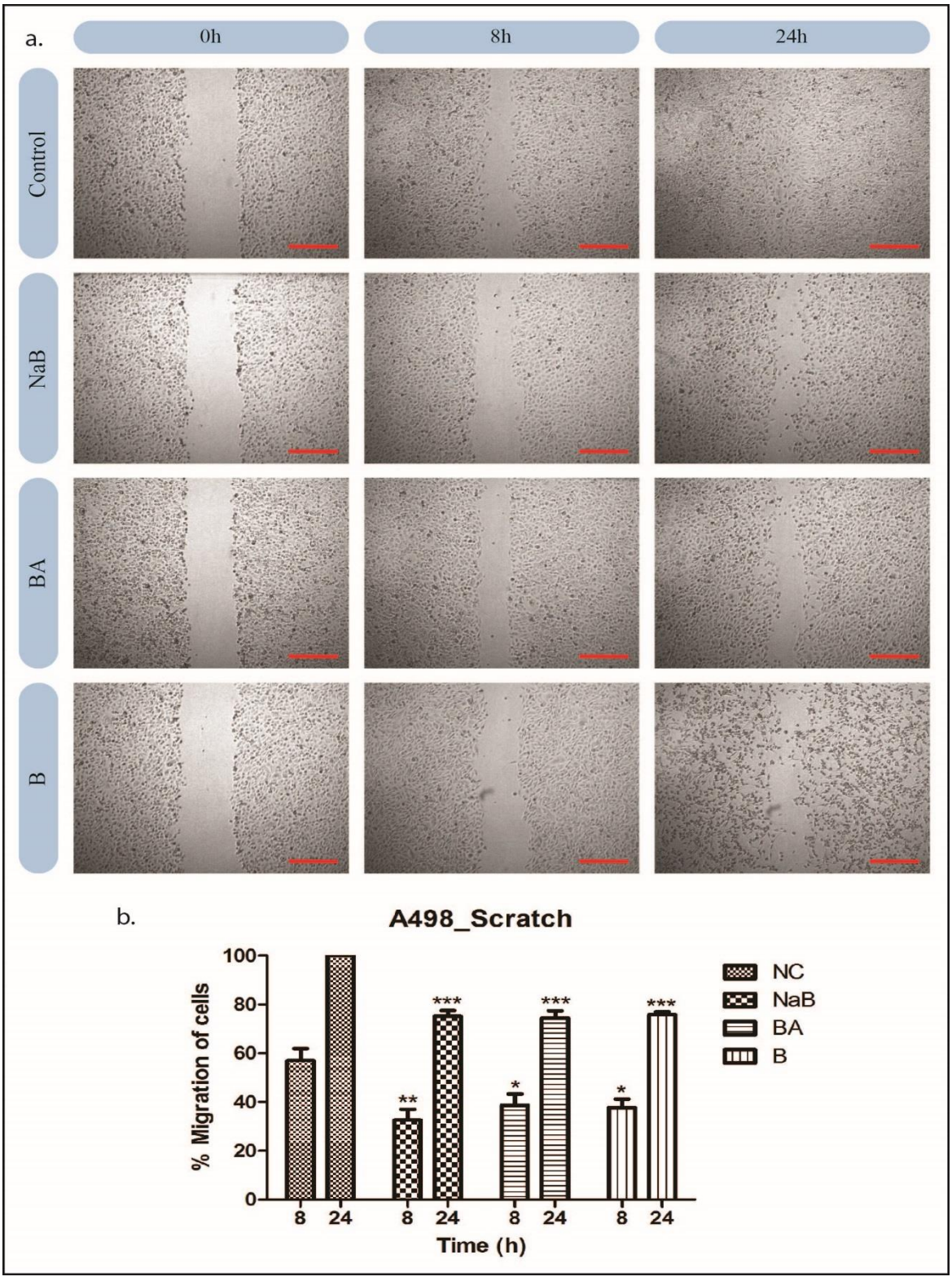

Figure 4.9. Scratch assay results for NaB, BA and B treated A498 cells. All groups were compared to their corresponding NC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, scale bar shows 500 μm

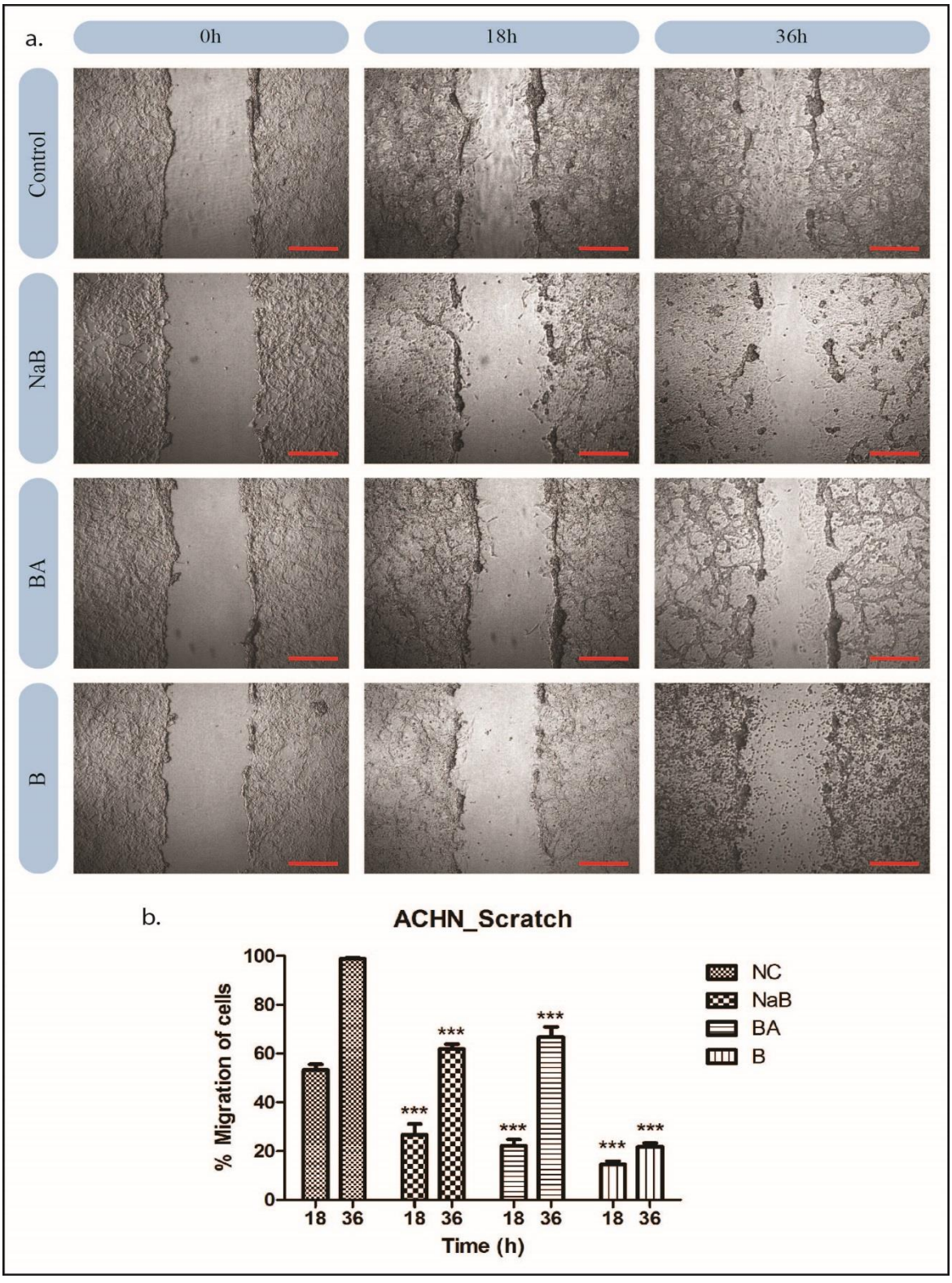

Figure 4.10. Scratch assay results for NaB, BA and B treated ACHN cells. All groups were compared to their corresponding NC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Scale bar shows 500 μm

per cent, 4,56 per cent and 2,16 per cent, respectively) and G2 phase (19,29 per cent, 15,56 per cent and 4,53 per cent, respectively) (Figure 4.13.b).

4.6. EFFECTS of NaB, BA and B on APOPTOSIS via ANNEXIN-V ASSAY

Analysis of the effects of NaB, BA and B on programmed cell death were investigated by both Annexin-V apoptosis detection assay as mentioned in the *Method Section 3.3* and RT-PCR analysis as mentioned in the *Method Section 3.5* at 72h. For this assay, defined IC50 concentrations of three chemicals for each cell line were used for ACHN and A498 cell lines. For analysis carried out on RPTEC cell line, the IC50 concentrations of NaB, BA and B on ACHN cell line were used as mentioned in *Results Section 4.1*. All experiments were conducted with three biological replicates.

Annexin-V results revealed that NaB and BA induced apoptosis through similar pathway which is opposite to B case.

For A498 cell line, there was a significant increase on early apoptosis marker on cells treated with NaB (41,53 per cent), BA (33,79 per cent) and B (23,25 per cent) compared to NC (1,51 per cent) (Figure 4.14). On the other hand, significant change only for late apoptosis marker was observed on B (10,39 per cent) treated cells compared to both NaB (7,97 per cent), BA (9,45 per cent) versus NC (2.27 per cent) (Figure 4.14). It may indicate that B was more toxic on A498 cell line.

For ACHN cell line, only BA and B significantly increased the early apoptosis marker (15 per cent and 26,21 per cent, respectively) compared to NC (4,16 per cent) and NaB (4,47 per cent) (Figure 4.15). However, late apoptotic marker was significantly increased only B treated cells (26,21 per cent) compared NC (4,16 per cent), NaB (4,47 per cent) and BA (3.66 per cent) (Figure 4.15). It could be stated that chemical treated ACHN cells prefers to undergo apoptosis through caspase dependent pathways, whereas A498 cells might use different pathways to regulate cell death when they are treated with NaB and BA.

For RPTEC cell line, slight increase on early-apoptotic markers and no significant change on late-apoptotic markers were monitored in both NaB and BA treated cells, since the non-

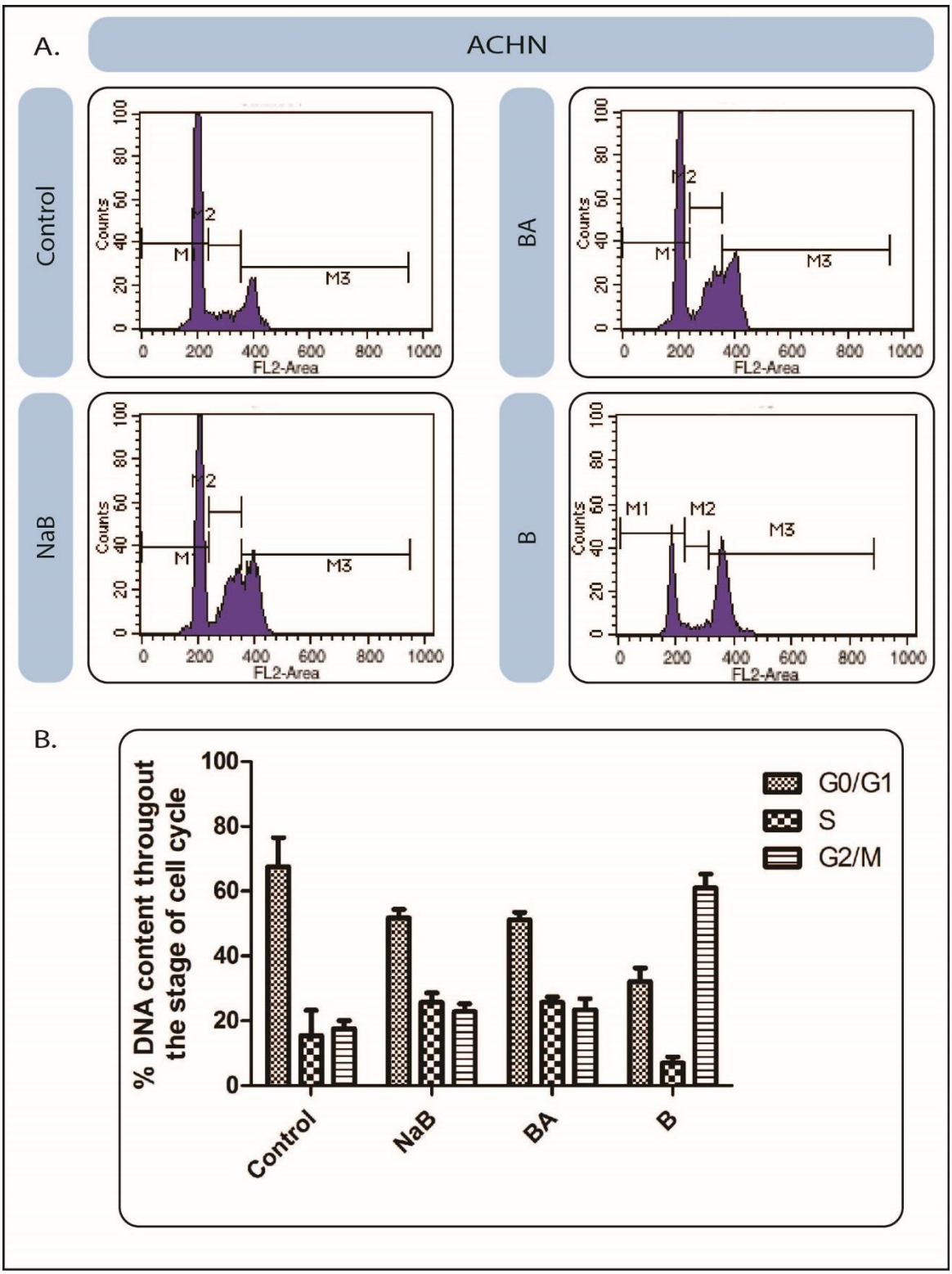

Figure 4.11. Cell cycle results for Control, NaB, Ba and B treated ACHN cells at 3th days.

