AMERICAN UNIVERSITY OF BEIRUT

CROSSTALK BETWEEN BRADYKININ AND RETINOIC ACID RECEPTORS SIGNALING PATHWAYS IN COLORECTAL CANCER CELLS

by
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AN ABSTRACT OF THE THESIS OF

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Title: Crosstalk between Bradykinin and Retinoic Acid Receptors Signaling Pathways in Colorectal Cancer Cells

Colorectal cancer is a main cause of morbidity and mortality worldwide and chronic inflammation is key in its development and progression. Bradykinin (BK), a vasoactive peptide, is implicated in inflammation-induced tumor progression of many types of cancer where it exerts its action through specific G-protein coupled receptor. On the other hand, all-*trans* retinoic acid (ATRA), the active metabolite of vitamin A, mediates essential cellular functions including an anti-inflammatory role in various cell types; and abnormalities in the retinoid receptors signaling are commonly observed in tumorigenesis. Hence, we investigated the potential crosstalk between BK and retinoic acid receptors (RARs) signaling pathways and how it is translated in various cellular functions related to colorectal cancer formation and progression.

In the present study, we tested the effect of BK and/or ATRA on cell proliferation and anchorage-independent growth of colorectal cells, and on the modulation of bradykinin receptor B2 (B2R) and RARγ protein profile and their subcellular localization. We also evaluated the effect of BK and ATRA on the modulation of inflammatory, differentiation, and mesenchymal markers, and on downstream signaling transduction proteins.

We showed that BK increased growth of colorectal cancer cell lines (HCT116 and HCT116 $p53^{-/-}$), while having an opposite effect on the normal-like colon cell line (NCM460) using the MTT assay. In addition, BK induced colony growth of the tested cancer cells, through B2R, while sparing the normal-like counterparts, while ATRA abrogated BK effect in soft agar colony formation assay. We observed by western blot analysis differential protein profile of B2R, RARγ, E-cadherin, and fibronectin upon treatment with BK, ATRA, and their combination. Interestingly, BK treatment resulted in an activation of ERK1/2 in NCM460 and HCT116 and activation of AKT in NCM460 cells only.

These findings might be of considerable significance in uncovering novel mechanism, biomarkers and therapeutic strategies in colorectal cancer that target BK and RARs signaling.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
9cRA	9-cis-retinoic acid
AKT	protein kinase B
AMP	adenosine monophosphate
APC	*Adenomatous Polyposis Coli*
APL	acute promyelocytic leukemia
ATRA	all-trans-retinoic acid
B1R	bradykinin receptor B1
B2R	bradykinin receptor B2
BK	bradykinin
ºC	Celsius
CAC	colitis-associated cancer
CAF	cancer-associated fibroblast
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary deoxyribonucleic acid
Cm	centimeter
COX-2	cyclooxygenase-2
CPM	carboxypeptidase M
CPN	carboxypeptidase N
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein

CT	cycle threshold
CVD	cardiovascular disease
CYP26	cytochrome P26
CYP450	cytochrome P450
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FN	fibronectin
g	gram
GPCR	G-protein-coupled receptor
GSK3	glycogen synthase kinase 3
HAT	histone acetyltransferase
HDAC	histone deacetylase
HKa	bradykinin-free-high molecular weight kininogen
HMWK	high molecular weight kininogen
IBD	inflammatory bowel disease
IL-6	interleukin-6
KD	kallidin
KKS	kallikrein-kinin system
KLK	kallikrein
KNG	kininogen

LMWK	low molecular weight kininogen
LPS	lipopolysaccharide
LRAT	lecithin retinol acyltransferase
LXR	liver X receptor
mg/ml	milligram per milliliter
mM	millimolar
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
mRNA	messenger ribonucleic acid
MSK1	mitogen- and stress-activated kinase 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NF-κB	nuclear factor κB
ng/ml	nanogram per milliliter
nM	Nanomolar
NP-40	nonidet P-40
NSAID	non-steroidal anti-inflammatory drug
NUP98	nucleoporin 98
O.D.	optical density
p38 MAPK	p38 mitogen-activated protein
PBMC	peripheral blood mononuclear cell
pg/ml	picogram per milliliter
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
pKLK	plasma kallikrein

PMA	phorbol-12-myristate-13-acetate
PML	promyelocytic leukemia
PPARγ	peroxisomal proliferator-activated receptor
RA	retinoic acid
RALDH	retinal dehydrogenase
RARE	retinoic acid response element
RAR	retinoic acid receptor
RBP	retinol-binding protein
RDH	retinol dehydrogenase
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
RXR	retinoid X receptor
SD	standard deviation
SDR	short-chain dehydrogenase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Src	sarcoma
SSP	sessile serrated polyp
TGF β1	transforming growth factor beta 1
TGF β2	transforming growth factor beta 2
tKLK	tissue kallikrein
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	thyroid hormone receptor
UC	ulcerative colitis

U/ml	unit per milliliter
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
μg	microgram
μg/ml	microgram per milliliter
μL	microliter
μM	micromolar

CHAPTER I

INTRODUCTION

A. Colorectal Cancer

1. Incidence and Mortality of Colorectal Cancer

Cancer is the second leading cause of death worldwide just after cardiovascular diseases (CVDs) (WHO, 2017). In particular, colorectal cancer is classified as the third most diagnosed malignancy and the fourth principal cause of cancer-related death throughout the world. One million new colorectal cancer cases are approximately occurring each year, and it is estimated to have more than 2.2 million new cases and 1.1 million cancer deaths by 2030 (Arnold et al., 2017, Arvelo et al., 2015). Half of the diagnosed cases are expected to progress to metastatic cancer, and the survival rates for 50 to 60% of colorectal cancer patients are approximately five years. Colorectal cancer incidence is slightly more dominant in men; it occurs third after lung and prostate cancer among males and second after breast cancer among females (Figure 1) (Arvelo et al., 2015). The risk for colorectal cancer rises with age yet taking into consideration that the percentage of those younger than 50 years and which are diagnosed with colorectal cancer has increased from 6% in 1990 to 11% in 2013 as determined by the American Cancer Society in 2017. Colorectal cancer incidence and mortality vary across the regions: the highest incidence rates reside in the more developed countries such as Australia, New Zealand, United States, and Western Europe, while the highest mortality rates are in Central and Eastern Europe (Figure 1) (Rabeneck et al., 2015). However, incidence and mortality of colorectal cancer are increasing rapidly in many

low and middle-income countries while unchanging or decreasing only in the high-income countries (Arnold et al., 2017).

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	746	374	1953	614	320	1590	1361	694	3544
More developed regions	399	175	1164	338	158	966	737	333	2130
Less developed regions	347	198	789	276	163	624	624	361	1414
WHO Africa region (AFRO)	16	11	32	15	11	31	31	22	63
WHO Americas region (PAHO)	125	57	362	121	55	342	246	112	705
WHO East Mediterranean region (EMRO)	18	12	40	15	10	33	33	21	73
WHO Europe region (EURO)	255	120	686	216	108	573	471	228	1258
WHO South-East Asia region (SEARO)	68	48	122	52	37	93	120	85	216
WHO Western Pacific region (WPRO)	264	125	711	195	100	518	460	225	1229
IARC membership (24 countries)	418	187	1181	351	167	976	769	353	2157
United States of America	69	29	214	65	27	199	134	55	413
China	147	79	338	107	60	245	253	139	583
India	37	28	50	27	21	37	64	49	87
European Union (EU-28)	193	83	536	152	69	417	345	152	953

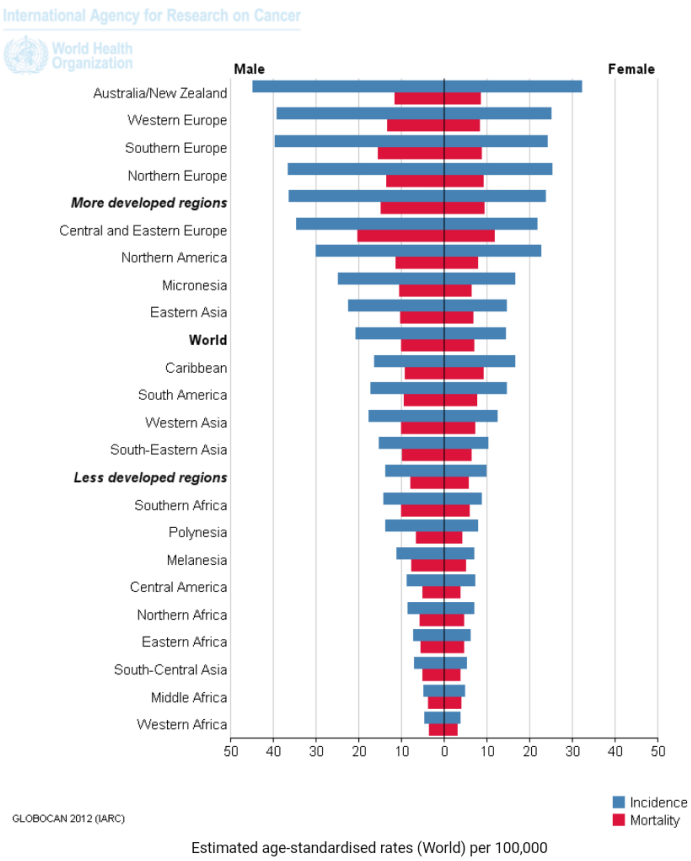


Figure 1. Estimated incidence, mortality, and prevalence of colorectal cancer in 2012. Colorectal cancer rates vary by gender and across regions. They are higher in men (746,000 cases, 10.0% of the total) than in women (614,000 cases, 9.2% of the total). Approximately, 55% of colorectal cancer cases reside in the more developed countries. (Adopted from (Bray et al., 2013, Ferlay J, 2012)).

2. Development of Colorectal Cancer

Development of colorectal cancer is, in general, a multistep process evolving from a slow accumulation of histological, morphological, genetic, and epigenetic

changes within benign polyps. These polyps start as aggregations of abnormal cells at the intestinal mucosa, the innermost lining of the large intestine, and protrude into the intestinal lumen. The growing cells inside the polyps can acquire with time enough mutations allowing them to invade the bowel wall, which is a hallmark of colorectal cancer. Finally, when these cells become more transformed, they can reach the regional lymph nodes and then other adjacent or distant metastatic sites (Figure 2A). Not all polyps become malignant, only a small percentage of them progress slowly to cancer over many years, and even a decade (Simon, 2016).

Adenomas and sessile serrated polyps (SSPs) are the main two types of malignant polyps. These two types differ by their histological appearance, malignant potential, and biological pathways leading to cancer formation. 60 to 70% of colorectal cancer develop from adenomas while the remaining progress from SSPs (Simon, 2016).

The accumulation of genetic and epigenetic mutations results in the histological changes from benign polyps to cancer. Two main pathways lead to colorectal cancer formation, each corresponding to the two types of polyps: adenomas and SSPs (Simon, 2016). The chromosomal instability pathway is, in general, related to traditional adenomas. The first mutations occurring are in the tumor suppressor *APC* (*Adenomatous Polyposis Coli*) gene that plays a central role in many cellular processes such as cell adhesion, migration and chromosome segregation, followed by mutations in the *KRAS* oncogene which are implicated in cell proliferation, differentiation, survival, and apoptosis (Aoki and Taketo, 2007, Migliore et al., 2011, Simon, 2016). With time, the loss of *p53* gene, the "guardian of the genome" and key regulator of cell cycle and apoptosis, can be a result of these genetic mutations leading eventually to carcinogenesis. On the other hand, serrated pathway begins with mutations in the *BRAF*

serine/threonine kinase gene involved in directing growth signaling. Alongside genetic mutations, aberrant gene promoter hypermethylation is common in serrated-based colorectal cancer. This epigenetic modification leads to the inhibition of gene transcription and deactivation of many genes including the regulators of other growth-promoting genes (Simon, 2016). In addition, microsatellite instability (Mishra et al.) is a hallmark of colorectal cancer. This genomic instability subtype is found in both adenomas and SSPs and is caused by defective DNA repair apparatus. As a result, uneven replication of microsatellites noncoding regions takes place increasing the chances for additional genetic mutations (Figure 2B) (Samowitz, 2008, Simon, 2016).