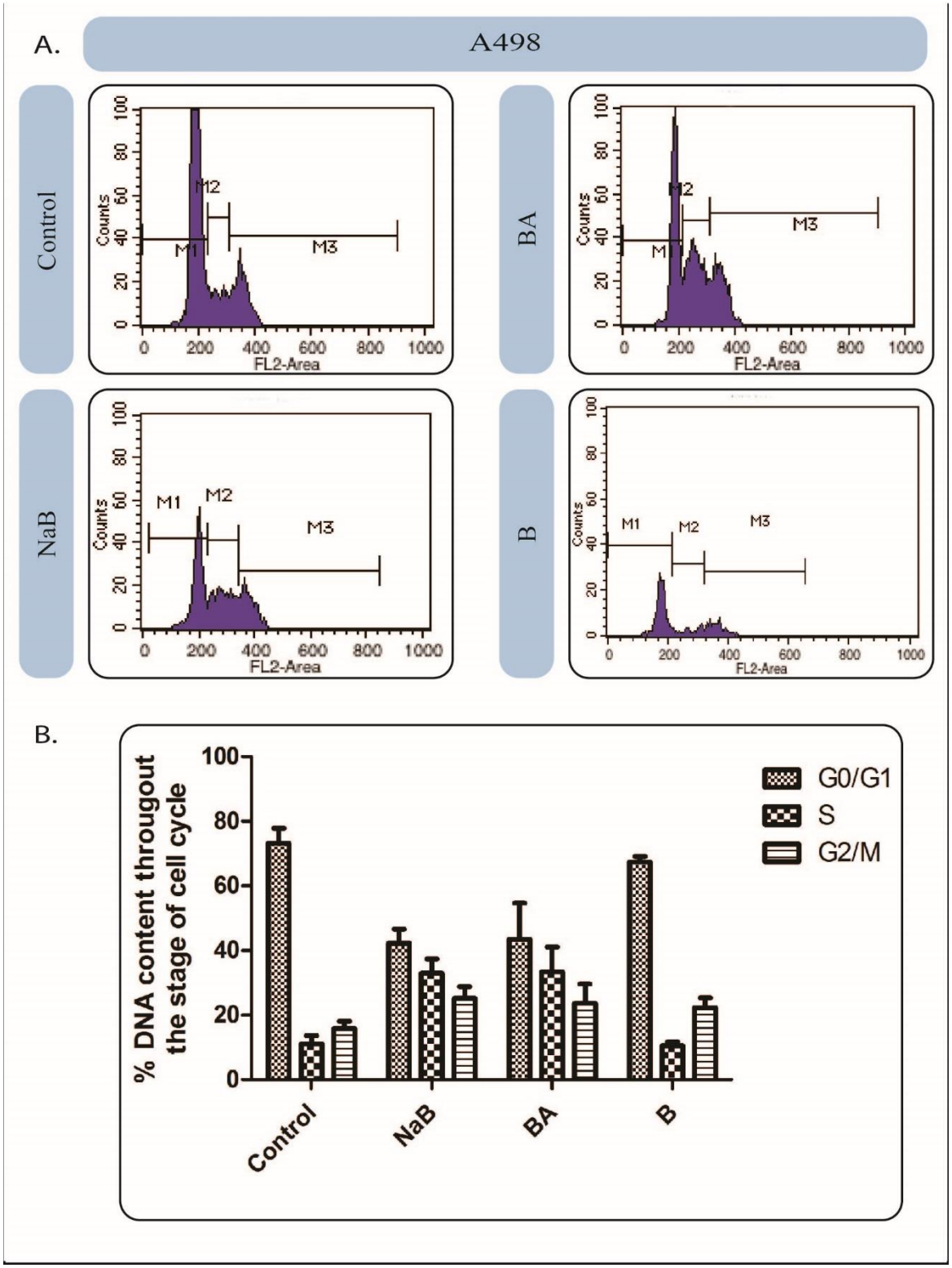

Figure 4.12. Cell cycle results for Control, NaB, Ba and B treated A498 cells at 3th days.

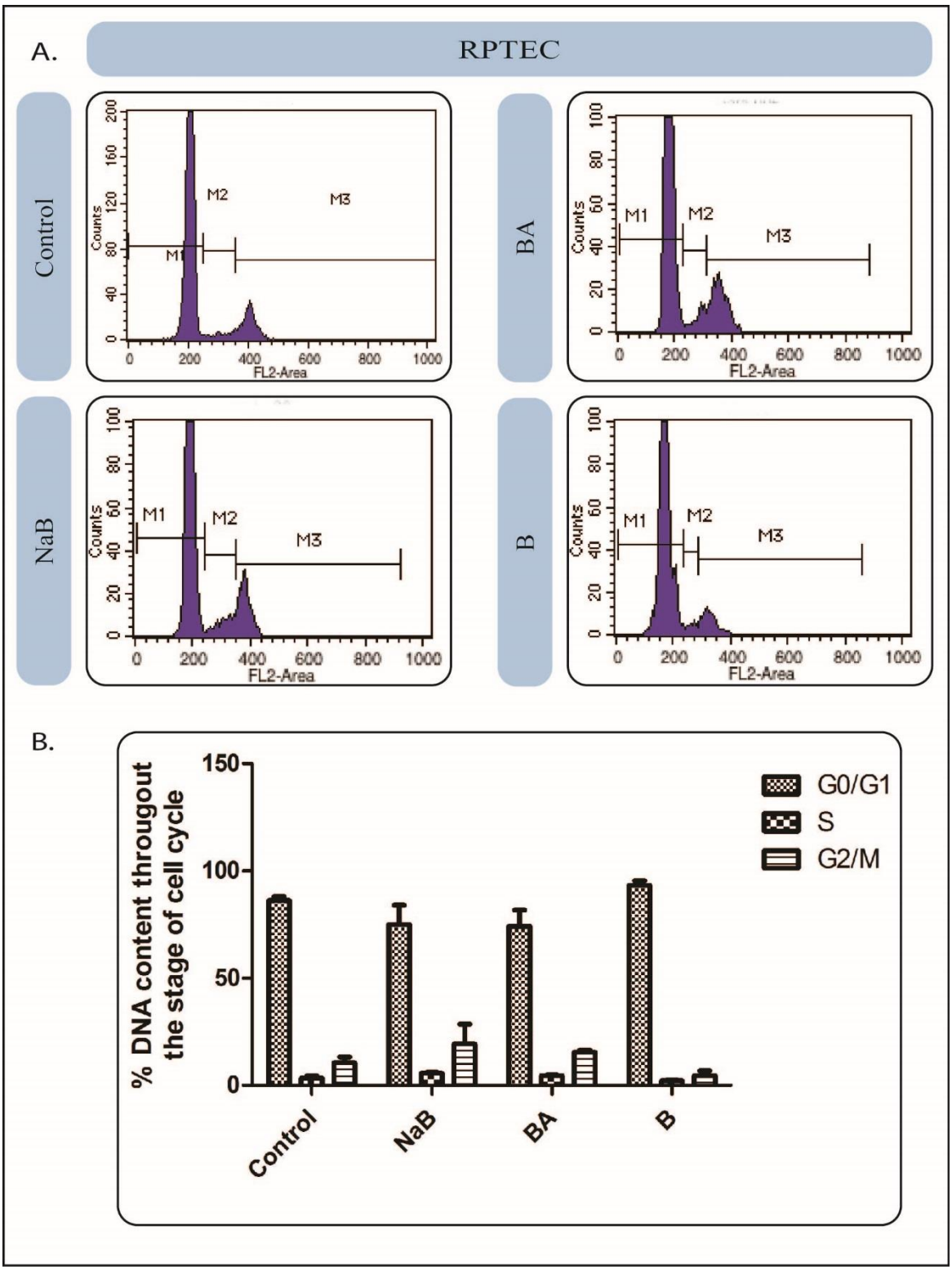

Figure 4.13. Cell cycle results for Control, NaB, Ba and B treated ACHN cells at 3th days.

toxic dose was used in this experiment (IC50 for cancer lines was used). On the contrary, B treated RPTEC cells showed significant increase in late apoptotic markers even though the dose used in this assay (100nM) was non-toxic according to the MTS assay results *(Result Section 4.1).*

Based on the results, it could be said that NaB and BA selectively increased apoptosis of ACHN and A498 cells, however did not affect the cell viability of RPTEC cells. On the other hand, B showed toxic effect to all three types of cells (Figure 4.16).

4.7. EFFECTS of NaB, BA and B on PROGRAMMED CELL DEATH PATHWAY

There are three types of programmed cell death (PCD) which were mentioned in introduction section 1.4.3. In this thesis, both mitochondria mediated apoptosis through the expressions of Bax, Bcl-2, p53 and caspase 3, and death receptor mediated apoptosis through the expression of NFκB were evaluated. Furthermore, in order to get a brief insight about the autophagy mediated cell death, any alteration on TORC1 expression was investigated by using real-time PCR method for three sample/biological replicates and each including three technical replicates.

Real-time results indicated that NaB and BA treatment of ACHN cell line induced apoptosis by upregulated the expressions of Bax (in a ratio of 44 per cent and 81 per cent compated to NC), TP53 (in a ratio of 96 per cent and 66 per cent compared to NC), caspase3 (in a ratio of 167 per cent and 201 per cent) and NFκB (in a ratio of 167 per cent and 201 per cent) but downregulated the expression of Bcl-2 (37 per cent and 34 per cent), respectively (Figure 4.17). Interestingly, B treatment have only significant upregulation on the expression level of caspase 3 as 44 per cent, however it decreased the expression levels

of TP53, NFκB, Bax and Bcl-2 in a ratio of 85 per cent, 39 per cent, 31 per cent and 99 per cent (Figure 4.17). It was seemed that NaB and BA treatment induced cell death by regulating similar pathways but differing from B in ACHN cell line. To check the autophagy induction capacity of all three compounds for ACHN cell line, TORC-1 expression level was analyzed (Figure 4.17.f). TORC1 is an autophagy-suppressor protein playing role on cell survival through PI3K/Akt/mTORK pathway. Results indicated that

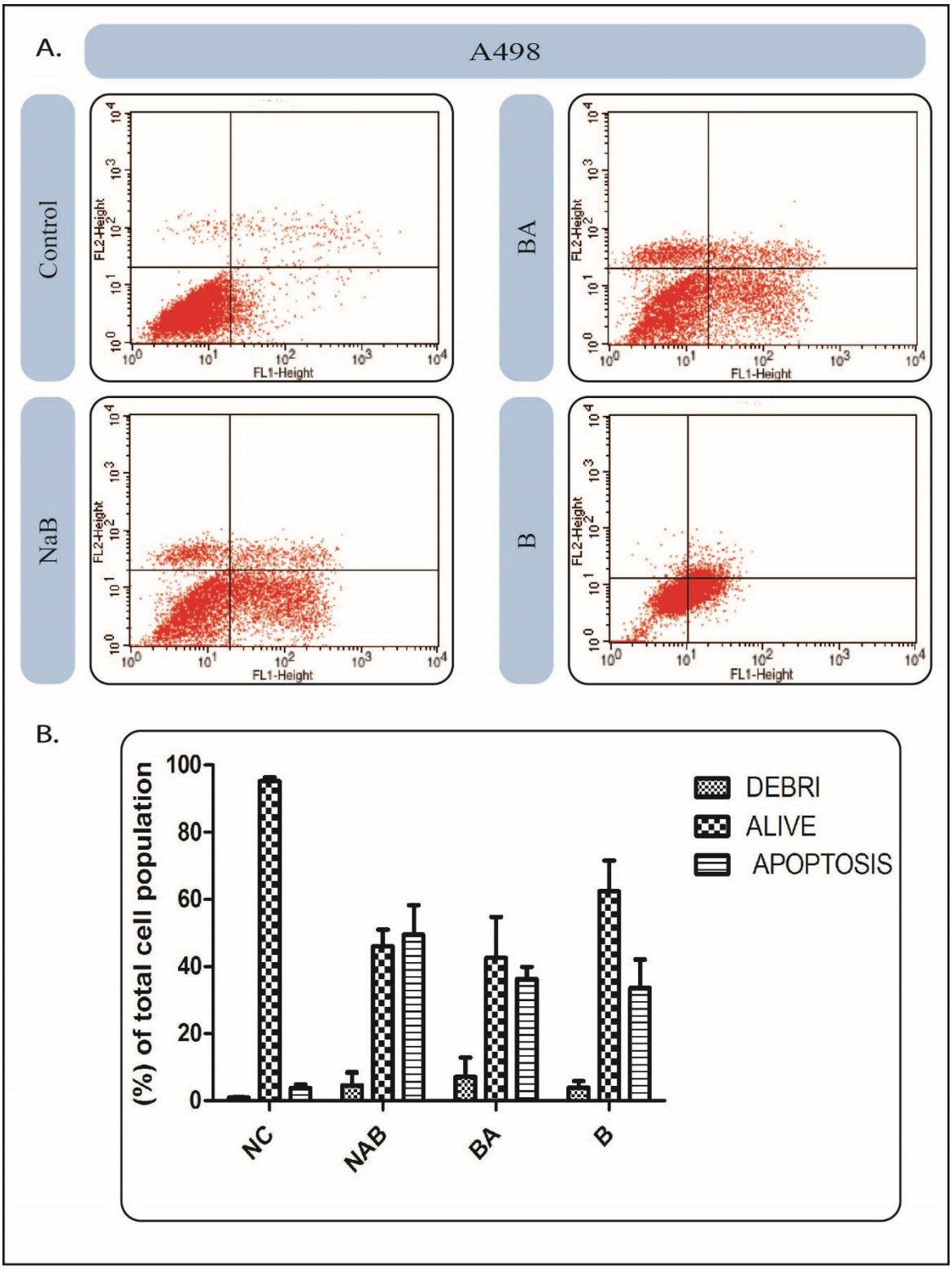

Figure 4.14. Annexin-V assay results for NaB, BA and B treated A498 cells at 3th days. All groups were compared to their corresponding NC

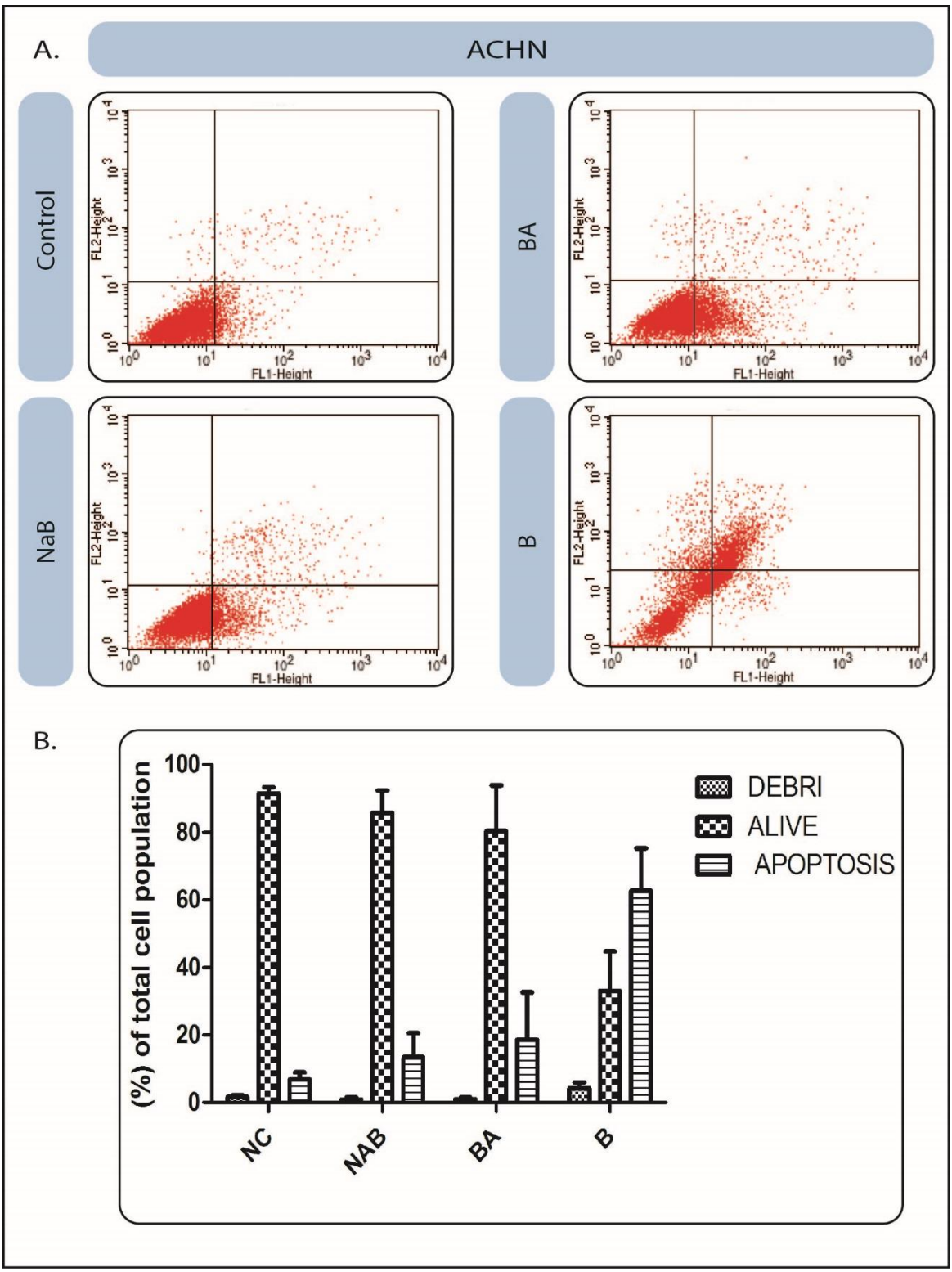

Figure 4.15. Annexin-V assay results for NaB, BA and B treated ACHN cells at 3th days. All groups were compared to their corresponding NC

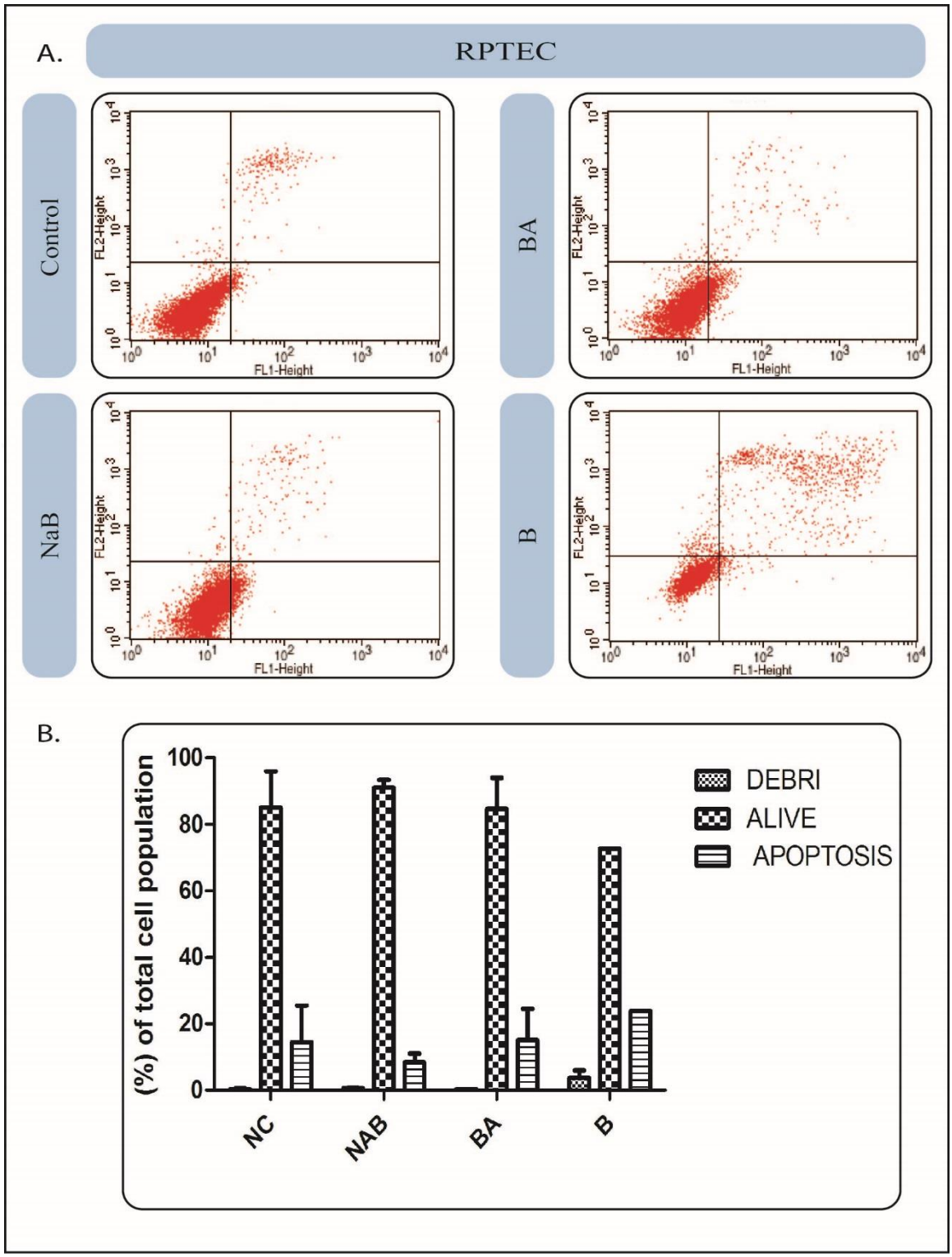

Figure 4.16. Annexin-V assay results for NaB, BA and B treated RPTEC cells at 3th days. All groups were compared with their corresponding NC

both the treatment of NaB and BA significantly increased the TORC1 gene expression in a ratio of 74 per cent and 84 per cent, respectively (Figure 4.17). Conversely, B did not affect the expression level of TORC1 (Figure 4.17). On the light of the results, it was interpreted that NaB and BA induced cell death via apoptosis but not autophagy. Additionally, in order to address the mechanism of cell death via B more experiments is required to be conducted.

Application of all three compounds separately did not induce apoptosis mediated cell death on A498 cell line, opposite to ACHN cell line. Both NaB and BA showed their anti-apoptotic effect through similar pathway by suppressing the expression of BAX (57 per cent and 68 per cent); P53 (54 per cent and 77 per cent), and Caspase-3 (56 per cent and 67 per cent); and strong activation of Bcl-2 (105 per cent, 90 per cent) respectively, while no effect was observed on NFκB and TORC1 expression levels (Figure 4.17). On the contrary, B application resulted in a little difference on gene expression profile in A498 via suppression of all the genes including BAX (76 per cent), Bcl-2 (75 per cent), Caspase3 (73 per cent), NFκB (60 per cent), P53 (64 per cent) and TORC-1 (79 per cent) (Figure 4.17). These results may suggest that another type of cell death possibility instead of caspase-dependent apoptosis or autophagy for A489 cell line, but to prove this hypothesis more experiment should be conducted on the pathways.

Moreover, real-time PCR analysis of RPTEC cell line revealed that NaB did not induce mitochondrial mediated apoptosis on healthy cells, as no significant change on expression levels of BAX, Caspase3 and TP53 were examined. Since Annexin-V assay showed that these cells undergo apoptosis in a certain level by chemical treatment, it is possible that cell death is induced with alternative pathway (caspase-independent) via upregulation of NFκB expression (112 per cent) and downregulation of Bcl2 (70 per cent) and TORC1 (73 per cent) expressions (Figure 4.17). On the other hand, BA treatment possibly triggered apoptosis by suppressing the expression of Bcl2 (81 per cent) TP53 (38 per cent) and TORC1 (79 per cent), while it had no significant effect on the expression levels of Bax and NFκB, although it upregulated Caspase 3 expression (49 per cent) (Figure 4.17). Moreover, the experiment which were conducted by B was shown that B triggered mitochondria mediated cell death by inducing BAX (73 per cent) and decreasing Bcl-2 (99 per cent), however the suppression of Caspase3 (91 per cent), P53 (83 per cent) and NFκB (79 per cent) meaned that the activation of apoptosis was controlled via another unknown

pathways (Figure 4.17). Additionally, the inhibition on the expression of TORC1 (44 per cent) was another supportive data on the hypothesis of B mediated cell death (Figure 4.17).