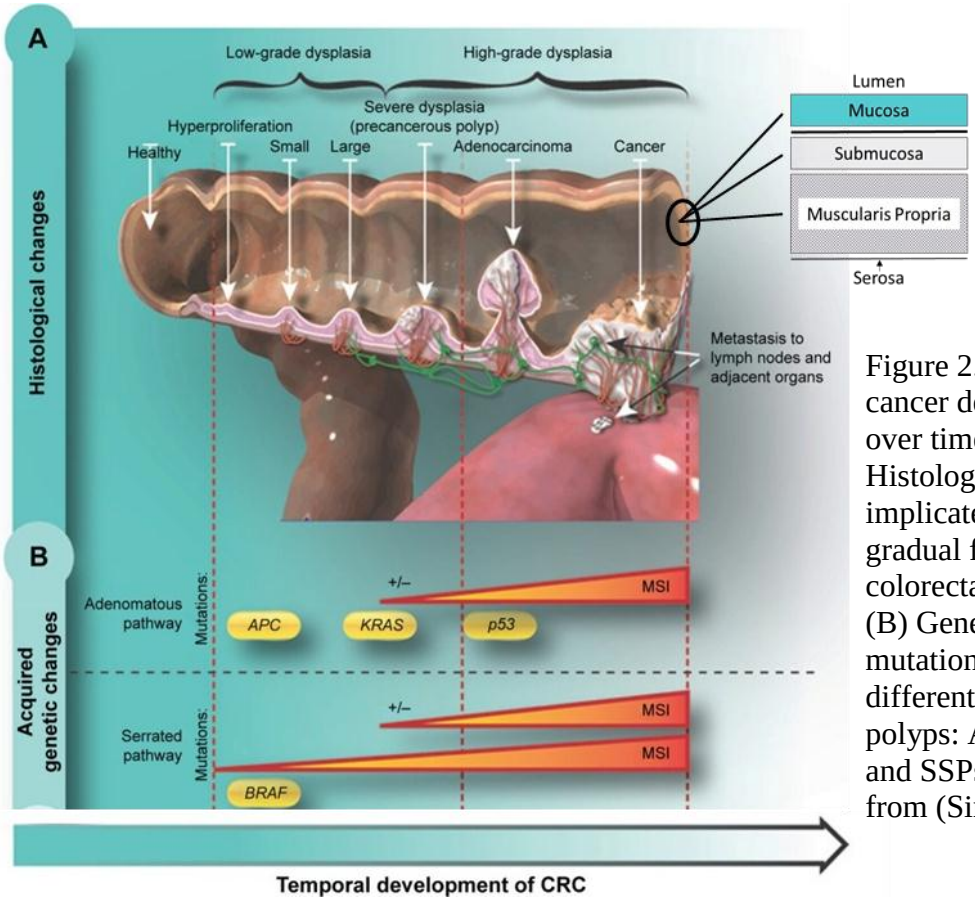


Figure 2. Colorectal cancer development over time. (A) Histological changes implicated in the gradual formation of colorectal cancer. (B) Genetic mutations in the two different types of polyps: Adenomas and SSPs. (Modified from (Simon, 2016)).

3. Risk Factors and Treatments for Colorectal Cancer

Some risk factors are associated with an increased chance of developing colorectal cancer. They can be divided as such: modifiable and nonmodifiable risk factors. The modifiable acquired risk factors include diet, obesity, lack of physical activity, tobacco use, and moderate-to-heavy alcohol use (Simon, 2016). On the other hand, the nonmodifiable risk factors are those of which the person cannot control such as personal and/or family history of polyps or colorectal cancer, personal history of inflammatory bowel disease (IBD) like ulcerative colitis (UC) (Blais et al., 2000) and Crohn's disease, familial syndrome (inheriting gene mutations), and type 2 diabetes. In addition, the risk of developing colorectal cancer increases with age, significantly after 50 years (Haggar and Boushey, 2009, Simon, 2016). Taking all those factors into consideration, regular screening and monitoring persons who are considered at risk and above 50 years offer a better chance for colorectal cancer prevention and early detection (Simon, 2016).

Treatment of colorectal cancer depends on its type and stage at diagnosis. There are several standard therapeutics methods for colorectal cancer that might be performed alone or in combination. Surgery is the most common therapy for colorectal cancer. Adjuvant therapy might be given after surgery to reduce the chances of colorectal cancer recurrence. Other therapeutic options include cryosurgery (freezing abnormal tissues), chemotherapy (giving drugs to stop cancer cell growth), radiation therapy (using radiations to destroy cancer cells), and targeted therapy (using treatments that are specific to cancer cells while sparing normal ones) (Board, 2002).

4. Colorectal Cancer and Inflammation

Rudolf Virchow, 150 years ago, first described a possible relation between inflammation and cancer when he identified infiltration of leukocytes within tumors. However, clear evidence elucidating the correlation between inflammation and tumorigenesis, and the molecular mechanisms underlying it were only revealed during the last decade (Grivennikov et al., 2010). Inflammation translated with the interaction between multiple players including immune cells, cytokines, chemokines, growth factors, reactive oxygen, and nitrogen leads to autocrine and paracrine signaling that might enhance the risk of cancer (Janakiram and Rao, 2014). In fact, these different inflammatory mediators can participate in tumorigenesis by sustaining continuous growth, signal molecules to evade apoptosis, proangiogenic factors, and enzymes involved in epithelial-mesenchymal transition (EMT). In addition, they can contribute to genome instability and reprogramming of cellular metabolism which ultimately serves cancer promotion and development (Landskron et al., 2014).

Several types of cancer arise due to prolonged inflammation or presence of characteristics of chronic inflammation during their progression; and it is now well established that an inflammatory microenvironment is a hallmark of cancer (Lasry et al., 2016). Even when linked to genetic mutations, colorectal cancer best exemplifies the correlation between inflammation and tumorigenesis (in particular chronic inflammation). This relationship is proved by the decrease in colorectal cancer mortality when non-steroidal anti-inflammatory drugs (NSAIDs) are regularly used (Janakiram and Rao, 2014). Moreover as previously mentioned, it is well established that patients with IBD are at higher risk of colorectal cancer (Landskron et al., 2014). Many inflammatory bioactive players can influence colorectal cancer establishment, one

of which is interleukin-6 (IL-6). This cytokine is secreted by many types of cells including fibroblasts, monocytes, macrophages, and various cancerous cells. In many studies, IL-6 levels were found to be increased in the serum and within the tumor of patients suffering from colorectal cancer (Waldner et al., 2012). Moreover, IL-6 levels were shown to correlate with the stage, size, metastasis, prognosis of colorectal cancer, and also survival of patients suffering from this disease. By driving several processes such as cell growth, migration, and angiogenesis, it is now well established that IL-6 is a tumor-promoting cytokine (Nagasaki et al., 2014, Waldner et al., 2012).

B. Kallikrein-Kinin System

1. Overview

The kallikrein-kinin system (KKS) was discovered in 1909 when Abelous and Bardier found signs of hypotension in dogs injected with human urine, a causal factor that was later recognized as the tissue kallikrein (Marceau and Regoli, 2004, Moreau et al., 2005). KKS is a complex system of a multi-protein cascade resulting in the release of vasoactive kinins (bradykinin-related peptides) (Moreau et al., 2005). This system includes a family of kallikrein serine proteases, precursor kininogens, kinin peptides, and B1 and B2 G-protein-coupled receptors. KKS has been extensively studied due to its implication in inflammation, smooth muscle contraction, cardiovascular homeostasis, vascular permeability, natriuresis, coagulation, fibrinolysis, pain, and regulation of the expression of some pro-inflammatory genes (Kashuba et al., 2013, Marceau and Regoli, 2004, Savard et al., 2008).

2. Structure and Organization of the Kallikrein-Kinin System

Kinin peptides are generated by two main classical pathways: the plasma and the tissue KKS, where each pathway can elucidate different physiological and pathological functions (Moreau et al., 2005). In general, kininogenase enzymes, known as kallikreins (KLKs), release kinin peptides by cleavage of kininogen substrates (KNGs). Kinins can further be processed by several kininases such as carboxypeptidases. These various released peptides in the KKS cascade mediate their actions through kinin G-protein-coupled receptors (GPCRs) (Figure 3) (da Costa et al., 2014).

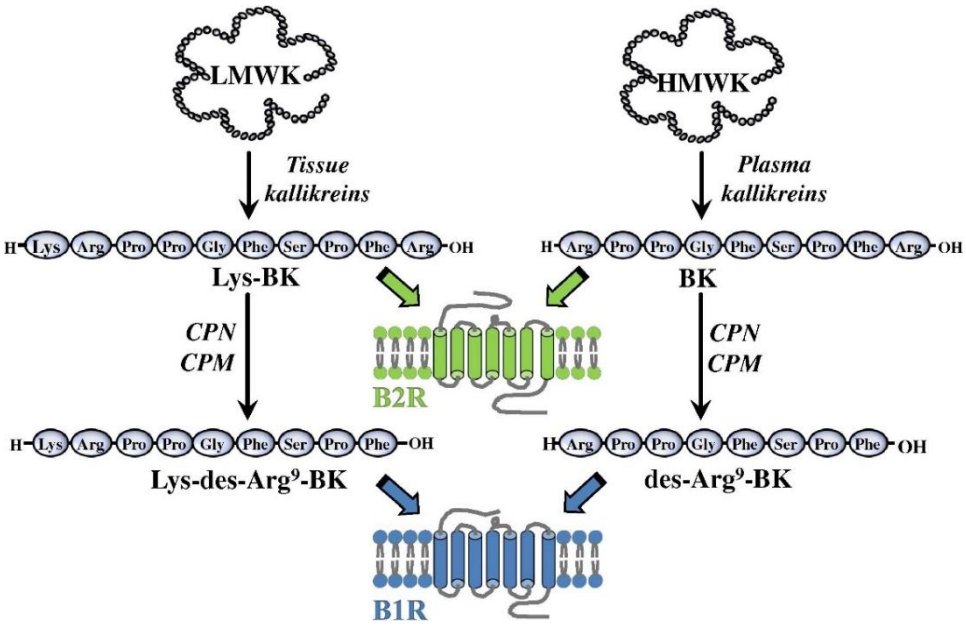


Figure 3. Schematic representation of the kallikrein-kinin system. Plasma and tissue kallikreins cleave HMWK and LMWK respectively generating the ligands of B2R: BK and Lys-BK respectively. BK and Lys-BK can further be cleaved by CPN and CPM kininases generating the agonists of B1R: des-Arg^9-BK and Lys-des-Arg^9-BK respectively.
HMWK - high molecular weight kininogen; LMWK - low molecular weight kininogen; BK - bradykinin; carboxypeptidase N (CPN); carboxypeptidase M (CPM); B2R - bradykinin receptor B2; B1R - bradykinin receptor B1. (Adopted from (da Costa et al., 2014)).

Human KNGs are multi-domain protein substrates synthetized predominately in the liver with minor expression in other extrahepatic tissues (Kashuba et al., 2013). In humans, two different isoforms of KNGs are known: the high-molecular-weight kininogen (HMWK) and the low molecular weight kininogen (LMWK), both of which are derived from the alternative splicing of a single gene (Howl and Payne, 2003, Kashuba et al., 2013). KNGs, circulating at high levels in plasma, are cleaved by mainly two known kininogenases: plasma KLK (pKLK) and tissue (glandular) KLK (tKLK) (Blais et al., 2000, Rhaleb et al., 2011).

pKLK and tKLK are two serine proteases that possess distinct biochemical and immunologic properties and may even elucidate different functions (Blais et al., 2000). pKLK is synthesized and secreted mainly by hepatocytes as an inactive precursor called prekallikrein circulating either as a heterodimer complexed to HMWK (about 80%) or as a free protein (about 20%) (Moreau et al., 2005). Upon activation, pKLK preferentially cleaves the HMWK liberating a nanopeptide called bradykinin (BK) and a BK-free-HMWK (HKa). The latter after cleavage adopt a new rearrangement translated into new properties and functions such as inhibiting endothelial cell proliferation, migration, and neovascularization. In fact, HKa is shown to have anti-apoptotic and anti-angiogenic functions that contradict BK effects and future studies are needed to validate the efficacy of HKa to be used in cancer therapy (Bryant and Shariat-Madar, 2009, Moreau et al., 2005).

BK is a bioactive metabolite secreted locally or in plasma. It is a positively charged nanopeptide with the following sequence: Arg^{1} - Pro^{2} - Pro^{3} - Gly^{4} - Phe^{5} - Ser^{6} - Pro^{7} - Phe^{8} - Arg^{9}. BK is rapidly degraded by several pathways; it has a short half-life of around 15 seconds along with low circulating levels (da Costa et al., 2014, Kashuba

et al., 2013, Marceau and Regoli, 2004). It has many different functions and is involved in wide range of cellular processes such as inducing vasodilation, vascular permeability, and inflammation (figure 4) (Maurer et al., 2011).