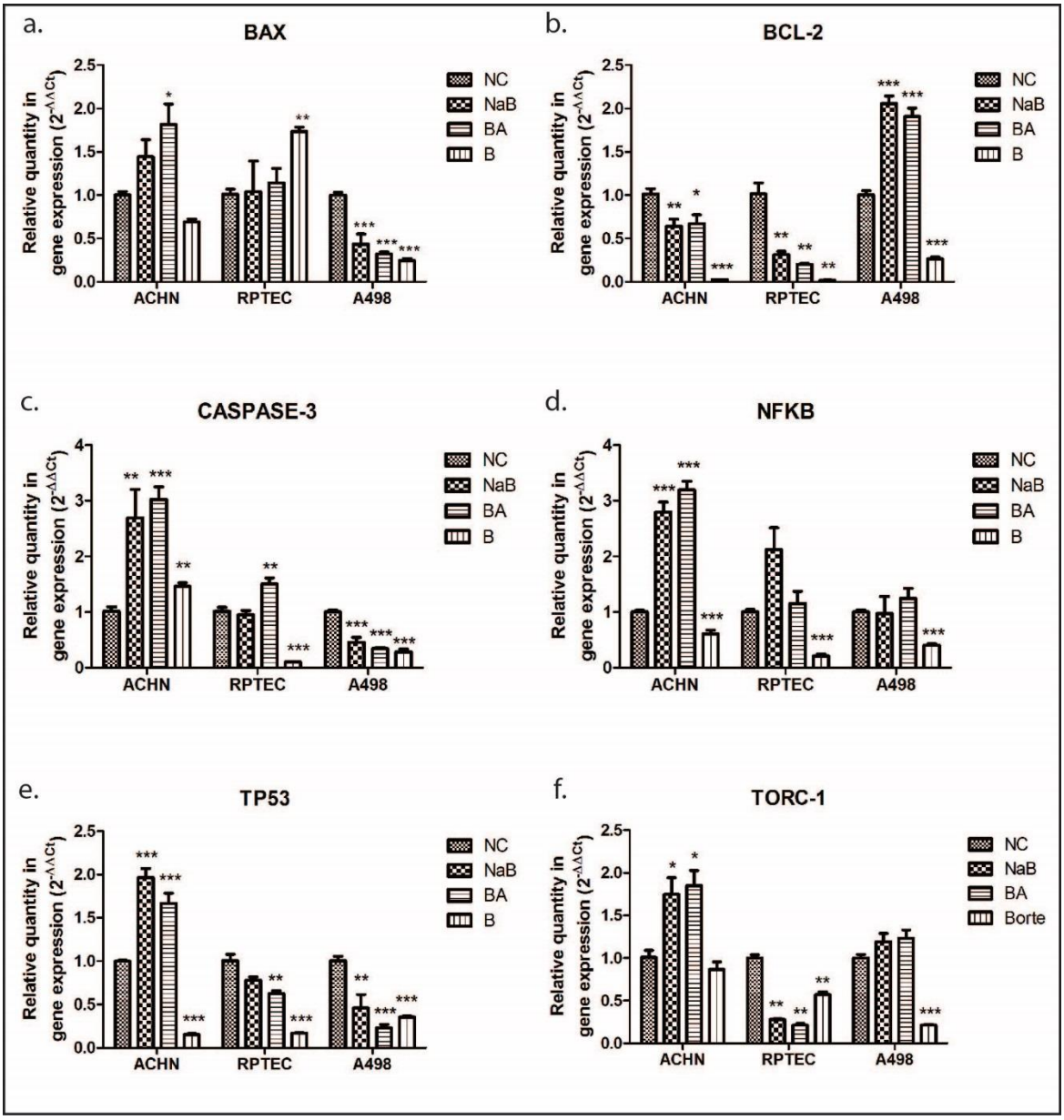

Figure 4.17. Real-time analysis results for NaB, BA and B treated ACHN, A498 and RPTEC cells. All groups were compared to their corresponding NC. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5. DISCUSSION

Cancer is the most common cause of mortality after heart and cardiovascular diseases [1]. It generally occurs due to number of molecular alternations of signaling molecules and dysregulation in the control mechanisms that causes abnormal growth and proliferation of the cells [1]. Cancer results in 8.2 million deaths among 14.1 million cases per year [2]. Approximately 3 per cent of all malignant tumors in adults arises in the kidney [6]. The most important and common type of kidney cancer is renal cell carcinoma (RCC) with high frequency and lethality because of highly vascularized construction of kidney and high metastatic capability of kidney cancer cells [6]. The main curative therapy method of renal cancer is surgery by removing whole organ or partial nephrectomy if the tumor is localized to kidney and smaller than 4 cm [12]. Chemotherapy and radiotherapy are another options, but unfortunately ineffective against renal cell carcinoma most of the time [17]. RCC is one of the most immune responsive human malignancy. Therefore, interferon-α and interleukin-2 is also used to increase immunity against tumor [18, 19]. However, this method is restricted because of tumor induced immune suppression. Another method that is used for RCC therapy is vaccine therapy. Although this type of therapy specifically induces immune system cells against to cancer cells and considered as clinically safe, it is effective only in a minority of patients [20]. In recent years, with the accumulation of information about molecular mechanisms of cancer progression, targeted therapies have been developed. These targeted drugs are especially important in chemo-drug resistant kidney cancers, since they occasionally work when the chemo-drugs fail. Furthermore, they often have less severe side effects. Several targeted drugs can be used to treat advanced kidney cancers to block angiogenesis or tyrosine kinases which have important roles in tumorigenesis [21].

Boron is a non-essential mineral that plays important roles in animal cells. The studies showed that low B diet associated with a number of health problems [163-165]. Most common symptoms of B deficiency include arthritis [166,167], memory loss [168,169], osteoporosis [170], degenerative and soft cartilage diseases [171], hormonal disequilibria and drop in libido [172].

Moreover, there are evidences indicating that boron intake decreases both cancer incidence and tumor progression [131-138]. One controlled case study which compared the boron intake of 95 prostate cancer cases with 8720 male controls showed that the risk of prostate cancer to be inversely proportional to dose-responsive boron intake [173]. In another study of mice which were implanted subcutaneously with human prostate adenocarcinoma cells showed that boron intake for 8 week decreased tumor size and serum PSA (prostate specific antigen) levels compared to control group. Based on the results, it concluded that, PSA inhibition via boron intake mediates inhibition of stored calcium release from cells and induction of apoptosis in prostate cancer [174,175].

It has shown that boron supplementation may increase the serum magnesium, testosterone and estrogen levels but have no adverse clinical effects. According to the literature, test subjects taken 100 mg of boron intravenously or 270 mg of boric acid orally reported no discomfort and showed no obvious sign of toxicity [176,177]. Additionally, drinking water with high boron concentration in Turkey has not been shown to cause untoward effect in human exposed over multiple generation. On the other hand, a fatal outcome was reported after ingestion of 1 g of boric acid by a child whereas adults survived acute intake of nearly 300g [178]. The food and nutrition board of the institute of medicine has established a tolerable upper intake level for boron of 20mg/day for adults <18 years of age and <6mg for children between 1-3 years of age [179]. Because of its non-toxic nature but anti-cancerogenic effects in human, it could be used as a new targeted therapeutic agent against cancer.

In addition to the case studies about correlation of boron intake with cancer incidence or prognosis, the exact mechanism of boron on cancer has not been clearly established, yet. In this study, the mechanism of three boron derivatives as sodium pentaborate pentahydrate (NaB), boric acid (BA) and a type of boronic acid called bortezomib (B) has been investigated on ACHN and A498 renal carcinoma cell lines and human primary renal proximal tubule epithelial cells (RPTEC). Effects of chemicals on angiogenesis, metastasis and programmed cell death were analyzed by cell viability assay, relative real-time PCR analysis, scratch assay analysis and Annexin-V apoptosis assay.

Before conducting the rest of the experiments, toxicity assay was performed to determine IC50 of NaB, BA and B to treat cells with. 500, 1000, 1500, 2000, 2500, 3000 and 3500 µg/ml of NaB and BA were tested to measure its effect on survival of A498 cells and ACHN cells for 3 days (Figure 4.1 and Figure 4.3). 1400 µg/ml of NaB and BA showed 50 per cent inhibition (IC50s) effect for A498 cells (Figure 4.2.a and Figure 4.2.b), while, 1550 µg/ml of NaB and BA exhibited IC50 for ACHN cells (Figure 4.4.a and Figure 4.4.b) in 3th day. Based on these results, it was concluded that A498 was more sensitive against NaB and BA treatment compared to ACHN. On the other hand, a wide concentration range as 500, 1000, 2500, 5000, 7500, 10000 µg/ml of NaB and BA were tested to find out IC50s for RPTECs, since its survival rate was higher than ACHN and A498 (Figure 4.5.a and Figure 4.5.b). Results showed that IC50s of NaB and BA was seen in the concentration of 5000 µg/ml in 3th day (Figure 4.6.a and Figure 4.6.b). Our results suggested that NaB and BA have anti-carcinogenic effect for A498 and ACHN cancer cells while non-toxic effect for RPTEC normal renal cells. Moreover, these results support the literature about the concentration of maximum intake for human health that NaB and BA have no toxicity for healthy kidney cells while excretion from kidney.

In cell viability assays, 30, 50, 80, 100, 130, 150 and 180 nM of B were tested to determine its effect on survival of ACHN cells for 3 days (Figure 4.3.b). 100 nM of B showed IC50 affect for ACHN at 3th day (Figure 4.4.c). Besides, 1, 5, 10, 20, 30, 40 and 50 nM of B were tested to figure out its effect on A498 cell for 3 days (Figure 4.1.c). 20nM of B presented IC50 affect for A498s at 3th day (Figure 4.2.c). Results revealed that A498 was highly sensitive to B treatment compared to ACHN. Investigating the B sensitivity for A498, we found that *VHL* mutation resulted with sensitivity against B via causing hypoxic condition on A498 cells [180]. On the other hand, RPTEC cells responded to B treatment similar to NaB and BA by high resistance. 50, 100, 250, 500, 750 and 1000 nM of B were tested to display its effect on survival of RPTEC for 3 days. Results indicated that 750nM of B affected cell survival with 50 per cent inhibition at 3th day. Based on these results, we concluded that B has highly toxic effect on ACHN and A498 cells compared to RPTECs similar to the results of NaB and BA.

Additionally, recent *in vitro* studies showed that boric acid inhibited the proliferation of the hormone dependent and independent human prostate cancer cell line in a dose dependent

manner [181]. Our results also supported that the survival rate was decreased with increasing boron concentration level in all cell types however stay similar after a threshold concentration (Figure 4.1, Figure 4.3 and Figure 4.5).

In the present study, the role of SLC4 $Na^{+}HCO^{-}$ co-transporter family member, *SLC4A11*, was evaluated based on relative real-time PCR analysis in order to understand the mechanism of boron derivatives used to get in through the mammalian cells. Results showed that both NaB and BA treatment increased *SLC4A11* expression highly significantly, however B treatment suppressed gene expression crucially compared with control in all cell lines (Figure 4.7). Based on the literature, the only established results about the transport of boron derivatives to kidney cancer cells showed similarity to our results. Boric acid treatment on human embryonic kidney 293T cells was responded with dose dependent toxicity and also up-regulation on SLC4A2 and SLC4A3 gene expression even in non-toxic dose of boric acid [182]. Additionally, in another article, up-regulation of SLC11A1 expression was associated with apoptotic processes via the depletion in iron content of the cell to cytosol antagonizes cell growth. By contrast, transcriptional repression of SLC11A1 resulted in cell survival [183]. Based on the results, it can be concluded that NaB similar with BA prefers to induce toxicity via SLC protein family member, SLC4A11. Moreover, SLC4A11 expression in ACHN was increased with NaB and BA treatment by 0.5 fold more compared to A498 and RPTEC. Therefore, our data suggested that NaB and BA might effect ACHN cells on molecular level more than A498. Hereby, our results are the first to establish SLC4A11 gene expression association with NaB, BA and B treatment. Additionally, it is important to underline that NaB and BA transport pathway and therefore downstream mechanisms are different from B in the renal cells.

To understand the cell death mechanism of ACHN and A498 with the treatment of boron derivatives, real-time PCR analysis was conducted to evaluate the expression of Bax, TP53, Bcl-2, Caspase3, NFKB and TORC1. As it was mentioned in *introduction section 1.4.3.1*, apoptosis is a programmed cell death mediated by either extrinsic pathway or intrinsic pathway. Briefly, the extrinsic pathway is activated by Fas receptor mediated pro-caspase-8 activation which proceeds activation of pro-caspase-3 and apoptosis. On the

other hand, intrinsic pathway is controlled by mitochondrial pro-enzymes and Bcl-2 family members including pro-apoptotic members such as Bax and Bcl-2. The reduction in expression of Bcl-2 is associated with apoptotic response against anticancer drugs, while up-regulation of it causes drug-resistance to chemotherapeutics. With the imminent death signaling, pro-apoptotic proteins go through posttranslational modifications and are translocated to mitochondria. By the contribution of Bax and Bak, outer mitochondrial membranes become permeable to internal cytochrome-c and release it into cytosol. Cytochrome-c interact with Apaf-1 and pro-caspase-9 to produce apoptosome, leading to activation of caspase-9 and caspase-3 cascade, subsequently generating apoptosis (96).

NF-κB is a transcription factor that promotes inhibition of apoptosis and resistance to chemotherapy. It is commonly believed that inhibition of NF-κB activity can increase sensitivity of cancer cells to chemotherapy. [184]. However, there is an evidence in the literature that NF-κB activation can sensitize cells to apoptosis and that inhibition of NF-κB results in resistance to chemotherapy [185]. According to the literature, NF-κB activation is associated with TP53 mediated apoptosis, therefore suppression the tumor progression [186]. Moreover, mTORC1 is a member of PI3K/Akt pathway and plays role to provoke autophagy by activation of Akt (107).

To support our hypothesis about cell death mechanism of cells, Annexin-V apoptosis assay were also conducted. The rapid translocation and accumulation of the membrane phospholipid phosphatidylserine (PS) is an early indicator of apoptosis from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by utilizing the preferentially binding properties of Annexin V, a calcium dependent phospholipid binding protein, to negatively charged phospholipids including PS.

Based on these informations, our results suggested that NaB and BA treatment significantly induced mitochondrial mediated apoptosis via increasing of Bax, TP53 and Caspase3 expression and suppressing of Bcl-2 expression on ACHN cell line (intrinsic pathway). Additionally, the up-regulation of the expression of NF-κB in NaB and BA treated ACHN cells may be supported our hypothesis. It is possible that upregulation of NFKB sensitizes cells against NaB and BA and with this way promotes mitochondrial mediated apoptosis. Also, the results of TORC1 expression strengthen our hypothesis that TORC1 activation presumably mediates cell to undergo apoptosis via obstructing autophagy.

On the contrary, B treatment only up-regulated expression of caspase-3 whereas decreased expressions of Bax, TP53 and Bcl-2. The induction of caspase-3 and suppression of Bcl-2 lead us to the conclusion of apoptosis. The suppression of Bax and TP53 also support our hypothesis and directed us to Fas ligand mediated caspase dependent apoptosis (extrinsic pathway).

Additionally, down-regulation of NFKB means that cell reacts against bortezomib which can be interpreted that cell were induced to undergo apoptosis in a low level. According to the literature, NF-kappa B signaling is regulated by binding to I kappa B. Ubiquitination and proteasome degradation of I kappa B releases NF-kappa B into the cytoplasm, which can then move into the nucleus and induces transcription of cytokine and chemokine signaling, cell proliferation, angiogenesis, and resistance to apoptosis. The proteasome inhibitor B, a boron-containing dipeptide, blocks I kappa B degradation in a dose-dependent manner, thereby blocking NF-kappa B activity and decreasing its downstream signaling effects [187]. Taken together with the lower amount of caspase activation, the results are consistent with the literature that bortezomib treatment on ACHN cells induced apoptosis but not as much as NaB and BA. Moreover, no-effect on TORC1 expression and decrease on TP53 expression level could lead us to assume that B treatment did not affect the autophagy mediated cell death pathway.

TP53 is a tumor suppressor gene and an important prognostic indicator in cancer. In renal cell carcinoma, there are contradicting data in the literature. Recent studies showed that high level of TP53 could be a predictor of poor outcome [188]. Interestingly, the results indicated that late apoptosis phase was associated with TP53 expression level on all three types of cells. Up-regulation of TP53 was playing role on induction of mitochondrial mediated apoptosis (intrinsic apoptosis) concluded with increase only early phase of apoptosis, on the other hand, down-regulation of TP53 induce extrinsic or caspase independent apoptosis as a result of increase late phase of apoptosis

Based on the results, NaB, BA and B treatment increased the early apoptosis marker compared to NC on the ACHN cells. However, late apoptotic marker was significantly increased only B treated cells. These results supported our hypothesis that NaB and BA treatment lets cells to undergo cell death via intrinsic apoptosis pathway, whereas B treatment triggers cell death via extrinsic apoptosis pathways. It is possible that cells

became more sensitive with NFKB suppression. It is also likely that another mechanisms lead to cell necrosis.

On the other hand, NaB and BA treatment on A498 cells should be activated cell death via different cell death mechanism compared with ACHN. The down-regulation of Bax, Caspase-3 and TP53 expression with upregulation of Bcl-2 expression suggested that the death of A498 is not mediated via caspase dependent apoptosis. No change in NFKB and TORC1 also suggested that A498 cells did not have a resistance for NaB and BA treatment or have no tendency to autophagy, respectively. Based on the results, we suggested that A498 cells was dead via Bcl-2 mediated caspase independent apoptosis pathway.

Beside the effects of NaB and BA on A498 cells, our results showed that B treatment leads the cells to die through different pathway. Down-regulation of the Caspase-3, Bax and TP53 could not be explained with inactivation of caspase-mediated apoptosis. On the other hand, Bcl-2, NFKB and TORC1 suppression pointed us that programmed cell necrosis or autophagy might be activated for cell death. Moreover, Annexin-V assay results showed that NaB, BA and B treated A498 cells significantly induced the early apoptosis, whereas significant effect for late apoptosis was seen only on B treated A498 cells. Compared to the real-time PCR results, increase of early apoptosis on NaB and BA treated cells supported our caspase-independent apoptosis hypothesis. However, significant increase of late apoptosis was seen only on B treated A498 cells parallel with our necrosis or autophagy mediated cell death hypothesis. Importantly, NaB, BA or B induced apoptosis was shown for the first time together with gene expression analysis and Annexin-V analysis on A498 by our group.

Moreover, real-time PCR analysis of RPTEC cell line revealed that NaB did not induce mitochondrial-mediated apoptosis on healthy cells, as no significant change on expression levels of BAX, Caspase-3 and TP53 was examined. Since Annexin-V assay showed that these cells undergo apoptosis in a certain level by chemical treatment, it is possible that cell death is induced with alternative pathway such as caspase-independent apoptosis by the up-regulation of NFκB expression and down-regulation of Bcl2 in addition to autophagy through suppression of mTORC1 (73 per cent) expressions.

On the other hand, BA treatment possibly triggered extrinsic caspase-dependent apoptosis by suppressing the expression of Bcl2, TP53 and TORC1, while it had no significant effect

on the expression levels of Bax and NFκB. Although, it up-regulated Caspase-3 expression. Parallel to the real-time PCR analysis, Annexin-V assay also gives indications about apoptosis of BA treated cells.

Lastly, B treatment probably induced autophagy or necrosis by suppression of TORC1 expression additional to inhibition of Bax, Caspase-3, TP53, NFKB and Bcl-2 expressions. Annexin-V assay results also supported necrosis or autophagy mediated cell death hypothesis with significant increase of late apoptotic markers on B treated RPTEC cells.

It should be highlighted that, our Annexin –V results for ACHN, A498 and RPTEC cells were the first in boron treatment researches for renal cancer therapy. On the other side, there are evidences about the apoptotic and anti-proliferative effect of boron derivatives such as boric acid (BA) and calcium fructoborate (CF) on breast cancer cells in the literature [189]. Previously, it was shown that both CF and BA had anti-proliferative effect in breast cancer cells but only apoptotic effect was seen on CF treated cancer cells via up-regulation of caspase 3 expressions and down-regulation of Bcl-2 and P53 expression. However, in that study, the effect of CF and BA on breast cancer cells were established via dose dependent manner. Their maximum concentration that used on the experiments was 5mM which was 5 fold less than our concentration for ACHN and approximately 4 fold less than our concentration of A498 which were used even if they conducted their experiment for 8 days compared our experiment for 3 days [189]. In another study, apoptotic effect of boric acid was analyzed on DU-145 prostate cancer cells dose-dependently. The results showed that boric acid exhibited its apoptotic effects by down-regulation of Bcl-2 expression causing an arrest in cell proliferation. These results are consistent with the literature [190]. Although, in order to clarify the effect of boron derivatives on kidney cancer cell lines, protein based experiments should be design.

To investigate the c-MET mediated metastasis on ACHN and A498 cells, real-time PCR analysis were conducted to analyze the expression levels of c-MET, Stat3 and Stat5a in addition to scratch assays and cell cycle assay.

c-MET is a receptor tyrosine kinase binding to a receptor, hepatocyte growth factor, and activates a wide range of different cellular signaling pathways including proliferation, motility, migration and invasion. (50-53). Although c-MET is important for controlling tissue homeostasis under normal physiological conditions, it has also been found to be

abnormally activated in human cancers through mutation, amplification or protein over-expression [50]. c-MET is activated with phosphorylation of a serine residue (Ser 985) at its juxtamembrane domain via serine kinases (62). Moreover, an increase in intracellular calcium levels may result in negative c-MET regulation [89]. c-Met mediated downstream signalling is activated by binding of ligand HGF to c-Met. This interaction induces a serial phosphorilation cascade and with this way activates downstream signalling pathways such as Src/FAK pathway regulaing cell adhesion, anchorage-independent growth and migration (85-87) and p120/STAT3 pathway stimulaing branching morphogenesis of cells (85-87). PI3K/Akt pathway activates cell motility and cell survival (85-88), while Ras/MEK pathway mediates HGF-induced cell scattering, cell proliferation and cell cycle progresion (85-87).

Boron derivatives act as a serine kinase inhibitor. Furthermore, they cause augmentation of Ca^{+2} level in the cells [134,135]. Therefore, our data suggested that NaB, BA and B may lead to inactivation of c-MET by down-regulation of its expression through both inhibition of serine kinases and with accumulation of intracellular calcium. Moreover, it has been shown in previous studies that boron decreases the expressions of five major cyclin proteins (A, B1, C, D1 and E) immensely important in the cell cycle regulation of prostate cancer cells [33, 55]. These studies highlight the effect of boron derivatives on cell cycle.

The results in this study revealed that NaB, BA and B treatment increased the expression level of c-MET and its downstream target, Stat3 on ACHN cells. Interestingly, BA, and NAB treatment up-regulated Stat5a expression level, while B treatment down-regulated its expression. Previous studies have shown that Stat3 plays a role in cell survival and migration [191], whereas Stat5a regulates cell proliferation [192]. Noted that all three chemicals might induce cell survival and increase migration capacity, B treatment suppressed cell proliferation. However, scratch assay analysis indicated that NaB, BA and B treated cells migration was ceased significantly *(Figure 4.9 and Figure 4.10*). This result was specifically important for ACHN cell treatment because of its highly metastatic capability compared to A498. In addition, B treatment restricted the migration of ACHN cells more than NaB and BA treatment. Furthermore, the cell cycle results demonstrated that NaB, BA and B also effect cell proliferation through arrested cell cycle of A498 and ACHN cell lines *(Figure 4.11 and Figure 4.12)* except RPTECs (Figure 4.13). This might be explained with its alternative transport system to cells, its working mechanism on cells

or its specific toxic effect on ACHN cells. NaB and BA treatment behaved similarly on migration of ACHN cells and cell cycle arrest compared to B. Therefore, it is possible to state that they showed their effect by using similar pathways.

On the other hand, A498 cells responded to NaB treatment with up-regulation of c-MET and steady-state regulation of Stat3 and Stat5a expressions. Beside this, BA treatment did not alter the expression level of c-MET and Stat3, but decreased the expression level of Stat5a. Moreover, B down-regulated the expression levels of c-MET and Stat5a but did not effect the Stat3 expression level. Based on the real time PCR results of A498 cells, we may suggest that Stat3 and Stat5a expression might be controlled via c-MET independent pathway similar to ACHN. Moreover, steady-state change on Stat3 for all three compounds may suggest that they have no effect on migration. However, decreases in Stat5a levels in BA and B treated cells may suggest that they may respress the cell proliferation. Our scratch assay results also showed that boron treatment ceased the cell migration significantly. However, scratch assay analysis revealed that A498 cells showed less sensitivity to boron derivatives compared to ACHN. Addionally, A498 cells were responded to NaB and BA treatment more than B treatment (Figure 4.11.b). On the other hand, cell cycle analysis showed that NaB and BA treatment significantly restricted cell cycle on S phase but no-effect was seen on B treated cells.

Based on the results, it was concluded that, B probaby transport into the cell via different mechanism compared to NaB and B therefore affect the cell migration and cell cycle mechanism with different pathway as we concluded for ACHN cells. Moreover, compared to B treated ACHN cells, A498 did not arrested the cell migration and cell proliferation as much as ACHN cells, even though ACHN cell line is known for metastatic characteristics. Previous studies showed that *VHL* mutation cause less sensitivity to B treatment [193]. Therefore, our findings suggested that A498 cell line was not affected from B treatment on both proliferation and migration stages.

As it emphasized before, although, the migration assay and real-time PCR analysis revealed the working mechanism of these chemicals on three cell lines in a certain extent, in order to decipher their real effect, protein based assay such as western blotting or protein

activity assays have to be conducted. The variation of real time PCR analysis with scratch assay might be caused by derivation of Stat's phosphorylation.

Consequently, in this thesis, we aimed to establish a new treatment approach for renal cancer cells. This study showed for the first time that, different boron derivatives might be an effective carcinogenic treatment for renal cancer. Moreover, our results were the first findings and evidences about the mechanism of boron derivatives on cell proliferation, cell migration and cell death for ACHN, A498 and RPTEC cell lines. We had data demonstrating that NaB showed similar effect on renal cancer treatment with BA, therefore either NaB or BA could be used as a therapeutic drug instead of B, because NaB and BA were effective as much as B, but less toxic. For this purpose, the cancer types which B has already used for treatment could be chosen as a model cancer type such as mantle cell and follicular non-Hodgkin's lymphoma, peripheral T-cell lymphoma, Waldenström's macroglobulinemia, chronic lymphocytic leukemia, head and neck / gastroesophageal junction / stomach /colorectal / prostate / non-small cell lung cancer [194]. However, additional to this thesis results, protein based analysis should be conducted to have wide range of information about the effects of boron derivatives. As it was mentioned before, NaB, BA and B may affect the phosphorylation of proteins at their serine residues and cause their inactivation. Moreover, to analyze the real amount of Ca level in the cells compared to dose dependent treatments of ACHN, A498 and RPTEC is necessary to prove our Ca+2 increase dependent migration arrest via inactivation of c-MET.

6. REFERENCES

1. G. I. Evan, K. H. Vousden. Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411(6835):342-348, 2001.

2. J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, DM. Parkin, D. Forman, F. Bray. Cancer Incidence and Mortality Worldwide: IARC *CancerBase No. 11. Lyon, France: International Agency for Research on Cancer; GLOBOCAN 2012* v1.0, 2013.

3. D. Hanahan, R. A. Weinberg. Hallmarks of cancer: the next generation. *Cell*. 144(5): 646-674, 2011.

4. A. Greenberg, A. K. Cheung. Primer on kidney diseases. *Elsevier Health Sciences*, 2005.

5. Iworx Web Based Labs, http://www.iworx.com.cn/company2/WebToolsCD/Illustrations

/human_kidney/human_kidney_web2.jpg.

6. J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent, S. Rosso, J. W. W. Coebergh, H. Comber, F. Bray. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *European Journal of Cancer*, 49(6), 1374-1403, 2013.

7. J. C. Cheville, C. M. Lohse, H. Zincke, A. L.Weaver, M. L. Blute. Comparisons of outcome and prognostic features among histologic subtypes of renal cell carcinoma. *The American Journal of Surgical Pathology*, *27*(5), 612-624, 2003.

8. O. Iliopoulos. Molecular biology of renal cell cancer and the identification of therapeutic targets. *Journal of Clinical Oncology*, 24(35): p. 5593-5600, 2006.

9. C. M. Lohse, J.C. Cheville. A review of prognostic pathologic features and algorithms for patients treated surgically for renal cell carcinoma. *Clinics in Laboratory Medicine*, 25(2): p. 433-464, 2005.