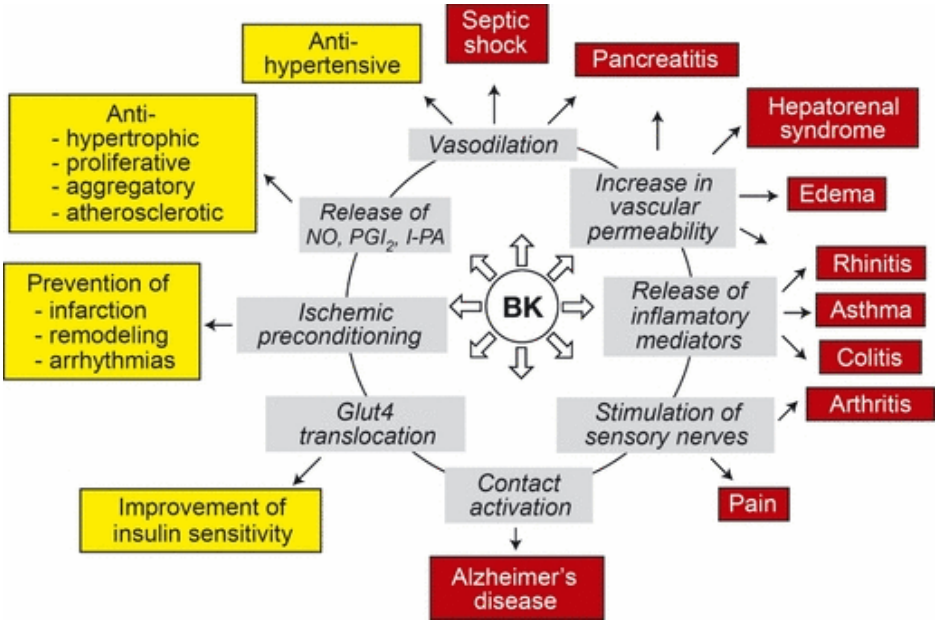


Figure 4. Schematic representation of the effect of bradykinin in physiological and pathological conditions. (Adopted from (Maurer et al., 2011)).

tKLK has a different catalytic activity and targeted proteins than pKLK (Kashuba et al., 2013). It is synthesized as an inactive proenzyme, known as prokallikrein, and expressed in various tissues of the human body including kidney, colon, central nervous system, and pancreas (Moreau et al., 2005). After activation, tKLK releases Lys-BK, a decapeptide know as kallidin (KD), from the LMWK. Worth mentioning that opposite of pKLK that preferentially cleaves HMWK, tKLK is

able to cleave the HMWK, besides the LMWK, to generate Lys-BK (Kashuba et al., 2013).

Lys-BK or KD differs from BK sequence by an additional lysine amino acid at N-terminus: Lys^{1} - Arg^{2} - Pro^{3} - Pro^{4} - Gly^{5} - Phe^{6} - Ser^{7} - Pro^{8} - Phe^{9} - Arg^{10}. Moreover, KD can further be converted to BK when the Lys-Arg bond is cleaved (Kashuba et al., 2013).

On the other hand, kininase carboxypeptidases catalyze the cleavage of arginine amino acid from the carboxy-terminus of kinin peptides that are released in circulation leading to the formation of des-Arg^{9}-BK and des-Arg^{10}-KD (Lys-des-Arg^{9}-BK). These carboxypeptidases are of two types with distinct catalytic activity: carboxypeptidase M (CPM) found in the cell membrane and carboxypeptidase N (CPN) present in the plasma. CPM is expressed in various human tissues such as placenta, kidney, and lungs. By turning BK to des-Arg-BK, CPM can regulate the activity of BK by abolishing the specific interaction of this peptide with its receptor. CPN is synthesized by hepatocytes and released into the intravascular compartment. This enzyme is constitutively active and can modulate the activity of kinins, growth factors, and cytokines by removing basic amino acid residues at the carboxy-terminus, hence maintaining homeostasis (Kashuba et al., 2013).

Kinins mediate their effects via two specific GPCRs: Bradykinin Receptor B1 (B1R) and Bradykinin Receptor B2 (B2R) that are coupled mainly to $G\alpha_{q/11}$ and $G\alpha_{i/o}$ (Maurer et al., 2011). While BK and KD bind B2R with higher affinity, des-Arg^{9}-BK and des-Arg^{10}-KD preferentially bind B1R. B2R is constitutively and widely expressed under normal conditions in the human tissues (da Costa et al., 2014, Kashuba et al., 2013). When B2R is in a ligand-free state, it is localized at the cell membrane, while

upon ligand binding B2R induce a rapid transient intracellular signaling followed by receptor internalization and recycling. On the contrary, B1R is an inducible receptor. Under normal physiological conditions, B1R has very low expression levels which are rapidly increased after certain stimuli such as its own agonist, inflammation, and cancer (Ehrenfeld et al., 2012). B1R is internalized and degraded when it is not bound to its ligand, yet once occupied with its Arg-truncated kinins it becomes stabilized at the cell membrane. Moreover, B1R agonist-induced response is sustained with slow desensitization process (Maurer et al., 2011). Finally, based on the cell type, B1R and B2R can activate diverse signaling cascades and induce various cellular responses including cell proliferation, migration, vascular permeability, and smooth muscle contraction. (Adopted from (da Costa et al., 2014, Ehrenfeld et al., 2012)).

3. Kallikrein-Kinin System and Cancer

Since KKS is involved in a wide range of cellular processes, deregulations affecting components of this system or its signaling cascade contribute to the progression of many diseases including cancer. First evidence suggesting a possible correlation between KKS and cancer was based on the facts that increase in vascular permeability, nitric oxide and prostaglandin production are mediated by the activation of KKS. Moreover, several peptides generated by the KKS were validated to possess pro-inflammatory properties and to be implicated in the initiation of inflammation (Kashuba et al., 2013). The association between the different components of the KKS (from KLKs, KNGs, kinins, and kinin receptors) and carcinogenesis offers a scope for the use of these molecules as novel cancer biomarkers or potential targets for anti-cancer therapy.

A number of studies reported considerable evidence on the role of kinins in tumorigenesis. BK and des-Arg^9-BK levels were found to be high in the sera of patients with breast cancer and low in patients with bladder cancer in comparison with normal subjects. Further studies supported the presence of des-Arg^9-BK at high levels in breast cancer patients, yet these levels went back to normal after removal of the tumor. These facts suggest a potential role of these deregulated kinins to be used as cancer biomarkers (da Costa et al., 2014, Figueroa et al., 2012).

Moreover, several studies revealed the role of BK in stimulating the growth of many types of cancer cells through B2R such as in primary normal and breast cancer cells, lung carcinoma, prostate, head, neck, breast, and colon carcinoma cell lines. For example, BK induced cell growth of PC3 prostate cancer cells along with other cells lines *in vitro* via activation of extracellular-signal-regulated kinase (ERK) signaling pathway (da Costa et al., 2014). It was also able to induce cell proliferation and migration of head and neck carcinoma through epidermal growth factor receptor (EGFR) transactivation (Kashuba et al., 2013). In MCF-7 cells, BK induced cell proliferation via ERK1/2, and co-treatment with tamoxifen and all-*trans* retinoic acid reversed BK-induced proliferation and decreased B2R levels (Searovic et al., 2009).

On the other hand, BK was shown to increase vascular endothelial growth factor (VEGF) expression in prostate cancer cell lines and to induce tube formation in endothelial cells, suggesting its role as a pro-angiogenic molecule. Furthermore, BK induced cell migration of chondrosarcoma cells through B1R and B2R pathway, and enhanced the migration of glioma and bladder cancer cells via B2R *in vitro* (da Costa et al., 2014). B1R agonist, on the other hand, induced the migration of glioma cells through upregulation of cyclooxygenase-2 (cox-2) and activation of phosphoinositide 3-

kinase (PI3K)/protein kinase B (AKT) pathway. In addition, when BK was used as a chemoattractant, it induced cell migration and invasion of glioma cells towards blood vessels in rat brain slices, an event that was decreased when B2R activity was inhibited either pharmacologically or by knockdown (Figueroa et al., 2012). Also, activation of B1R in breast cancer cells *in vitro* induced the secretion of matrix metalloproteinase-2 (MMP-2) and MMP-9 via ERK1/2 transduction pathway and EGFR transactivation, which can facilitate tumor migration, invasion, and angiogenesis (da Costa et al., 2014). In colorectal cancer, BK was shown to increase the expression and secretion of IL-6 and to induce cell migration and invasion of SW480 cells through B2R and ERK1/2 pathway (Wang et al., 2014). In addition to the above, BK can generate the four basic signs of inflammation: redness, local heat, swelling, and pain; and increased levels of BK are found in chronic inflammation. In fact, this effect of BK creates a favorable environment for tumor development and progression (da Costa et al., 2014, Searovic et al., 2009).

Several studies proposed contributions of kinin receptors in tumorigenesis and in cancer pain. These receptors can enhance tumor growth and metastasis by activation of inflammatory pathways that cancer cells rely on (da Costa et al., 2014). Kinin receptor levels are subject to changes in many pathological conditions, which implicate that kinin receptors profile might regulate the total effect of the KKS in disease states such as in inflammation and cancer. (Zelawski et al., 2006). B1R was shown to be overexpressed in prostate cancer and B2R had high expression levels in lung, liver, brain, gastric, head, and neck cancer samples. Both of these receptors were overexpressed in human chondrosarcoma when compared to normal cartilage cells (Kashuba et al., 2013). In addition, B1R and B2R were found to be overexpressed in

human colorectal adenomas and in hyperplastic polyps, respectively (Zelawski et al., 2006).

Finally, it is well established now that cancer tissue does not consist of tumor malignant cells only, yet it is also composed of tumor stroma (immune cells, fibroblasts, extracellular matrix, and vasculature…). Many reports emphasize the importance of the interaction between tumor cells and the tumor-associated stroma to enhance cancer growth, angiogenesis, and metastasis. In a study performed by Ikeda and colleagues, BK secreted by tumor cells was found to act on stromal cells such as fibroblasts that express B2R receptor and subsequently promote tumor growth and angiogenesis (Figure 5) (da Costa et al., 2014, Ikeda et al., 2004).

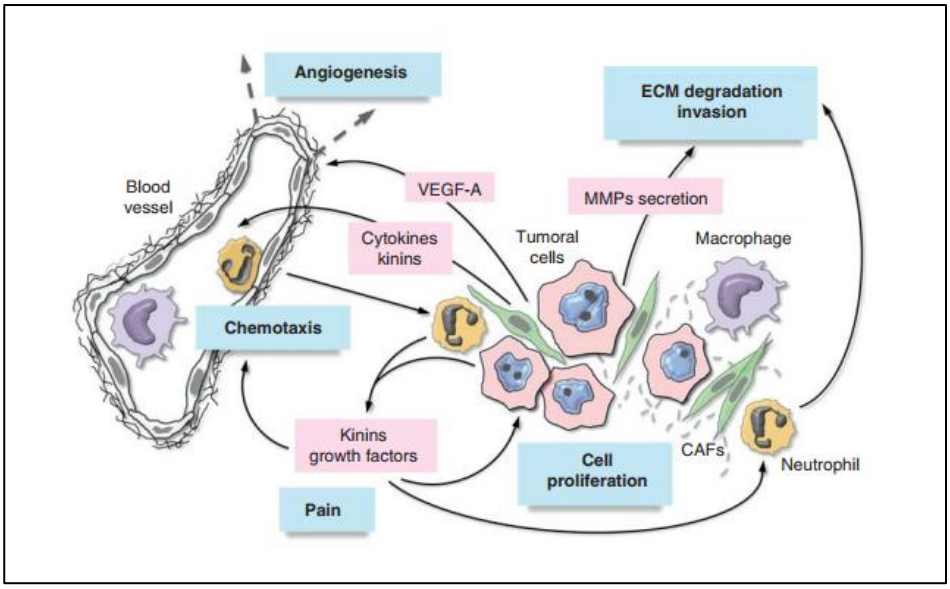


Figure 5. Schematic representation of the interplay between the different components of the tumor microenvironment. Tumor cells or other cells from the tumor stroma generate kinin peptides that can stimulate in an autocrine and/or paracrine fashion kinin receptors expressed on tumoral cells, immune cells, blood vessels, and cancer-associated fibroblasts (CAFs). Activation of kinin receptors can induce several processes such as cell proliferation, angiogenesis, chemotaxis, extracellular matrix degradation, invasion, and pain. (Adopted from (Figueroa et al., 2012)).

C. Vitamin A and Retinoids

1. Overview

In 1817, Magendie conducted one of the first experiments involving vitamin A while Lunin in 1881 first described the effect of vitamin A on growth; both scientists performed experiments on animals fed with different diets. Later on, McCollum, as well as Mendel and Osborne, discovered independently the "fat-soluble factor", crucial for growth and development, that was later named as vitamin A (Bushue and Wan, 2010).

The human body cannot synthesize vitamin A de novo, rather it has to be obtained through diet as retinyl-ester from animal sources and β-carotene from plant sources (Bushue and Wan, 2010, Das et al., 2014). Over four thousand molecules, natural or synthetic, are related to vitamin A structurally and/or functionally (Bushue and Wan, 2010). Vitamin A along with its analogue derivatives (retinoids) are involved in diverse physiological functions such as vision, embryonic growth and development, cell growth and differentiation, apoptosis, and immune competence (Alvarez et al., 2014). This crucial role of vitamin A is mediated mainly via all-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (9cRA), whereby binding their cognate biological receptors they regulate transcription of many genes related to various cellular functions (Li et al., 2014). Retinoids have a basic structure that consists of three major parts: a trimethylated cyclohexene ring (hydrophobic group), a conjugated tetraene side chain (linker), and a carbon-oxygen functional group (polar) (Figure 6) (Das et al., 2014).