10. J. A. Shapiro, M. A. Williams, N. S. Weiss, A. Stergachis, A. Z. LaCroix, W. E. Barlow. Hypertension, antihypertensive medication use, and risk of renal cell carcinoma. *American Journal of Epidemiology*, *149*(6), 521-530, 1999.

11. M. Gago-Dominguez, J. M. Yuan, J. E. Castelao, R. K. Ross, M. C. Yu. Regular use of analgesics is a risk factor for renal cell carcinoma. *British Journal of Cancer*, *81*(3), 542, 1999.

12. M. Peycelon, V. Hupertan, E. Comperat, R. Renard-Penna, C. Vaessen, P. Conort, M. Rouprêt. Long-term outcomes after nephron sparing surgery for renal cell carcinoma larger than 4 cm. *The Journal of Urology*, *181*(1), 35-41, 2009.

13. A. Mancuso, C. Sternberg. New treatments for metastatic kidney cancer. *The Canadian Journal of Urology*, 12: p. 66-70; discussion 105, 2005.

14. R. C. Flanigan, Steven C. Campbell, Joseph I. Clark, Maria M. Picken. Metastatic renal cell carcinoma. *Current Treatment Options in Oncology*, 4(5): p. 385-390, 2003.

15. A. Yagoda, D. Petrylak, and S. Thompson. Cytotoxic chemotherapy for advanced renal cell carcinoma. *The Urologic Clinics of North America*, 20(2): p. 303-321, 1993.

16. M. Kjaer, P. L. Frederiksen, S. Engelholm. Postoperative radiotherapy in stage II and III renal adenocarcinoma. A randomized trial by the Copenhagen Renal Cancer Study Group. *International Journal of Radiation Oncology Biology Physics*, *13*(5), 665-672, 1987.

17. H. Juusela, K. Malmio, O. Alfthan, K. J. Oravisto. Preoperative irradiation in the treatment of renal adenocarcinoma. *Scandinavian Journal of Urology and Nephrology*, *11*(3), 277-281, 1977.

18. J. C. Yang, R. M. Sherry, S. M. Steinberg, S. L. Topalian, D. J. Schwartzentruber, P. Hwu, S. A. Rosenberg. Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer. *Journal of Clinical Oncology*, *21*(16), 3127-3132, 2003.

19. M. P. Upton, R. A. Parker, A. Youmans, D. F. McDermott, M. B. Atkins. Histologic predictors of renal cell carcinoma response to interleukin-2-based therapy. *Journal of Immunotherapy*, 28(5): p. 488-495, 2005.

20. A. Kugler, G. Stuhler, P. Walden, G. Zöller, A. Zobywalski, P. Brossart, R. H. Ringert. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell–dendritic cell hybrids. *Nature Medicine*, 6(3): p. 332-336, 2000.

21. H. Haddad, and B. I. Rini. Current treatment considerations in metastatic renal cell carcinoma. *Current Treatment Options in Oncology*, 13(2): p. 212-229, 2012.

22. B. Zbar. Von Hippel-Lindau disease and sporadic renal cell carcinoma. *Cancer Surveys*, 25: p. 219-232, 1994.

23. S. C. Clifford, A. H. Prowse, N. A. Affara, C. H. Buys, E. R. Maher. Inactivation of the von Hippel–Lindau (VHL) tumour suppressor gene and allelic losses at chromosome arm 3p in primary renal cell carcinoma: Evidence for a VHL-independent pathway in clear cell renal tumourigenesis. *Genes, Chromosomes and Cancer*, 22(3): p. 200-209, 1998.

24. R. R. Raval, K. W. Lau, M. G. Tran, H. M. Sowter, S. J. Mandriota, J. L. Li, P. J. Ratcliffe. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Molecular and Cellular Biology*, 25(13): p. 5675-5686, 2005.

25. P. Vaupel, A. Mayer. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Reviews*, 26:225-39, 2007.

26. A. J. Pantuck, G. Zeng, A. S. Belldegrun, R. A. Figlin. Pathobiology, prognosis and targeted therapy for renal cell carcinoma: exploiting the hypoxia-induced pathway. *Clinical Cancer Research*, 9:4641-52, 2003.

27. W. Linehan, R. Srinivasan, L. Schmidt. The Genetic Basis of Kidney Cancer: a Metabolic Disease. *Nature Reviews Urology*, 7(5), 277-285, 2010.

28. G. L. Semenza. Targeting HIF-1 for cancer therapy. *Nature Reviews Cancer*, 3:721-32, 2003.

29. W. G Jr. Kaelin. The von Hippel-Lindau protein, HIF hydroxylation and oxygen sensing. *Biochemical and Biophysical Research Communucations.*, 338:627-38, 2005.

30. W. G. Kaelin, The von Hippel-Lindau tumor suppressor protein: roles in cancer and oxygen sensing. *Cold Spring Harbor Symposia Quantitative Biology*, 70:159-66, 2005.

31. L. C. Kim, L. Song, E. B. Haura. Src kinases as therapeutic targets for cancer. *Nature Reviews Clinical Oncology*, 6:587-95, 2009.

32. WY. Kim, WG. Kaelin. Molecular pathways in renal cell carcinoma-rationale for targeted treatment. *Seminars in Oncology*, 33: 588-95, 2006.

33. A. C. Mena, E. G. Pulido, C. Guillen-Ponce. Understanding the molecular-based mechanism of action of the tyrosine kinase inhibitor: Sunitinib. *Anticancer Drugs*, 21(suppl 1):S3–S11, 2010.

34. E. Laughner, P. Taghavi, K. Chiles, P. C. Mahon, G.L. Semenza. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1 (HIF-1) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Molecular and Cellular Biology*, 21:3995–4004, 2001.

35. R. Fukuda, K. Hirota, F. Fan, Y. D. Jung, L. M. Ellis, G. L. Semenza. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *The Journal of Biologial Chemistry*, 277:38205–11, 2002.

36. E. Metzen, J. Zhou, W. Jelkmann, J. Fandrey, B. Brune. Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. *Molecular and Cellular Biology*, 14:3470–81, 2003.

37. T. Hagen, C. T. Taylor, F. Lam, S. Moncada. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF-1α. *Science*, 302:1975–8, 2003.

38. M. Callapina, J. Zhou, S. Schnitzer, Eric Metzen, Christian Lohr, Joachim W. Deitmer, Bernard Brüne. Nitric oxide reverses desferrioxamine- and hypoxia-evoked HIF-1α accumulation—implications for prolyl hydroxylase activity and iron. *Experimental Cell Research*, 306:274–84, 2005.

39. B. L. Krock, N. Skuli and M. C. Simon. Hypoxia-Induced Angiogenesis: Good and Evil, *Genes & Cancer*, 2(12) 1117–1133, 2011.

40. M. Sun, S. F. Shariat, C. Cheng, V. Ficarra, M. Murai, S. Oudard, P. I. Karakiewicz. Prognostic factors and predictive models in renal cell carcinoma: a contemporary review. *European Urology*, *60*(4), 644-661, 2011.

41. B. I. Rini. VEGF-Targeted Therapy in Metastatic Renal Cell Carcinoma, *The Oncologist*, 10(3), 191-197, 2011.

42. Y. Ben-Yosef, A. Miller, S. Shapiro, N. Lahat. Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. *American Journal of Physiology Cell Physiology*, 289:C1321-31, 2005.

43. K. Kaira, H. Murakami, M. Serizawa, Y. Koh, M. Abe, Y. Ohde, N. Yamamoto. MUC1 expression in thymic epithelial tumors: MUC1 may be useful marker as differential diagnosis between type B3 thymoma and thymic carcinoma. *Virchows Archiv*, 458:615–20, 2011.

44. H. K. Zhang, Q. M. Zhang, T. H. Zhao, Y. Y. Li, Y. F. Yi. Expression of mucins and E-cadherin in gastric carcinoma and their clinical significance. *World Journal of Gastroenterology*, 10:3044–7, 2004.

45. Y. Hinoda, Y. Ikematsu, M. Horinochi, S. Sato, K. Yamamoto, T. Nakano. Increased expression of MUC1 in advanced pancreatic cancer. *Journal of Gastroenterology*, 38:1162–6, 2003.

46. T. Arai, K. Fujita, M. Fujime, T. Irimura. Expression of sialylated MUC1 in prostate cancer: relationship to clinical stage and prognosis. *International Journal of Urology*, 12:654–61, 2005.

47. E. Lacunza, M. Baudis, A. G. Colussi, A. Segal-Eiras, M. V. Croce, M. C. Abba. MUC1 oncogene amplification correlates with protein overexpression in invasive breast carcinoma cells. *Cancer Genetics and Cytogenetics*, 201:102–10, 2010.

48. L. Wang, J. Ma, F. Liu, Q. Yu, G. Chu, A. C. Perkins, Y. Li. Expression of MUC1 in primary and metastatic human epithelial ovarian cancer and its therapeutic significance. *Gynecologic Oncology*, 105:695–702, 2007.

49. S. Nagai, K. Takenaka, M. Sonobe, E. Ogawa, H. Wada, F. Tanaka. A novel classification of MUC1 expression is correlated with tumor differentiation and postoperative prognosis in non-small cell lung cancer. *Journal of Thoracic Oncology*, 1:46–51, 2006.

50. S. L. Organ, M. S. Tsao. An overview of the c-MET signaling pathway. *Therapeutic Advances in Medical Oncology*, *3*(1 suppl), S7-S19, 2011.

51. S. Pennacchietti, P. Michieli, M. Galluzzo, M. Mazzone, S. Giordano, P. M. Comoglio. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*, *3*(4), 347-361, 2003.

52. T. R. Hartman, E. Nicolas, A. Klein-Szanto, T. Al-Saleem, T. P. Cash, M. C. Simon, E. P. Henske. The role of the Birte-HoggeDubé protein in mTOR activation and renal tumorigenesis. *Oncogene*, 28:1594 –1604, 2009.

53. J. Chen, K. Futami, D. Petillo. Deficiency of FLCN in mouse kidney led to development of polycystic kidneys and renal neoplasia. *PLoS One*, 3:e3581, 1– 8, 2008.

54. E. M. Boon, R. van der Neut, M. van de Wetering, H. Clevers, S.T. Pals. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. *Cancer Res*earch 62: 5126-5128, 2002.

55. J. A. Epstein, D. N. Shapiro, J. Cheng, P. Y. Lam, and R. L. Maas. Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proceeding of the National Academy of Science*, 93: 4213-4218, USA, 1996.

56. G. Gambarotta, C. Boccaccio, S. Giordano, M. Ando, M. C. Stella, and P. M. Comoglio. Ets upregulates MET transcription. *Oncogene*, 13: 1911-1917, 1996.

57. C. Boccaccio, G. Gaudino, G. Gambarotta, F. Galimi, P. M. Comoglio. Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *The Journal of Biological Chemistry*, 269: 12846-12851, 1994.

58. D. P. Bottaro, J. S. Rubin, D. L. Faletto, A. M. Chan, T. E. Kmiecik, G. F. Vande Woude, S. A. Aaronson. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science*, 251, 802-804, 1991.

59. L. Naldini, K. M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R. P. Narsimhan, G. Hartmann, R. Zarnegar, G. K. Michalopoulos, W. Birchmeier, P. M. Comoglio. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *The EMBO Journal.* 10, 2867-2878, 1991.

60. G. A. Rodrigues, M. A. Naujokas, M. Park. Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. *Molecular and Cellular Biology*, 11, 2962-2970, 1991.

61. A. Bardelli, C. Ponzetto, P. M. Comoglio. Identification of functional domains in the hepatocyte growth factor and its receptor by molecular engineering. *Journal of Biotechnology*. 37, 109-122, 1994.

62. L. Gandino, P. Longati, E. Medico, M. Prat, P. M. Comoglio. Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. *The Journal of Biological Chemistry*. 269, 1815-1820, 1994.

63. P. Peschard, N. Ishiyama, T. Lin, S. Lipkowitz, M. Park. A conserved DpYR motif in the juxtamembrane domain of the Met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required for suppression of oncogenic activation. *The Journal of Biological Chemistry*. 279, 29565-29571, 2004.

64. P. Longati, A. Bardelli, A. Petrelli, G.F. Gilestro, S. Lanzardo, P.M. Comoglio, N. Migone, S. Giordano. The endophilin-CIN85-Cbl complex mediates liganddependent downregulation of c-Met. *Nature.* 416: 187-190, 2002.

65. P. Peschard, T.M. Fournier, L. Lamorte, M.A. Naujokas, H. Band, W.Y. Langdon. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Molecular Cell* 8: 995-1004, 2001.

66. G. Maulik, A. Shrikhande, T. Kijima, P. C. Ma, P. T. Morrison, R. Salgia. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine and Growth Factor Reviews*, 13, 41-59, 2002.

67. C. Birchmeier, W. Birchmeier, E. Gherardi, G. F. Vande Woude. Met, metastasis, motility and more. *Nature Reviews Molecular Cell Biology*, 4, 915-925, 2003.

68. P. M. Comoglio, S. Giordano, L. Trusolino. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nature Reviews Drug Discovery*, 7, 504-516, 2008.

69. K. Kajiya, S. Hirakawa, B. Ma, I. Drinnenberg, M. Detmar. Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO Journal.* 24, 2885-2895, 2005.

70. S. Ding, T. Merkulova-Rainon, Z. C. Han, G. Tobelem. HGF receptor up-regulation contributes to the angiogenic phenotype of human endothelial cells and promotes angiogenesis in vitro. *Blood* 101, 4816-4822, 2003.