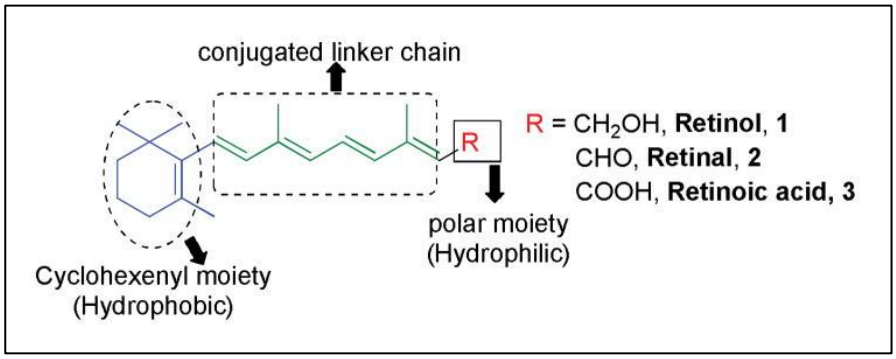


Figure 6. Basic structure of the retinoids. (Adopted from (Das et al., 2014)).

2. Production and Metabolism of Vitamin A and Retinoids

Retinoids or carotenoids are turned into retinoic acid (RA) through successive bioconversions taking place first in the intestine, then in the liver, and ending up in the targets cells (Das et al., 2014). Retinyl-ester, uptaken by diet, is hydrolyzed by pancreatic and intestinal enzymes to retinol that is subsequently absorbed by enterocytes. On the other hand, 50% of the ingested β-carotene is oxidized to retinal and afterward reduced to retinol in these epithelial cells (Kim, 2011). Retinol is esterified and incorporated, along with intact carotenoids, in chylomicrons. These latter are uptaken by the hepatocytes where their content can be stored in the form of retinyl-esters in the liver stellate cells or hydrolyzed again to retinol. Free retinol binds to retinol binding protein before being secreted into the circulation to reach target cells (Figure 7) (Henning et al., 2015).

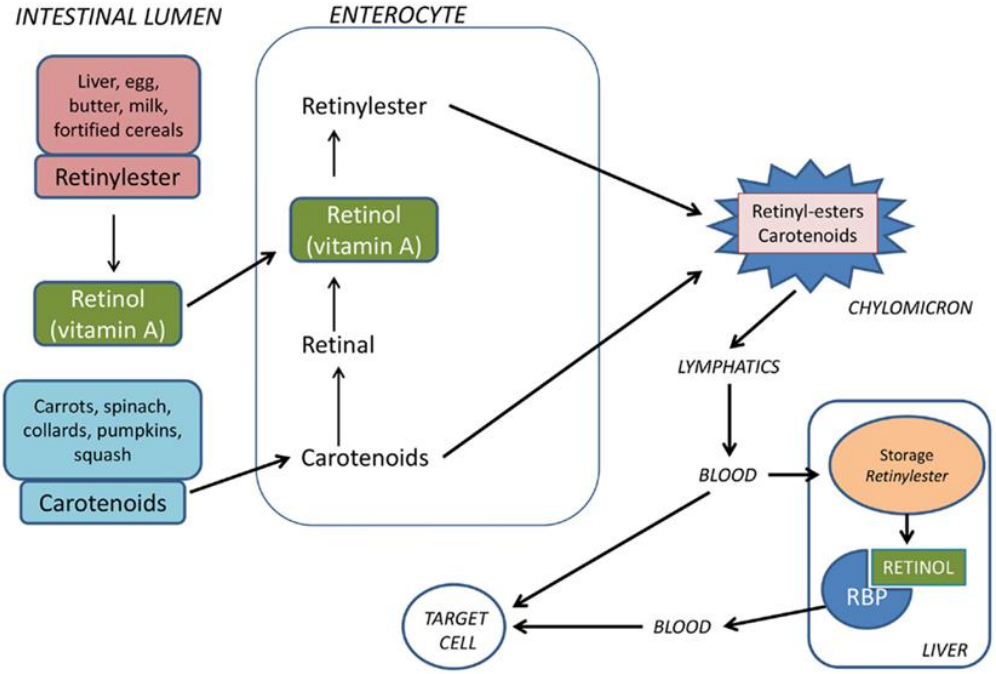


Figure 7. Schematic representation of vitamin A uptake and its metabolism. Retinol-esters and carotenoids are uptaken by the enterocytes and then are packaged in chylomicrons. These are taken by the hepatocytes via endocytosis. In the liver, vitamin A can be stored in the form of retinyl-ester or can be hydrolyzed to retinol. Finally and before being released into the circulation, retinol binds to retinol-binding protein (RBP). (Adopted from (Henning et al., 2015)).

ATRA, also known as tretinoin, is the main biologically and physiologically active metabolite of vitamin A (Al Tanoury et al., 2013, Bushue and Wan, 2010, Siddikuzzaman et al., 2011). In the target cells, ATRA is generated via two steps. First retinol is oxidized by retinol dehydrogenase (RDH) in a reversible reaction that leads to the formation of retinal which is oxidized in an irreversible reaction by retinal dehydrogenase (RALDH) that yields ATRA. This metabolite can further be oxidized by cytochrome P450 (CYP450) enzyme, mainly CYP26. The balance between these two reactions mediated by RALDH (biosynthesis) and CYP450 (catabolism) regulates the cellular levels of ATRA. Eventually, ATRA binds the cellular retinoic acid-binding

protein (CRABP) and translocates to the nucleus to interact with its biological receptors (Figure 8) (Bushue and Wan, 2010, Henning et al., 2015).

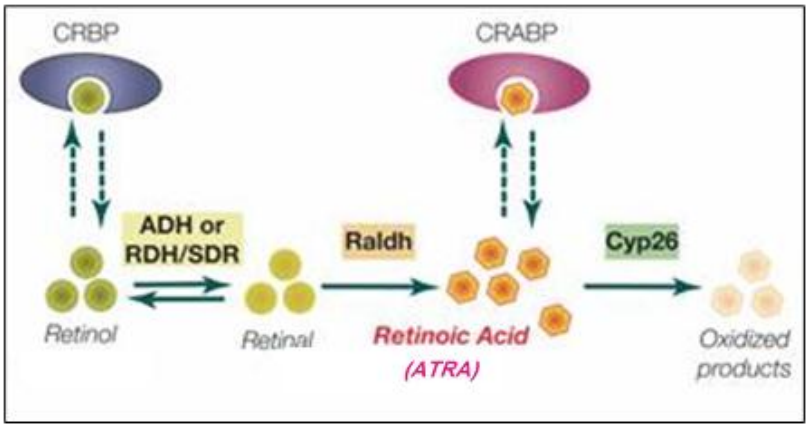


Figure 8. Schematic representation of all-*trans* retinoic acid metabolism. Cellular retinol-binding protein (CRBP) binds to free retinol. Retinal is formed by the action of aldehyde dehydrogenase or retinol dehydrogenase (RDH)/short-chain dehydrogenase (SDR) in a reversible reaction. Then retinal is oxidized by retinaldehyde dehydrogenases (RALDH) in an irreversible reaction that yields all-*trans*-retinoic acid (ATRA). This active metabolite can bind to cellular retinoic acid binding protein (CRABP). Ultimately, endogenous ATRA is degraded by the action of cytochrome P26 (CYP26). (Modified from (Marlétaz et al., 2006)).

3. Vitamin A and Retinoid Signaling

Retinoids mediate their biological functions by binding two primary distinct types of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Tanaka et al., 2004). Each of these nuclear receptors exists in three subtypes (α, β, and γ) that are encoded by separate genes, and each subtype has at least two isoforms (Duong and Rochette-Egly, 2011). Worth noting that the retinoid receptors belong to the family of steroid-thyroid-vitamin D receptors. In general, RARα is ubiquitous and widely expressed, while RARβ and RARγ are tissue-specific (Duong

and Rochette-Egly, 2011). RARs are able to bind both ATRA and 9cRA metabolites, while RXRs preferentially bind 9cRA (Tanaka et al., 2004). RARs and RXRs have distinct ligand-binding domains, therefore these two retinoid receptors can have different pharmacological targets. Moreover, RARs heterodimerize with RXRs, however RXRs can homodimerize and heterodimerize with other types of nuclear receptors such as the thyroid hormone receptors (TRs), vitamin D receptors (VDRs), liver X receptor (LXRs), and peroxisomal proliferator-activated receptor (PPARγ) (Bushue and Wan, 2010, Freemantle et al., 2002).

Retinoids regulate gene expression and mediate their actions by two mechanisms: the classical genomic and the non-genomic signaling pathways (Henning et al., 2015).

a. Genomic Action of Retinoids

In the absence of ligand binding, RAR/RXR heterodimer, complexed with transcriptional co-repressors and accompanied with histone deacetylase (HDAC) activity, binds to the retinoic acid response element (RARE) at the promoters of target genes. As a result, chromatin is maintained in a condensed form and target genes are silenced. Upon ligand binding, the heterodimer exhibits conformational changes by which the co-repressors are released and the co-activators are recruited endowed with histone acetyltransferase (HAT) activity. Subsequently, these events lead to chromatin decompaction and activation of the transcriptional machinery. Finally, to end the transcriptional response, nonconventional coactivators and HDAC are recruited and/or RARs are degraded by the ubiquitin-proteasome system (Al Tanoury et al., 2013, Das et al., 2014).

b. Non-Genomic Action of Retinoids

In addition to the well-established genomic effects of RAs, non-genomic signaling is also mediated by these metabolites independently of the classical mechanism of nuclear receptors (Schenk et al., 2014). Upon RA treatment, several studies demonstrated a rapid, transient, and cell-specific activation of different protein kinases implicated in signal transduction (Al Tanoury et al., 2013, Schenk et al., 2014). In fibroblasts, breast cancer, leukemia, and mouse embryo carcinoma cells, RARα localized in the lipid rafts of the plasma membrane forms complexes with $G\alpha_q$ after stimulation with ATRA. Consequently, RhoGTPase, p38 mitogen-activated protein kinase (p38MAPK), and mitogen- and stress-activated kinase 1 (MSK1) are activated. Thus, phospho-MSK1 phosphorylates the histones and activates the transcription of RARα and its target genes (Figure 9) (Al Tanoury et al., 2013, Schenk et al., 2014). On the other hand, in neuronal cells, Sertoli cells, and embryonic stem cells, ERK is activated upon RA stimulation which can afterward activate downstream effectors that still need to be identified. ERK activation is mediated by RARα present in the lipid rafts or RARγ that is bound to sarcome (Src) kinase (Figure 9) (Al Tanoury et al., 2013). In addition, ATRA can cause a rapid accumulation of cyclic adenosine monophosphate (AMP) in acute promyelocytic leukemia (APL). This effect might be mediated by stimulation of adenyl cyclase and activation of protein kinase A (PKA), which in turn can phosphorylate RARα and ultimately increase the expression of specific target genes and differentiation (Schenk et al., 2014).

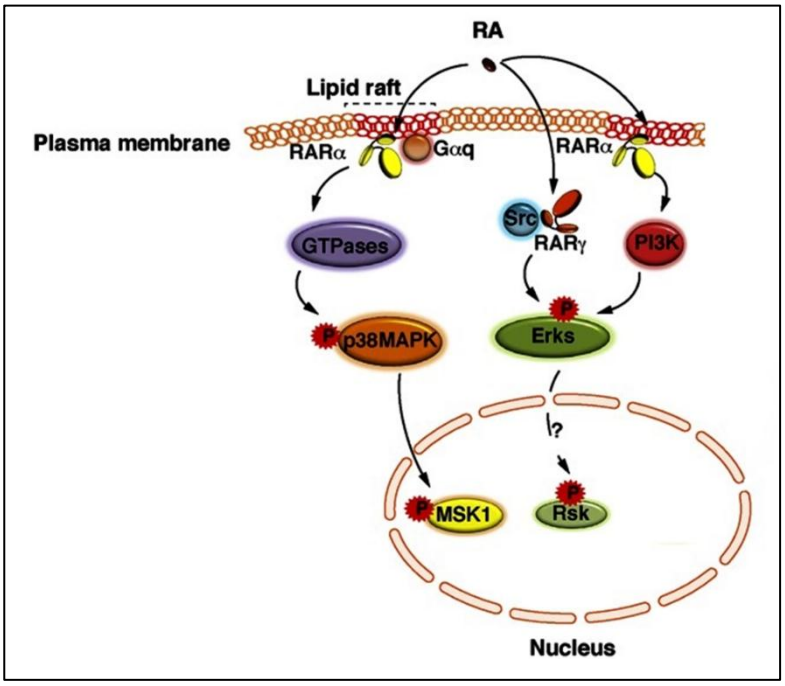


Figure 9. Schematic representation of the non-genomic action of retinoids. (Modified from (Al Tanoury et al., 2013)).

4. Retinoids and Cancer

a. Aberrant Retinoid Signaling and Metabolism in Cancer

RARs, in general, are not mutated nor take part of fusion proteins in cancer, except the case where RARα forms a fusion protein with promyelocytic leukemia (PML) in APL patients (Tang and Gudas, 2011). Rather abnormalities in retinoid signaling and metabolism are often seen in carcinoma cells when compared to the normal epithelial cells (Applegate and Lane, 2015, Tang and Gudas, 2011). Thus, deciphering the mechanisms leading to deregulations in retinoid signaling might allow to reverse these causes and subsequently inhibit tumor formation (Tang and Gudas, 2011).

In several epithelial-derived adenomas and carcinomas, the expression of one or even more than one type of RAR is lost (Applegate and Lane, 2015). Based on several studies, RARβ is thought to be considered as a tumor suppressor since its expression is frequently decreased in several types of tumors (head and neck tissues, basal skin cells, breast, lung, esophagus, prostate, thyroid, larynx, endometrium, and oral tissues) (Perraud et al., 2011). Moreover, RARβ in colorectal cancer tissues was found to be significantly lower than in normal tissues using immunohistochemistry technique in gross specimens. Also, RARβ expression rate correlated with colorectal cancer stage where it decreased in patients in an advanced stage and with lymph node metastasis in comparison with patients in early stage and without metastasis (Fang et al., 2010). On the other hand, there are conflicting results about RARα and RARγ expression that seems to depend on the type of pathology and the technique used in the study (Perraud et al., 2011). In keratinocytes, loss of RARγ led to skin tumorigenesis and was consequently considered as a tumor suppressor. In contrast, RARγ was overexpressed in hepatocellular carcinoma and correlated with cell growth due to its abnormal accumulation in the cytoplasm. RARγ in the cytosol led to the activation of the PI3K/AKT pathway which is well-known as the main survival pathway in tumor cells (Duong and Rochette-Egly, 2011).

On the other hand, colorectal cancer cells seem to lose the ability to generate ATRA while favoring its degradation through CYP450 enzyme. Experimental evidence shows that gene expression of the enzymes implicated in ATRA synthesis was decreased in colorectal cancer tumors and cell lines. Subsequently, little ATRA is produced; being crucial for the differentiation of epithelial cells, colonocytes thus become less differentiated. In addition to the above-mentioned deregulations, retinoid

storage metabolism might also be altered in cancer. In colorectal cancer, the promotor of lecithin retinol acyltransferase (LRAT) gene, the enzyme that esterifies retinol to retinyl esters, was found to be hypermethylated. Consequently, gene expression of LRAT would decrease leading to a decrease in intracellular retinoids (Applegate and Lane, 2015).

b. Retinoids Use in Anticancer Therapy

The use of retinoids in cancer prevention and treatment have captivated the attention of a lot of researchers (Bushue and Wan, 2010). Given their potential in inducing apoptosis and cell differentiation and their antiproliferative and anti-oxidant effect, retinoids have been used as chemotherapeutic or chemopreventive agents (Das et al., 2014). In humans, retinoids can induce cell differentiation of myeloid cells, prevent the formation of lung, liver, and breast cancer, and reverse premalignant epithelial lesions (Bushue and Wan, 2010). ATRA treatment was developed to be used on several types of tumors such as lung cancer, kidney cancer, cervical cancer, neuroblastoma, glioblastoma, lymphoma, and leukemia (Das et al., 2014). In fact, ATRA potency in cancer therapy was best exemplified in the case of APL, where it is employed regularly and efficiently only in the case of this rare leukemia (Bushue and Wan, 2010, Schenk et al., 2014). Finally, the use of ATRA in combination with other drugs has emerged during these past recent years in various precancerous and cancer conditions (Schenk et al., 2014). As an example, ATRA treatment when combined with dexamethasone, a corticosteroid, inhibited proliferation and induced the differentiation of human osteosarcoma cells (Schenk et al., 2014). Worth noting that few studies to date have been performed using combination of ATRA and inhibitors of signal transduction pathways. Combining inhibitors of glycogen synthase kinase 3 (GSK3) with ATRA

was shown to inhibit cell growth and induce cell differentiation of various leukemic cells. Since GSK3 by phosphorylating RARα reduces its transcriptional activity and induces its degradation by the proteasome; inhibiting this kinase increases back the intracellular levels of RARα and subsequently the responsiveness to ATRA (Schenk et al., 2014).

CHAPTER II

OBJECTIVE, SPECIFIC AIMS, AND SIGNIFICANCE

The correlation between inflammation and colorectal cancer development and progression is well-established in many studies (Janakiram and Rao, 2014). Kinins, secreted within the inflammatory tissue microenvironment, may induce diverse cellular functions related to tumorigenesis namely cell proliferation, migration, endothelial cell activation, and immune cell activation. Thus, BK and kinin receptors are implicated in many pathologies including inflammation and cancer. In fact, kinins and their receptors are promising targets for cancer therapy (da Costa et al., 2014). On the other hand, retinoids are well-known for their essential role in the development and function of many systems; therefore, deregulation in the retinoid signaling is linked to several diseases including cancer. Numerous retinoids, whether natural or synthetic, are being tested in clinical trials or used in the clinic for treating cancer (Das et al., 2014). Moreover, during immune reactions, production of RA is induced in cells as a response to certain cytokines, and ATRA was shown to have immunomodulatory and anti-inflammatory functions in UC and colitis-associated cancer (CAC) (Kim, 2011, Rafa et al., 2017).

Based on these facts, we aim to characterize the crosstalk between kinin and retinoic acid receptors signaling which may subsequently control cell growth, transformation, differentiation, and immune responses of colorectal cancer. Accordingly, we will use *in vitro* human cancer models harboring mutations relevant to colorectal tumorigenesis (mainly HCT116 and HCT116 $p53^{-/-}$) and normal human colon

cells (NCM460) to characterize the potential crosstalk between bradykinin and retinoic acid systems and its implication on various cellular processes involved in colorectal cancer development and progression. Specifically, we will target the following aims:

- Profile the basal protein levels of B1R, B2R, RARα, RARβ, RARγ, and RXRα.
- Assess the effect of BK and ATRA, as single or combination treatments, on the regulation of B2R and RARγ protein levels.
- Investigate the effect of BK on the subcellular localization of B2R and RARγ.
- Determine the downstream signaling mediators of BK.
- Test the effect of BK and ATRA single or combination treatments on cell growth and transformation.
- Study the modulation of inflammation and differentiation markers by BK and/or ATRA.

Our findings may lead to novel biomarkers and therapeutic strategies in colorectal cancer by targeting the deregulated KKS players and its crosstalk with the retinoid signaling. This is the first study, to our knowledge, that addresses this novel crosstalk between BK and retinoic acid signaling and its implications on tumorigenesis.

CHAPTER III

MATERIALS AND METHODS

A. Cell Culture

1. In Vitro Human Model of Colorectal Cancer

Human colorectal cancer cell lines: HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo were used.

a. HCT116

HCT116 is a human epithelial malignant cell line; one of three subpopulations of colonic carcinoma primary cell culture isolated from a male. HCT116 cells are able to form colonies in semisolid medium (agar) or on a feeder layer of mouse fibroblasts (Brattain et al., 1981). They are invasive, tumorigenic in nude mice, and can be used as transfection hosts (Rajput et al., 2008). This cell line possesses a wild-type *p53* gene (Waldman et al., 1995). It has a mutation in codon 13 of the *ras* proto-oncogene and expresses the transforming growth factor beta 1 (TGF β1) and beta 2 (TGF β2) (HCT116 (ATCC® CCL-247™)).

b. HCT116 $p53^{-/-}$

p53 protein is a key regulator of the cell cycle, programmed cell death, DNA repair mechanism, and cellular metabolism. As observed in 40 to 50% of colorectal cancer cases, many human cancer types have mutated *p53* gene. In this cell line, *p53* was knockout from the parent cell line by homologous recombination. HCT116 $p53^{-/-}$ cells are relatively more resistant to drugs than their parental cells (Boyer et al., 2004).

c. HCT116 $p21^{-/-}$

Cyclin-dependent kinase inhibitor (CDKI) 1A, known as p21, mediates the p53-dependent cell cycle G1 arrest. It also plays different functions such as inducing differentiation and inhibiting apoptosis (Ferrandiz et al., 2009). *p21* gene was knockout in HCT116 $p21^{-/-}$ cell line by homologous recombination (Waldman et al., 1995).

d. HT-29

HT-29 is a human epithelial colorectal adenocarcinoma cell line isolated from a 44 years old Caucasian female. These cells show characteristics of mature intestinal cells and are suitable for transfection (Martínez-Maqueda et al., 2015) (HT-29 (ATCC® HTB-38™)). They are tumorigenic as they form tumors in nude mice and in steroid-treated hamsters. HT-29 cells have mutated *APC*, *BRAF*, and *p53* genes (HT29 (ATCC® HTB38™)).

e. SW480

SW480 is a human epithelial malignant cell line. These cells were isolated from a primary colon adenocarcinoma of a 50 years old Caucasian male (SW480 [SW-480] (ATCC® CCL228™)). Initially, they grew as epithelial and bipolar cells, but then epithelial cells dominated (Leibovitz et al., 1976). SW480 cells can be used for transfection. They are tumorigenic as they have the ability to form tumors in nude mice (SW480 [SW480] (ATCC® CCL228™)). Several mutations have been reported in SW480, namely a point mutation in codon 12 of *c-K-ras* gene, *myc* amplification, loss of the *APC* gene on chromosome 5, and point mutations in the *p53* gene (Rochette et al., 2005).

f. LoVo

LoVo is a human epithelial cell line derived from a metastatic nodule of a grade 4 colorectal adenocarcinoma of a 56 years old male. These cells are suitable for transfection. They are tumorigenic as they can form tumors in nude mice. LoVo cells have mutations in the *APC* and *K-ras* genes (LoVo (ATCC® CCL229™)).

2. In Vitro Human Model of Normal Colon Cells

NCM460

NCM460 is an epithelial cell line isolated from the normal mucosa of a male with gastric cancer (Moyer et al., 1996). These cells are immortalized but not transformed. Moreover, NCM460 cells are unable to form tumors in nude mice (Table 1). They grow in two ways as attached and in suspension (Sahi et al., 1998). In this work, we focused our studies on the attached cells.

3. In Vitro Human Model of Monocytes

THP-1

THP-1 is an immortalized monocyte-like cell line, derived from the peripheral blood of a one-year-old infant male with acute monocytic leukemia (Bosshart and Heinzelmann, 2016). THP-1 cells are round in shape, growing in suspension, and showing distinct monocytic markers. When treating with phorbol-12-myristate-13-acetate (PMA, also known as TPA, 12-O-tetradecanoylphorbol-13-acetate), these cells can be induced to differentiate into a macrophage-like phenotype, during of which many phenotypic changes can occur such as adhering to culture plates and becoming flat and amoeboid in shape (Qin, 2012). These differentiated cells can be used as an alternative

to human peripheral blood mononuclear cells (PBMCs) regarding cytokine production and macrophage surface markers (Chanput et al., 2014). THP-1 cells are suitable for transfection (THP-1 (ATCC® TIB202™)).

CHARACTERIZATION OF NCM460	
Phenotypic Characteristics	Observations
Growth in soft agar[a]	Negative
Tumorigenicity[b]	Negative
Periodic acid–Schiff mucin staining	Positive
Immunocytochemistry assays[c]	
Cell type: Marker	
Epithelial cells: pancytokeratin	Positive (>90%)
GI epithelial cells	
Villin: cytoskeleton	Positive (>90%)
5E113: Cell surface	Positive (>90%)
Secretory epithelial cells	
Human secretory component	Positive (>90%)
Endothelial cells: Factor VIII	Negative (>90%); Positive (1–2%)
Lymphocytes (CD4; CD23: others)	Negative
Mesenchymal: other cells: Vimentin	Negative (>90%); Positive (10–15%)
Neuroendocrine cells	Positive (80–90%; suspension cells)
Chromogranin	Positive (20–40%; monolayer cells)
Neural cells	
Glial fibrillary acidic protein (GFAP)	Negative
Neurofilament (NF)	Negative
Galactosyl ceramide glycolipid (Gal-Cer)	Negative

Table 1. Phenotypic characterization of NCM460 cells. (Adopted from (Moyer et al., 1996)).

4. Cell Culture Conditions

HCT116, HT-29, SW480, and THP-1 cells were maintained in RPMI 1640 medium (Lonza) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Sigma-Aldrich), and 1 mM sodium pyruvate (Lonza). HCT116 *p53*$^{-/-}$, HCT116 *p21*$^{-/-}$, and LoVo cells were cultured in DMEM (Lonza) containing 10% FBS, 100 U/ml penicillin-streptomycin, 1 mM sodium

pyruvate, and 1x MEM non-essential amino acid (Sigma-Aldrich). NCM460 cells were maintained in M3:BaseA media (INCELL) containing 10% FBS. All cells were incubated at 37˚C and 5% CO_2 in a humidified atmosphere. Cells were serum starved for 24 hours in DMEM or RPMI media containing 0.5% FBS to reach quiescence before adding the corresponding treatments for each experiment.