71. K. Kajiya, S. Hirakawa, B. Ma, I. Drinnenberg, M. Detmar. Hepatocyte growth factor promotes lymphatic vessel formation and function. *Embo Journal*, 24, 2885-2895, 2005.

72. W. Jung, E. Castren, M. Odenthal, G. F. Vande Woude, T. Ishii, H. P. Dienes, D. Lindholm, P. Schirmacher. Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain. *Journal of Cell Biology* 126, 485-494, 1994.

73. J. Okano, G. Shiotaand, H. Kawasaki. Expression of hepatocyte growth factor (HGF) and HGF receptor (c-met) proteins in liver diseases: an immunohistochemical study. *Liver* 19, 151-159, 1999.

74. T. E. Kmiecik, J. R. Keller, E. Rosen, G. F. Vande Woude. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. *Blood* 80, 2454-2457, 1992.

75. Y. Liu, F. L. Wilkinson, J. P. Kirton, M. Jeziorska, H. Iizasa, Y. Sai, E. Nakashima, A. M. Heagerty, A. E. Canfield, M. Y. Alexander. Hepatocyte growth factor and c-Met expression in pericytes: implications for atherosclerotic plaque development. *Journal of Pathology*. 212, 12-19, 2007.

76. C. T. Miller, L. Lin, A. M. Casper, J. Lim, D. G. Thomas, M. B. Orringer, A. C. Chang, A. F. Chambers, T. J. Giordano, T. W. Glover, D. G. Beer. Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. *Oncogene* 25, 409-418, 2006.

77. C. M. Stellrecht, C. J. Phillip, F. Cervantes-Gomez, V. Gandhi, Multiple myeloma cell killing by depletion of the MET receptor tyrosine kinase. *Cancer Research*. 67, 9913-9920, 2007.

78. S. Garcia, J. P. Dales, E. Charafe-Jauffret, S. CarpentierMeunier, L. Andrac-Meyer, J. Jacquemier, C. Andonian, M. N. Lavaut, C. Allasia, P. Bonnier, C. Charpin. Overexpression of c-Met and of the transducers PI3K, FAK and JAK in breast carcinomas correlates with shorter survival and neoangiogenesis. *International Journal of Oncology*, 31, 49-58, 2007.

79. N. Puri, S. Ahmed, V. Janamanchi, M. Tretiakova, O. Zumba, T. Krausz, R. Jagadeeswaran, R. Salgia. c-Met is a potentially new therapeutic target for treatment of human melanoma. *Clinical Cancer Research*. 13, 2246-2253, 2007.

80. P. L. Peghini, M. Iwamoto, M. Raffeld, Y. J. Chen, S. U. Goebel, J. Serrano, R. T Jensen. Overexpression of epidermal growth factor and hepatocyte growth factor receptors in a proportion of gastrinomas correlates with aggressive growth and lower curability. *Clinical Cancer Research*. 8, 2273-2285, 2002

81. R. Abounader, J. Laterra. Scatter factor/hepatocyte growth factor in brain tumor growth and angiogenesis. *Neuro-Oncology*, 7, 436-451, 2005.

82. G.A. Rodrigues, M. Park. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. *Oncogene* 9: 2019-2027, 1994.

83. C. Ponzetto, A. Bardelli, Z. Zhen, F. Maina, P. dalla Zonca, S. Giordano, A. Graziani, G. Panayotou, P. M. Comoglio. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* 77, 261-271, 1994.

84. C. Ponzetto, A. Bardelli, F. Maina, P. Longati, G. Panayotou, R. Dhand, M. D. Waterfield, P. M. Comoglio. A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. *Molecular and Cellular Biology*, 13, 4600-4608, 1993.

85. G. Maulik, A. Shrikhande, T. Kijima, P. C. Ma, P. T. Morrison, R. Salgia. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Reviews*, 13, 41-59, 2002.

86. C. Birchmeier, W. Birchmeier, E. Gherardi, G. F. Vande Woude. Met, metastasis, motility and more. *Nature Reviews Molecular Cell Biology*, 4, 915-925, 2003.

87. P. M. Comoglio, S. Giordano, L. Trusolino. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nature Reviews Drug Discovery*, 7, 504-516, 2008.

88. G. Maulik, P. Madhiwala, S. Brooks, P.C. Ma, T. Kijima, E.V. Tibaldi. Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer. *Journal of Cellular and Molecular Medicine*, 6: 539 553, 2002.

89. L. Gandino, L. Munaron, L. Naldini, R. Ferracini, M. Magni, P.M. Comoglio. Intracellular calcium regulates the tyrosine kinase receptor encoded by the MET oncogene. The *Journal of Biological Chemistry,* 266: 16098-16104, 1991.

90. S. J. Martin, D. R. Green. Protease activation during apoptosis: death by a thousand cuts? *Cell* 82, 349–352, 1995.

91. G. M. Cohen, X. M. Sun, H, Fearnhead M. MacFarlane, D. G. Brown, R. T. Snowden Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. *Journal of Immunology*, 153, 507–516, 1994.

92. K. H. Eum, M. Lee. Crosstalk between autophagy and apoptosis in the regulation of paclitaxel-induced cell death in v-Ha-rastransformed fibroblasts. *Molecular and Cellular Biochemistry*, 348, 61–68, 2011.

93. J. F. R. Kerr, A. H. Wyllie, A. R. Currie. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer,* 26, 239–257, 1972.

94. T. Engel, D.C. Henshall. Apoptosis, Bcl-2 family proteins and caspases: the ABCs of seizure-damage and epileptogenesis? *International Journal of Physiology, Pathophysiology and Pharmacology*, 1, 97–115, 2009.

95. A. Shamas-Din, H. Brahmbhatt, B. Leber, D. W. Andrews. BH3-only proteins: orchestrators of apoptosis. *Biochimica Biophysica Acta*, 1813, 508–520, 2011.

96. X. Wen, Z. Q. Lin, B. Liu, Y. Q. Wei. Caspase-mediated programmed cell death pathways as potential therapeutic targets in cancer. *Cell Proliferation,* 45, 217–224, 2012.

97. M. Karin F. R. Greten. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology*. 5, 749–759, 2005.

98. D. E. Nelson, A. E. Ihekwaba, M. Elliott, J. R. Johnson, C. A. Gibney, B. E. Foreman. Oscillations in NF-κB signaling control the dynamics of gene expression. *Science,* 306, 704–708, 2004.

99. L. Ouyang, Z. Shi, S. Zhao, F. T. Wang, T. T. Zhou, B. Liu, J. K. Bao. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation*, 2012, 45, 487–498

100. S. Benchimol. P53-dependent pathways of apoptosis. *Cell Death Differentiation*, 8, 1049–1051, 2001.

101. J. Yu, Z. Wang, K. W. Kinzler, B. Vogelstein, L. Zhang. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proceedings of the National Academy of Sciences*, 100, 1931–1936, 2003.

102. B. Liu, Y. Cheng, Q. Liu, J. K. Bao, J. M. Yang. Autophagic pathways as new targets for cancer drug development. *Acta Pharmacologica Sinica,* 31, 1154–1164, 2010.

103. J. J. Liu, M. Lin, J. Y. Yu, B. Liu, J. K. Bao. Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Letter,* 300, 105–114, 2011.

104. A. Huett, G. Goel, R. J. Xavier. A systems biology viewpoint on autophagy in health and disease. *Current Opinion in Gastroenterology*, 26, 302–309, 2010.

105. M. Kundu, C. B. Thompson. Autophagy: basic principles and relevance to disease. *Annual Review of Pathology,* 3, 427–455, 2008.

106. C. He, D. J. Klionsky. Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics,* 43, 67–93, 2009.

107. H. Akca, A. Demiray, M. Aslan, I. Acikbas, O. Tokgun. Tumour suppressor PTEN enhanced enzyme activity of GPx, SOD and catalase by suppression of PI3K/AKT

pathway in non-small cell lung cancer cell lines. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *28*(3), 539-544, 2013.

108. A. M. Rojas, G. Fuentes, A. Rausell, A. Valencia. The Ras protein superfamily: evolutionary tree and role of conserved amino acids. *The Journal of Cell Biology*, 196, 189–201, 2012.

109. Y. Jin, Y. Shima, M. Furu, T. Aoyama, T. Nakamata, T. Nakayama. Absence of oncogenic mutations of RAS family genes in soft tissue sarcomas of 100 Japanese patients. *Anticancer Research*, 30, 245–251, 2010.

110. T. K. Schlesinger, G. R. Fanger, T. Yujiri, G. L. Johnson. The TAO of MEKK. *Frontiers in Bioscience*, 3, 1181–1186, 1998.

111. L. Galluzzi, G. Kroemer, Necroptosis: a specialized pathway of programmed necrosis. *Cell* 135, 1161–1163, 2008.

112. P. Golstein, G. Kroemer. Cell death by necrosis: towards a molecular definition. *Trends in Biochemical Sciences*, 32, 37–43, 2007.

113. W. Wu, P. Liu, J. Li. Necroptosis: an emerging form of programmed cell death. *Critical Reviews in Oncology/Hematology*, 82, 249–258, 2012.

114. K. McCall. Genetic control of necrosis – another type of programmed cell death. *Current Opinion in Cell Biology*, 22, 882–888, 2010.

115. M. Leist, M. Jaattela, Four deaths and a funeral: from caspases to alternative mechanisms. *Nature Reviews Molecular Cell Biology*, 2, 589–598, 2001.

116. S. Bialik, E. Zalckvar, Y. Ber, A. D. Rubinstein, A Kimchi. Systems biology analysis of programmed cell death. *Trends in Biochemical Sciences*, 35, 556–564, 2010.

117. B. C. Das, P. Thapa, R. Karki, C. Schinke, S. Das, S. Kambhampati, T. Evans. Boron chemicals in diagnosis and therapeutics. *Future Medicinal Chemistry*, 5(6), 653-676, 2013.

118. K. Warington. The effect of boric acid and borax on the broad bean and certain other plants. *Annals of Botany*, 37:629-72, 1923.

119. D. G. Blevins, K. M. Lukaszewski. Born in plant structure and function. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49:481-500, 1988.

120. M. A. O Neil, S. Eberhand, P. Albersheim, A. G. Darvill. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science*, 294:846-9, 2001.

121. R. Barr, M. Bottger, F. L. Crane. The effect of boron on plasma memebrane electron transport and associated proton section by cultured carrot cells. *Biochemistry and Molecular Biology International*, 31:31-9, 1993.

122. T. A. Devirian, S. L. Volpe. The physiological effects of dietary boron. *Critical Reviews in Food Science and Nutrition*, 43:219-31, 2003.

123. K. Noguchi, M. Yasumori, T. Imai, S. Naito, T. Matsunaga, H. Oda, H. Hyashi, M. Chino, T. Fujiwara. *bor1-1*, an Arabidopsis thaliana mutant that requires a high level of boron. *Plant Phsysiology*, 115:901-6, 1997.

124. J. Takano, J. Takano, K. Noguchi, M. Yasumori, M. Kobayashi, Z. Gajdos, K. Miwa, H. Hayashi, T. Yoneyama, T. Fujiwara. Arabidopsis boron transporter for xylem loading. *Nature*, 420:337-40, 2002.

125. M. Park, Q. Li, N. Shcheynikov, W. Zeng, S. Muallen. NaBC1 isa ubiquitious electrogenic Na^{+}-Coupled borate transporter essential for boron homeostasis and cell growth and proliferation. *Molecular Cell*, 16:331-41, 2004.

126. M. Park, Q. Li, N. Shcheynikov, S. Muallem, W. Zeng. Borate transport and cell growth and proliferation: not only in plants. *Cell Cycle*, 4(1), 24-26, 2004.

127. X. C. Liu, J. L. Hubbard, W. H. Scouten. Synthesis and structural investigation of two potential boronate affinity chromatography ligands catechol [2-(diisopropylamino)carbonyl] phenylboronate and catechol [2-(diethylamino)carbonyl, 4-methyl]phenylboronate. *Journal of Organometallic Chemistry*, 493:91–94, 1995.

128. G. Springsteen, B. Wang. A detailed examination of boronic acid-diol complexation. *Tetrahedron*; 58:5291–5300, 2002.

129. S. L. Wiskur, J. J. Lavigne, H. Ait-Haddou, V. Lynch, Y. H. Chiu, J. W. Canary, E. V. Anslyn. pKa values and geometries of secondary and tertiary amines complexed to boronic acids—Implications for sensor design. *Organic Letters*, 3:1311–1314, 2001.

130. T. Devirian, S.Volpe, The physiological effects of dietary boron. *Critical Reviews in Food Science and Nutrition*, 43, 219-231, 2003.

131. C. Yan, M. I. Winton, Z. F. Zhang, C. Rainey, J. Marshall, J. B. De Kernion, C. D. Eckhert. Dietary boron intake and prostate cancer risk. *Oncology Reports*, 11, 887-892, 2004.

132. NHNES III, National Health and Nutrition Examination Survey, www.cdc.gov/nchs/nhanes/htm

133. M. T. Gallardo-Williams, R. E. Chapin,, P. E. King, G. J. Moser, T. L. Goldworthy, J. P. Morrison, R. R. Maronpot. Boron supplementation inhibits the growth and local expression of IGF-1 in human prostate adenocarcinoma (LNCaP) tumors in nude mice. *Toxicologic Pathology*, 32, 73-78, 2004.

134. R. I Scorei, R. Popa. Boron-containing compounds as preventive and chemotherapeutic agents for cancer. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents*, 10(4), 346-351, 2010.