THP-1 cells were differentiated into macrophage-like phenotype by adding a final concentration of 50 ng/ml PMA. Cells were left for 48 hours to differentiate and rest prior to serum starvation.

B. MTT Proliferation Assay

HCT116 and HCT116 $p53^{-/-}$ cells were seeded in triplicates in a 96-well plate at a density of 5×10^3 cells/well, while NCM460 cells were seeded in triplicates at a density of 1×10^4 cells/well. Following serum starvation, cells were treated with different concentrations of BK ranging from 0.01 μM to 1 μM for 24 and 48 hours. 5 mg/ml thiazolyl blue tetrazolium bromide dye (MTT, Sigma-Aldrich) was added at each time point (final concentration 0.5mg/ml). After 3 hours, the resultant intracellular formazan crystals were dissolved by adding 100 μL of SDS-based solubilizing agent and left for overnight incubation. Absorbance (O.D.) was measured at 595 nm using the ELISA microplate reader (Multiskan Ex).

C. Soft Agar Colony Formation Assay

Soft agar colony formation assay was performed using CytoSelect™ 96-Well Cell Transformation Assay kit (Cell Biolab, Inc.) as described (Aouad et al., 2017). HCT116 and HCT116 $p53^{-/-}$ cells were suspended in triplicates at a density of 5×10^3/well in 0.4% agar laying on 0.6% agar. Both agar layers were diluted with

media containing 10% FBS. The different treatment conditions were added to the 0.4% agar layer and on top of it. The following treatments were employed for this assay: 0.1 μM and 0.5μM BK, 1μM ATRA, and 1 μM Hoe 140. The plate was incubated for 8 days (at 37˚C, 5% CO_2, and in a humidified atmosphere), and treatments were replenished along with control media every other day. Colonies were photographed using a Zeiss axiovert light microscope (Carl Zeiss) and were quantified using the CyQuant GR Dye (Cell Biolab, Inc.), then fluorescence was measured at 485/520 nm (Fluoroskan Ascent™).

D. ELISA Assay

HCT116 cells were seeded in 6-well plates at approximately 80% confluency. 0.1 μM BK, 1 μM ATRA, 0.1μM BK/1 μM ATRA, and 1 μg/ml lipopolysaccharide (LPS) treatments were used for this assay. Cell supernatants were collected at 3, 6, 12, 24, and 48 hours. To detect IL-6, enzyme-linked immunosorbent assay (ELISA) was performed using Human IL-6 ELISA Ready-SET-Go!® (Affymetrix eBioscience) according to the manufacturer's instructions.

E. RNA Extraction and Reverse Transcription Quantitative Real-Time PCR

HCT116 and THP-1 cells were seeded in 6-well plates and treated with 0.1 μM BK, 1 μM ATRA, 0.1μM BK/1 μM ATRA, and 1 μg/ml LPS for 6 hours. Extraction of total RNA from the different cell lines was done using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to manufacturer's instructions. RNA concentrations were measured using DeNovix DS-11 spectrophotometer. Total RNA (1 μg) was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-

Rad), according to manufacturer's instructions. Primer sequences used to detect IL-6 transcripts (Macrogen) are 5'-GGAGACTTGCCTGGTGAA-3' for forward sequence and 5'-GCATTTGTGGTTGGGTCA-3' for reverse sequence. The sequences for β-actin (TIB MOLBIOL) are 5'-CTCACCATGGATGATGATATCGC-3' for forward sequence and 5'- AGGAATCCTTCTGACCCATGC-3' for reverse sequence. Real-time PCR (qPCR) amplification reactions were performed using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) and CFX384 Touch™ Real-Time PCR Detection System. Cycle parameters were set to 95˚C for 3 minutes, followed by 40 cycles programmed at 95˚C for 15 seconds, 57˚C for 1 minute, and 72˚C for 30 seconds. Gene expression was quantified using comparative CT quantification method.

F. Western Blotting

Total cell protein extracts (30-60 µg) were obtained using Nonidet™ P 40-based lysis buffer and were quantified using Bradford Protein Assay (Bio-Rad). Proteins were separated by SDS-PAGE (8-12%) under reducing conditions and transferred to nitrocellulose membranes. The latter were blocked for 1 hour at 37°C in 5% non-fat milk, then immunoblotted against B2R (1:2000), B1R (1:2000), RARα (1:250), RARβ (1:250), RARγ (1:500), RXRα (1:250), p-ERK (1:2000), total ERK (T-ERK, 1:2000), p-AKT (1:2000), total AKT (T-AKT, 1:2000), p-EGFR (1:500), Total EGFR (T-EGFR, 1:1000), fibronectin (1:1000), E-cadherin (1:1000), β-actin (1:3000) primary antibodies and against the corresponding secondary antibodies at different optimized dilutions. The immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz) and the Clarity™ enhanced chemiluminescence reagent (ECL, Bio-Rad), and using Chemidoc™ MP Imaging System (Bio-Rad).

Signals were measured by densitometry and quantified by the ImageJ program. In some experiments, the representative immunoblot corresponds to the same western blot for different targeted antibodies and shows the same actin.

G. Nuclear/Cytoplasmic Fractionation

Subcellular fractionation was performed using modifications of the method as recently described (Heckler et al., 2016). Briefly, cells were seeded in 10 cm cell culture plates, treated with 0.1 μM BK for 30 minutes, washed and scraped with ice-cold PBS with Ca^{++}/Mg^{++}. After centrifugation at 5000 g, the pellet was resuspended in 0.4% Nonidet® P-40 Substitute (NP-40, amresco), then centrifuged at 7500g for 5 minutes. The supernatant was collected as the cytoplasmic fraction and Laemmli 4x was added to it. The pellet was resuspended in 0.4% NP-40 and centrifuged at 10,000 g for 5 minutes. This process was repeated several times then the nuclear pellet was resuspended in 0.4% NP-40 at which Laemmli 4x was added. Both fractions were heated at 95˚C for 10 minutes and then were quantified using DeNovix DS-11 spectrophotometer. Western blotting was performed as described above, and membranes were immunoblotted against RARγ (1:500), B2R (1:2000), and the loading controls β-tubulin (1:1000) for the cytoplasmic fraction, and nucleoporin 98 (NUP98) (1:1000) for the nuclear fraction.

H. Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least 3 independent experiments unless stated otherwise. Results were analyzed by analysis of variance ANOVA and Tukey as post hoc, and by Student's t-test for two-tailed unpaired

analysis using GraphPad Prism 6 and IBM SPSS Statistics 23 software. Differences were considered significant if $P < 0.05$.

CHAPTER IV

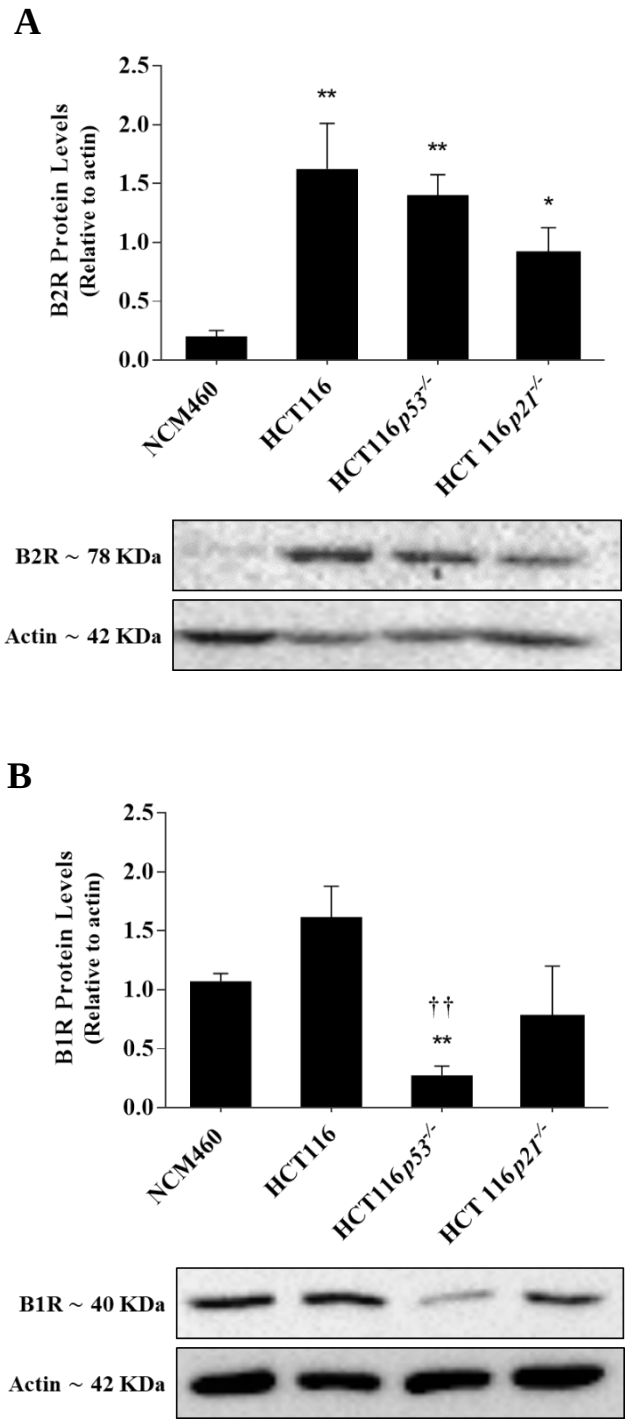
RESULTS

A. Basal Protein Profile of Kinin Receptors in Human Colorectal Cells

First, basal protein levels of B2R and B1R were determined by western blot analysis relative to actin in a human model of normal colon cells (NCM460) and in colorectal cancer cell lines harboring different *p53* and *p21* status (HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$). B2R basal levels were significantly increased in these colorectal cancer cells relative to their normal counterparts independently of *p53* or *p21* (Figure 10A). On the other hand, basal B1R levels did not significantly change in HCT116 and HCT116 $p21^{-/-}$ when compared with NCM460 (Figure 10B). However, B1R basal levels were significantly lower in HCT116 $p53^{-/-}$ *versus* NCM460 cells (Figure 10B). Interestingly, B1R basal levels in colorectal cancer cells seem to be modulated by *p53*, where B1R was significantly lower in the *p53* knockout cell line *versus* its wild-type counterpart (Figure 10B).

Since basal B2R levels in HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ were significantly higher than their levels in their nomal-like counterparts and since BK preferentially binds B2R, we investigated whether this expression pattern is observed in other human colorectal cancer cell lines having different characteristics and mutations. Using western blot analysis, we determined the basal profile of B2R in NCM460 and compared it to its profile in these different colorectal cancer cells: HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo. B2R basal levels were significantly

higher in the different tested colorectal cancer cells relative to the normal colon cells, except in LoVo cells which did not differ significantly (Figure 10C).