135. W. T. Barranco, H. T. Kim, S. L, Stella Jr, C. D. Eckhert. Boric acid inhibits stored Ca2+ release in DU-145 prostate cancer cells. *Cell Biology and Toxicology*, 25, 309-320, 2009

136. S. L. Meacham, K. E. Elwell, S. Ziegler, S. W. Carper. Boric acid inhibits cell growth in breast and prostate cancer cell lines. *Advances in Plant and Animal Boron Nutrition*, S*pringer*, 299-306, 2007.

137. A. C. W. Pike, A. M. Brzozowski, E. R. Hubbard, T. Bonn, A. G. Thorsell, O. Engström, J. Ljunggren, J. A. Gustafsson, M. Mats-Carlquist. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO Journal, 18, 4608-4618, 1999.

138. S. Mahabir, M. R. Spitz, S. L. Barrera, Y. Q. Dong, C. Eastham, M. R. Forman. Dietary boron and hormone replacement therapy as risk factors for lung cancer in women. *American Journal of Epidemiology*, 167, 1070-1080, 2008.

139. W. T. Barranco, C. D. Eckhert, Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letter*, 216, 21-29, 2004.

140. A. S. Acerbo, L. Miller. Assessment of the chemical changes induced in human melanoma cells by boric acid treatment using infrared imaging. Analyst, 2009, 134, 1669-1674

141. K. Henderson, S. L. Stella Jr., S. Kobylewski1, C. D. Eckhert. Receptor activated Ca2+ release is inhibited by boric acid in prostate cancer cells. *Plos One*, 4(6), 1-10, 2009.

142. N. Shomron, G. Ast. Boric acid reversibly inhibits the second step of pre-mRNA splicing. *FEBS Letter*, 552, 219-224, 2003.

143. R. I Scorei, & R. Popa. Boron-containing compounds as preventive and chemotherapeutic agents for cancer. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *10*(4), 346-351, 2010.

144. Y. Wang, Y. Zhao, X. Chen. Experimental study on the estrogenlike effect of boric acid. *Biological Trace Element Research*, 121, 160- 170, 2008.

145. A. C. W. Pike, A. M. Brzozowski, E. R. Hubbard, T. Bonn, A. G. Thorsell, O. Engström, J. Ljunggren, J.A. Gustafsson, M. Mats-Carlquist. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO Journal*, 18, 4608-4618, 1999.

146. D. M. Parkin, C. S. Muir, S. L. Whelan, Y. T. Gao, J. Ferlay, J. Powell. *Cancer incidence in five continents, volume VI* (No. 120). International Agency for Research on Cancer, 1992.

147. G. Ursin, M. C. Pike, S. Preston-Martin, R. K. Peters. Sexual, reproductive, and other risk factors for adenocarcinoma of the cervix: results from a population-based case-control study (California, United States). *Cancer Causes & Control*, *7*(3), 391-401, 1996.

148. B. A. Teicher, G. Ara, R. Herbst, V. J. Palombella, J. Adams. The proteasome inhibitor PS-341 in cancer therapy. *Clinical Cancer Research, 5*(9), 2638-2645, 1999.

149. A. Palumbo, F. Gay, S. Bringhen, A. Falcone, N. Pescosta, V. Callea, T. Caravita, F. Morabito, V. Magarotto, M. Ruggeri, I. Avonto, P. Musto, N. Cascavilla, B. Bruno, M. Boccadoro. Bortezomib, doxorubicin and dexamethasone in advanced multiple myeloma. *Annals of Oncology*, 19, 1160-1165, 2008.

150. S. Gatto, B. Scappini, L. Pham, F. Onida, M. Milella, G. G Ball, C. Ricci, V. Divoky, S. Verstovsek, H. M. Kantarjian, M. J. Keating, J. E. Cortes-Franco, M. Beran. The proteasome inhibitor PS-341 inhibits growth and induces apoptosis in Bcr/Abl-positive cell lines sensitive and resistant to imatinib mesylate. *Haematology*, 88, 853-63, 2003.

151. A. M. Davies, P. N. Lara Jr., C. P. Mack, D. R. Gandara. Novel agents in the treatment of lung cancer. Incorporating Bortezomib into the treatment of lung cancer. *Clinical Cancer Research*, 13, 4647s-4651s, 2007.

152. A. Frankel, S. Man, P. Elliott, J. Adams, R. S. Kerbel. Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. *Clinical Cancer Research*, 6, 3719-3728, 2000.

153. S. T. Nawrocki, J. S. Carew, M. S. Pino, R. A. Highshaw, R. H. I. Andtbacka, K. Dunner, Jr., A. Pal, G. H. Bornmann, P. J. Chiao, P. Huang, H. Xiong, J. L. Abbruzzese, D. J. McConkey. Aggresome disruption: a novel strategy to enhance bortezomib- induced apoptosis in pancreatic cancer cells. *Cancer Research*, 66, 3773-3781, 2006.

154. R. J. Bold, S. Virudachalam, D. J. McConkey. Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. *Journal of Surgical Research*, 100, 11-17, 2001.

155. J. Adams. Inhibitors in Cancer Therapy, *Cell Death and Differentiation,* Humana Press; Totowa, New Jersey, 2004.

156. J. B. Sunwoo, Z. Chen, G. Dong, N. Yeh, B. C. Crowl, E. Sausville, J. Adams, P. Elliott, C. Van Waes, Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clinical Cancer Research*, 7, 1419-1428, 2001.

157. R. Z. Orlowski, D. J. Kuhn. Proteasome inhibitors in cancer therapy: lessons from the first decade. *Clinical Cancer Research*, 14(6), 1649–1657, 2008.

158. P. G. Richardson, P. Sonneveld, M. W. Schuster Reversibility of symptomatic peripheral neuropathy with bortezomib in the phase III APEX trial in relapsed multiple myeloma: impact of a dose-modification guideline. *British Journal of Haematology*, 144(6), 895–903, 2009.

159. L. R. Dick, P. E. Fleming. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discovery Today*, 15(5–6), 243–249, 2010.

160. J. R. Gnarra, K. Tory, Y. Weng, L. Schmidt, M. H. Wei, H. Li, F. Latif, S. Liu, F. Chen, F. M. Duh, I. Lubensky, D. R. Duan, C. Florence, R. Pozzatti, M. M. Walther, N. H. Bander, H. B. Grossman, H. Brauch, S. Pomer, J. D. Brooks, W. B. Isaacs, M. I. Lerman, B. Zbar, W. M. Linehan. Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nature Genetics*, 7:85–90, 1994.

161. T. Acker, A. Diez-Juan, J. Aragones, M. Tjwa, K. Brusselmans, L. Moons, D. Fukumura, M. P. Moreno-Murciano, J. M. Herbert, A. Burger, J. Riedel, G. Elvert, I. Flamme, P. H. Maxwell, D. Collen, M. Dewerchin, R. K. Jain, K. H. Plate, P. Carmeliet, Genetic evidence for a tumor suppressor role of HIF-2alpha, *Cancer Cell*, 8(2):131-41, 2005.

162. M. W. Roomi, J. C. Monterrey, T. Kalinovsky, M. Rath, A. Niedzwiecki, Patterns of MMP-2 and MMP-9 expression in human cancer cell lines. *Oncology reports*, *21*(5), 1323-1333, 2009.

163. C. Yan, M. I. Winton, Z. F. Zhang, C. Rainey, J. Marshall, J. B De Kernion, C. D. Eckhert. Dietary boron intake and prostate cancer risk. *Oncology Reports*, 11, 887-892, 2004.

164. F. Xu, H. E. Goldbach, P. H. Brown, R. W. Bell, T. Fujiwara, C. D. Hunt, L. Shi. *Advances in Plant and Animal Boron Nutrition*. Dordrecht: Springer, 2007.

165. S. Mahabir, M. R. Spitz, S. L. Barrera, Y. Q. Dong, C. Eastham, M. R. Forman. Dietary boron and hormone replacement therapy as risk factors for lung cancer in women american. *Journal of Epidemology*, 167, 1070-1080, 2008.

166. R. E. Newnham. Agricultural practices affect arthritis. *Nutrition and Health*, 7, 89-100, 1991.

167. R. E. Newnham. Essentiality of boron for healthy bones and joints. *Environmental Health Perspectives*, 102, 83-85, 1994.

168. J. G. Penland. Ditary boron, brain function, and cognitive performance. *Environmental Health Perspectives*, 102, 65-72, 1994.

169. J. G. Penland. The importance of boron nutrition for brain and psychological function. *Biological Trace Element Research*, 66, 299-317, 1998.

170. P. Xu, W. B. Hu, X. Guo, Y. G. Zhang, Y. F. Li, J. F. Yao, O. K. Cai. Therapeutic effect of dietary boron supplement on retinoic acid-induced osteoporosis in rats. *Nan Fan Yi Ke Da Xue Xue Bao*, 26, 1785-1788, 2006.

171. X. Peng, Z. Lingxil, G. N. Schrauzer, G. Xiong. Selenium, boron and germanium deficiency in the etiology of Kashin-Beck disease. *Biological Trace Element Research*, 77, 193-197, 2000.

172. J. H. Beattie, S. H. Peace. The influence of a low-boron diet and boron supplementation on bone, major mineral and sex steroid metabolism in postmenopausal women. *British Journal of Nutrition*, 69, 871-884, 1993.

173. Y. Cui, M. I. Winton, Z. F. Zhang. Dietary boron intake and prostate cancer risk. *Oncology Reports*, 11:887-892, 2004.

174. K. Henderson, S. L. Stella, S. Kobylewski. Receptor activated Ca (2+) release is inhibited by boric acid in prostate cancer cells. *PLoS One*, 4:e6009. 2009

175. I. R. Scorei. Calcium fructoborate: plant based dietary boron as potential medicine for cancer therapy. *Frontiers in Bioscience*, 3:205-215, 2011.

176. J. A. Jansen, J. Anderson, J. S. Schou. Boric acid single dose pharmacokinetics after intravenous administration to man. *Archives of Toxicology*, 55:64-67, 1984.

177. J. A. Jansen, J. S. Schou, B. Aggerbeck. Gastro-intestinal absorbtion and in vitro release of boric acid from water-emulsifying ointment. *Food and Chemical Toxicology*, 22:49-53, 1984.

178. R. Von Burg. Boron, boric acid and boron oxide and boric acid dusts. *Journal of Occupational Medicine*, 26:584-586, 1984.

179. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Food and Nutrition Board, Institute of Medicine Washington, DC: National Academies Press, 2001.

180. J. An, M. Fisher, M. B. Rettig. VHL expression in renal cell carcinoma sensitizes to bortezomib (PS-341) through an NF-kappaB-dependent mechanism. *Oncogene* 24:1563–1570, 2005.

181. W. T. Barranco, C. D. Eckhert. Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letters*, 216(1):21-9, 2004.

182. F. Akbas, Z. Aydin. Boric acid increases the expression levels of human anion exchanger genes SLC4A2 and SLC4A3, *Genetics and molecular research*, 11 (2): 847-854, 2012.

183. J. Masumoto, N. Inohara. The molecular functions of Nod proteins and their associated diseases. *Current Medicinal Chemistry. Anti-inflammatory & Anti-allergy Agents*, 4: 43–51, 2005.

184. V. Baud, M. Karin. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nature Reviews Drug Discovery*, 8:33-40, 2009.

185. N. D. Perkins, T. D. Gilmore. Good cop, bad cop, the different faces of NF-kappaB. *Cell Death & Differentiation*, 13:759-72, 2006.

186. K. M. Ryan M. K. Ernst, N. R. Rice, K. H. Vousden. Role of NF-kappaB in p53-mediated programmed cell death. *Nature*, 404:892-7, 2000.

187. P. J. Elliot, J. S. Ross. Proteasome: A New Target for Novel Drug Therapies. *American Journal of Clinical Pathology*, 116(5), 2001.

188. H. E. Warburton, M. Brady, N. Vlatković , W. M. Linehan, K. Parsons, M. T. Boyd. p53 regulation and function in renal cell carcinoma. Cancer Research, 65(15):6498-503, 2005.

189. R. Scorei, R. Ciubar, C. M. Ciofrangeanu, V. Mitran, A. Cimpean, D. Iordachescu. Comparative effects of boric acid and calcium fructoborate on breast cancer cells. *Biological Trace Element Research*, 122(3), 197-205, 2008.

190. T. M. Bradke, C. Hall, S. W. Carper, G. E. Plopper. Phenylboronic acid selectively inhibits human prostate and breast cancer cell migration and decreases viability. *Cell Adhesion & Migration*, 2(3), 153-160, 2008.

191. G. Konjević, S. Radenković, A. Vuletić, K. M. Martinović, V. Jurišić, T. Srdić. STAT Transcription Factors in Tumor Development and Targeted Therapy of Malignancies, 2013.

192. M. Boissinot, C. Cleyrat, M. Vilaine, Y. Jacques, I. Corre, S. Hermouet. Anti-inflammatory cytokines hepatocyte growth factor and interleukin-11 are over-expressed in Polycythemia vera and contribute to the growth of clonal erythroblasts independently of JAK2V617F. *Oncogene,* 30(8), 990-1001, 2010.

193. J. An, M. Fisher, M. B. Rettig. VHL expression in renal cell carcinoma sensitizes to bortezomib (PS-341) through an NF-kappaB-dependent mechanism. *Oncogene,* 24:1563–1570, 2005.

194. A. M. Roccaro, A. Vacca, D. Ribatti. Bortezomib in the treatment of cancer. *Recent patents on anti-cancer drug discovery*, *1*(3), 397-403, 2006.