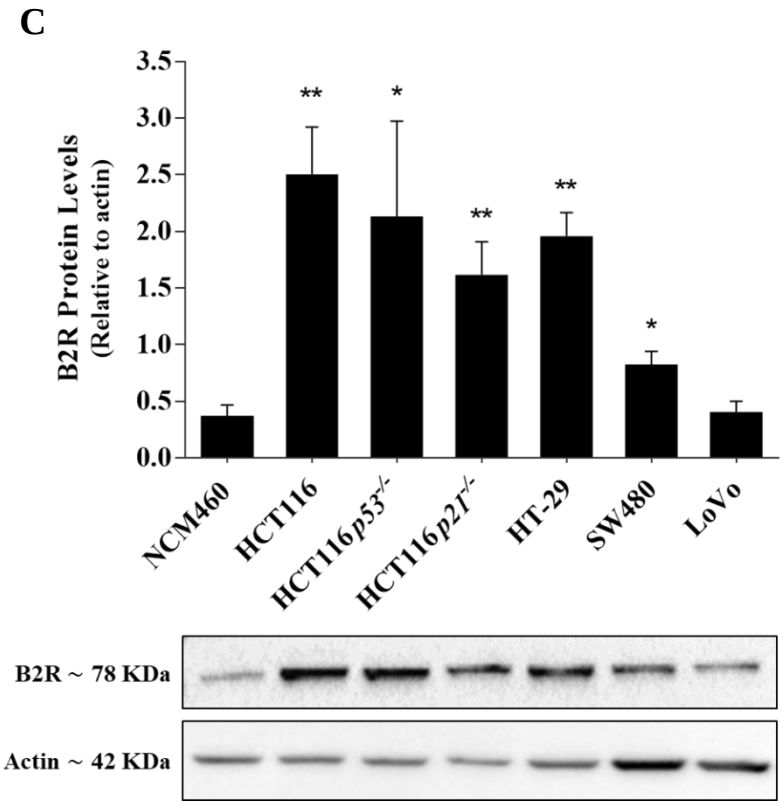
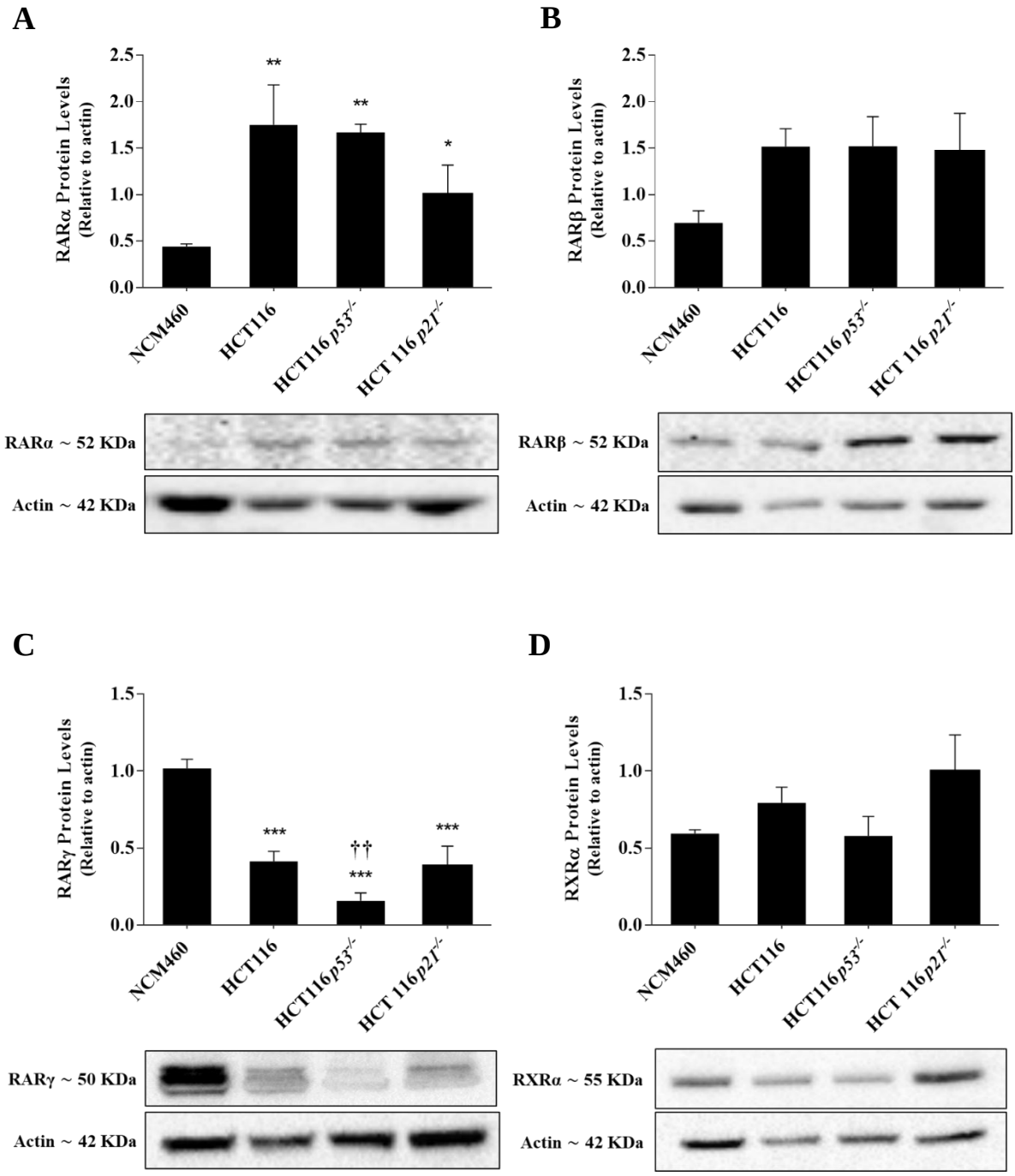


Figure 10. Differential basal protein profile of kinin receptors in human colorectal cells. (A, B) The bar graph represents the ratio of the basal protein levels of B2R and B1R relative to actin in NCM460, HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells. (C) The bar graph represents the ratio of the basal protein levels of B2R relative to actin in NCM460, HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo cells. Results were determined by immunoblotting and are representative of the mean of 3 independent experiments ± SEM. Statistical significance is reported by one-way ANOVA, post hoc Tukey indicating differences between HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ *versus* NCM460 (*, $P < 0.05$; **, $P < 0.01$) and between HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ *versus* HCT116 (††, $P < 0.01$). Student's t-test was used to check differences between HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo *versus* NCM460 cells (*, $P < 0.05$; **, $P < 0.01$).

B. Basal Protein Profile of Retinoid Receptors in Human Colorectal Cells

Basal protein levels of the different retinoid receptors were also assessed by western blot analysis relative to actin. RARα, RARβ, RARγ, and RXRα basal levels were measured in HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ and were compared to their levels in NCM460 cells. RARα basal protein levels were significantly higher in these colorectal cancer cells in comparison with NCM460 independently of *p53* or *p21* status (Figure 11A). However, RARγ basal levels were significantly lower in these tested colorectal cancer cells *versus* their normal counterparts (Figure 11C). Moreover, RARγ basal profile in colorectal cancer cells was *p53* dependent since knocking out *p53* led to a significant decrease in RARγ levels when compared to HCT116 cells (Figure 11C). As for RARβ and RXRα, basal protein levels of these receptors did not differ significantly in HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ *versus* NCM460 cells (Figure 11B, 11D).

Based on the finding that RARγ basal levels were lower in the tested colorectal cancer cells compared to the normal counterparts and that this profile was *p53* modulated, we further investigated whether this decrease was also observed in other human colorectal cancer cells. Western blot analysis was employed and basal profile of RARγ in NCM460, HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo cells was determined relative to actin. RARγ was significantly decreased in all the different studied colorectal cancer cells when compared to NCM460 (Figure 11E).


A
RARα Protein Levels (Relative to actin)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
NCM460
HCT116
HCT116 p53-/-
HCT 116 p21-/-
RARα ~ 52 KDa
Actin ~ 42 KDa
B
RARβ Protein Levels (Relative to actin)
2.5
2.0
1.5
1.0
0.5
0.0
NCM460
HCT116
HCT116 p53-/-
HCT 116 p21-/-
RARβ ~ 52 KDa
Actin ~ 42 KDa
C
RARγ Protein Levels (Relative to actin)
1.5
1.0
0.5
0.0
***
††
***
***
NCM460
HCT116
HCT116 p53-/-
HCT 116 p21-/-
RARγ ~ 50 KDa
Actin ~ 42 KDa
D
RXRα Protein Levels (Relative to actin)
1.5
1.0
0.5
0.0
NCM460
HCT116
HCT116 p53-/-
HCT 116 p21-/-
RXRα ~ 55 KDa
Actin ~ 42 KDa

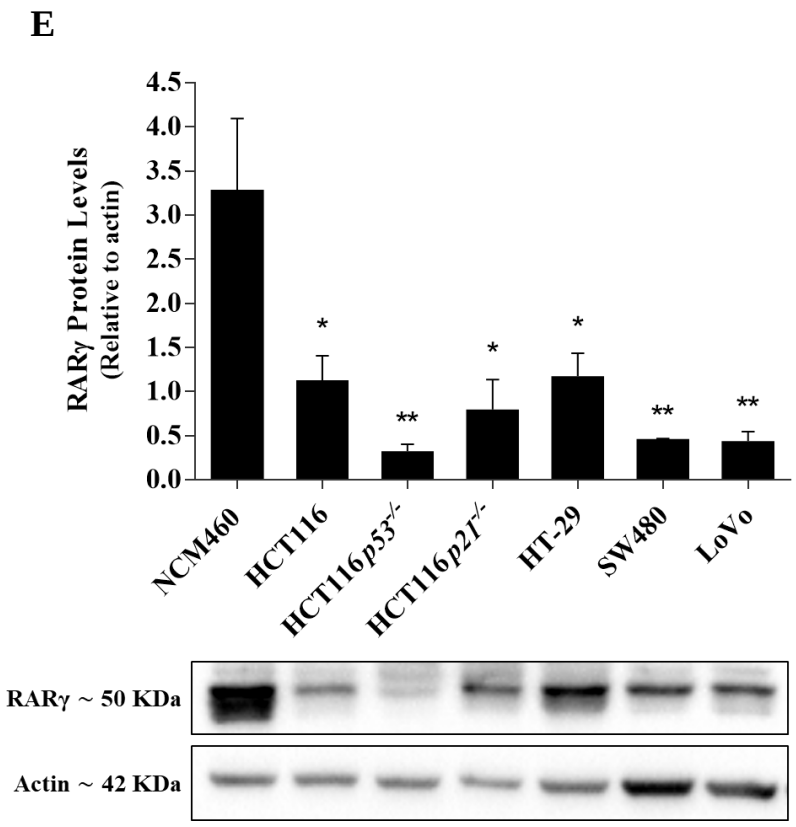


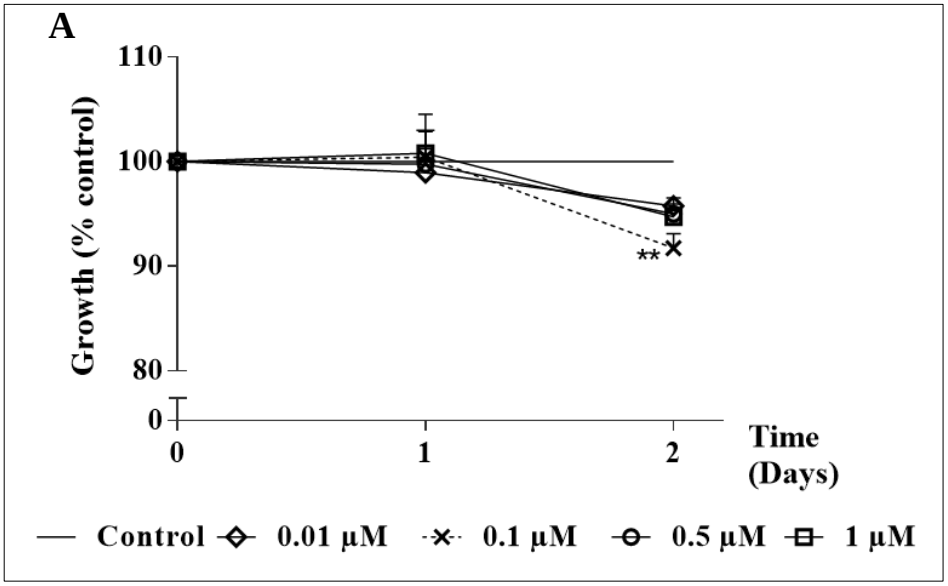
Figure 11. Differential basal protein profile of retinoid receptors in human colorectal cells. (A, B, C, D) The bar graph represents the ratio of the basal protein levels of RARα, RARβ, RARγ, and RXRα relative to actin in NCM460, HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells. (E) The bar graph represents the ratio of the basal protein levels of RARγ relative to actin in NCM460, HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo cells. Results were determined by immunoblotting and are representative of the mean of at least 3 independent experiments ± SEM. Statistical significance is reported by one-way ANOVA, post hoc Tukey indicating differences between HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ *versus* NCM460 cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) and between HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ *versus* HCT116 (††, $P < 0.01$). Student's t-test was used to test the difference between HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$, HT-29, SW480, and LoVo *versus* NCM460 cells (*, $P < 0.05$; **, $P < 0.01$).

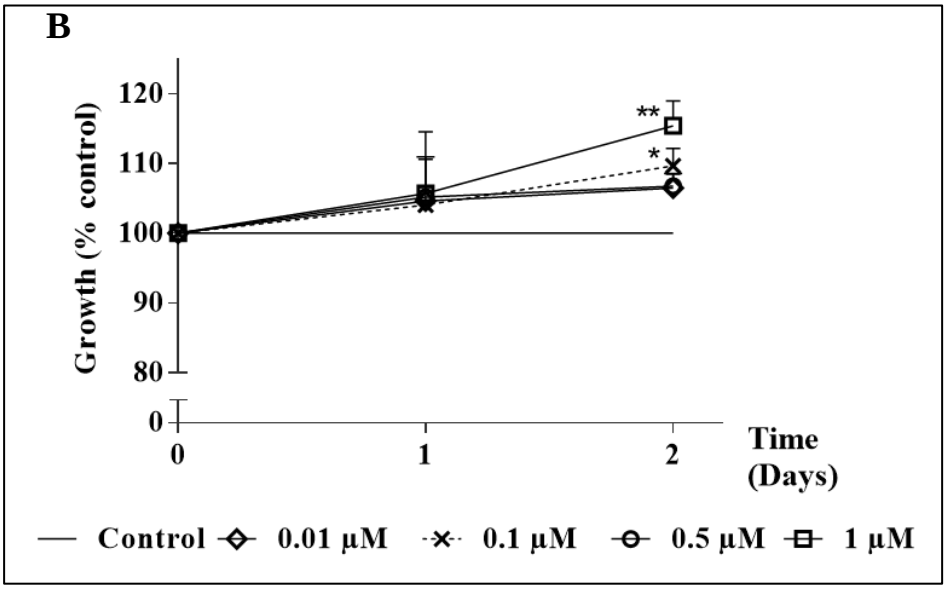
C. Effect of Bradykinin Treatment on the Growth of Human Colorectal Cells

To check whether BK has a proliferative effect on colorectal cancer cells and whether this effect is similar in normal colon cells, several concentrations of BK ranging from 0.01 μM to 1 μM were tested on NCM460, HCT116, and HCT116 $p53^{-/-}$ cells for 24 and 48 hours following serum starvation, and cell growth was assessed by MTT assay. This colorimetric assay measures cell metabolic mitochondrial activity which is an indicator of cellular viability. Cell culture media containing 10% serum was used as positive control for cell growth.

BK had no effect on the growth of serum-starved NCM460 cells as evaluated by MTT at 24 hours in comparison with the control-treated cells (Figure 12A). At 48 hours, growth of these cells decreased at most by 8% when treating with 0.1 μM BK (92% growth relative to control ± 1.4; **; $P < 0.01$) (Figure 12A). Cells grown in 10% FBS containing media, considered as a positive control, had an incremental increase in growth rising from 124% ± 3.2 at 24 hours to 141% ± 7.1 at 48 hours (data not shown). However, growth of BK-treated serum-starved HCT116 cells increased slightly at 24 hours at all tested BK concentrations and continued to rise at 48 hours reaching significance at concentrations of 0.1 and 1 μM BK (110% growth relative to control ± 2.6 for 0.1 μM BK; *, $P < 0.05$, and 115% growth relative to control ± 3.6 for 1 μM BK; **, $P < 0.01$) (Figure 12B). HCT116 positive control cells grew normally with a higher percentage of proliferation than BK induced-growth in serum starved cells (163% ± 11.4 at 24 hours and 241% ± 11.8 at 48 hours, data not shown). In HCT116 $p53^{-/-}$ cells, BK induced the growth of these serum starved cells in a dose-dependent manner at 24 hours where it reached its maximum at 1 μM BK treatment (117% ± of 14) (Figure 12C). However, growth of these cells decreased at 48 hours (Figure 12C).

HCT116 p53$^{-/-}$ cells grown in 10% FBS were still viable and proliferating at 24 hours and 48 hours (157% ± 7.7 and 216% ± 23.2, respectively, data not shown).





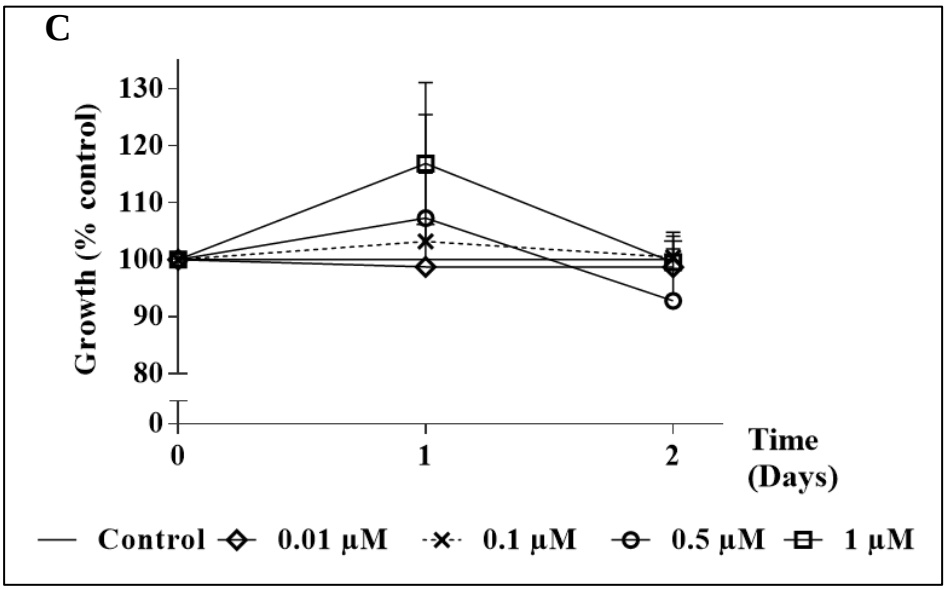


Figure 12. Effect of bradykinin on the growth of human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ were treated with the indicated concentrations of bradykinin (BK). Results of MTT assay, performed in triplicates, were expressed as percentage of control representing the average of at least three independent experiments ± SEM for NCM460 and HCT116 cells, and the average of two independent experiments ± SD for HCT116 $p53^{-/-}$ cells. Statistical significance is reported by two-way ANOVA indicating differences between treated cells and control at various time points (*, $P < 0.05$; **; $P < 0.01$).

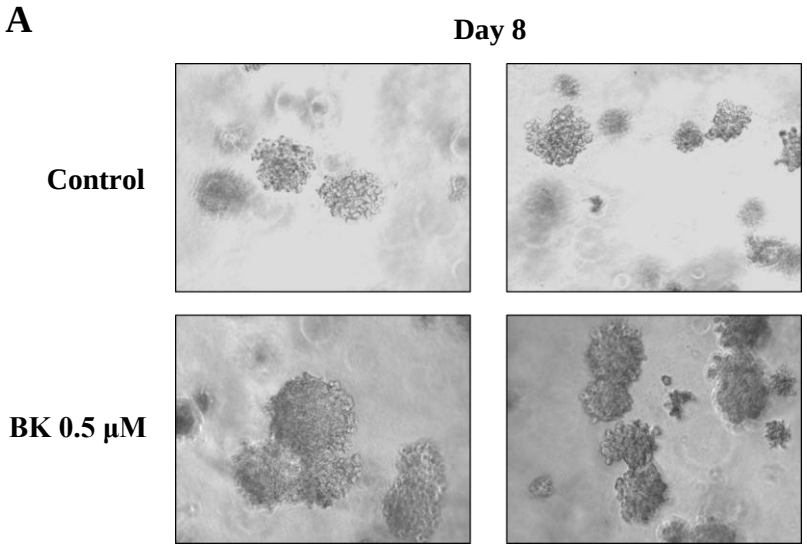
D. Effect of Bradykinin, ATRA, and Hoe 140 on Anchorage-Independent Growth of Human Colorectal Cancer cells

Although widely employed for cell-based assays, 2D cell culture lacks the natural surroundings of the cells such as cell-cell and cell-ECM interactions. On the other hand, several studies demonstrate that 3D cell cultures are more physiologically related to the environment of the cells than 2D which makes 3D cultures closer to the *in vivo* setting (Adcock, 2015).

Soft agar colony formation assay, a 3D cell culture model, was used to assess anchorage-independent growth of colorectal cancer HCT116 and HCT116 $p53^{-/-}$ cells upon treatment with 0.1 and 0.5 µM BK, 1 µM ATRA, and 1 µM Hoe 140. This assay

reflects the ability of anchorage independent-growth of cells that undergo transformation allowing them to grow independently of adhering to a solid surface.

BK treatment in HCT116 cells grown in 10% serum induced colony growth in a dose-dependent manner where it reached 212% relative to control values after treatment with 0.5 μM BK for 8 days (Figure 13A, 13B). Hoe 140, the selective B2R antagonist, at 1 μM concentrations increased slightly colony growth by 118%. However, pre-treatment with Hoe 140 abrogated BK-induced HCT116 colony growth at both BK concentrations (99% colony growth relative to control) (Figure 13A, 13B). Interestingly, ATRA treatment alone almost had no effect on colony growth, yet it completely abolished BK-induced HCT116 anchorage-independent growth at both BK concentrations (85% and 90% colony growth relative to control when treating with 1 μM ATRA/0.1 μM BK and 1 μM ATRA/0.5 μM BK respectively) (Figure 13B, 13C).



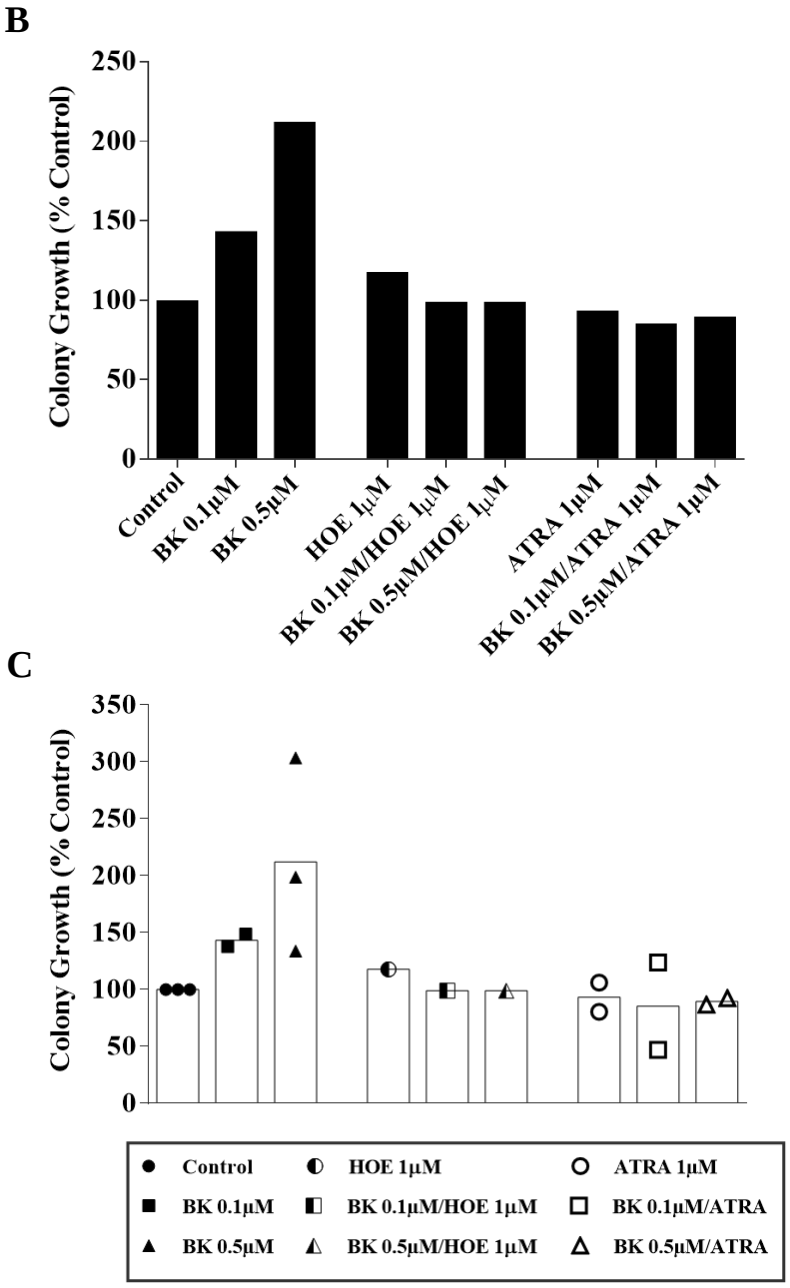


Figure 13. Effect of bradykinin, ATRA, and Hoe 140 on anchorage-independent growth of human colorectal cancer cells. HCT116 were suspended in triplicates in soft agar with the corresponding vehicles or with the indicated concentrations of bradykinin (BK), ATRA, and Hoe 140. Treatment and fresh media were replenished every other day. (A, B) Colony growth was photographed and quantified after 8 days of seeding using the CytoSelect TM 96-Well Cell Transformation Assay kit. Representative phase contrast images of HCT116 colonies control cells and treated with BK were acquired using Zeiss axiovert light microscope. Results are expressed as percentage of control cells. (C) Colony growth for the biological replicates of each condition was represented in a scatter plot.

Preliminary data on HCT116 $p53^{-/-}$ cells shows that BK induced colony growth at both BK concentrations for 8 days (261% and 190% colony growth relative to control when treated with 0.1 μM and 0.5 μM BK respectively) (Figure 14). Hoe 140, at 1 μM, induced colony growth by 123% relative to control-treated cells. Pre-treatment with Hoe 140 decreased BK-induced colony growth only when 1 μM BK was used, yet the percentage of colony growth remained higher than control levels (170% colony growth relative to control) (Figure 14). However, Hoe 140 had no effect on BK-induced colony growth when 0.5 μM BK was used (191% colony growth relative to control). ATRA treatment alone had no effect on colony growth however, it abolished BK-induced anchorage-independent growth at both tested-BK concentrations (Figure 14). When ATRA was used in combination with 0.1 μM BK, colony growth was reduced till below control levels (30% colony growth relative to control) but did not affect colony growth when combined with 0.5 μM BK (Figure 14).

Of interest when the soft agar cell transformation assay was performed on NCM460 and BK treatment was used, these cells barely formed colonies giving absorbance values close to the background (O.D. of 442 for the NCM460 cells *versus* 365 for the background) and treatment with BK slightly increased their growth (O.D. of 576 for BK treated cells) (Figure 15).

Altogether, these results indicate that bradykinin augments anchorage-independent growth of colorectal cancer cells in a B2R-dependent manner and ATRA treatment abolishes this effect.

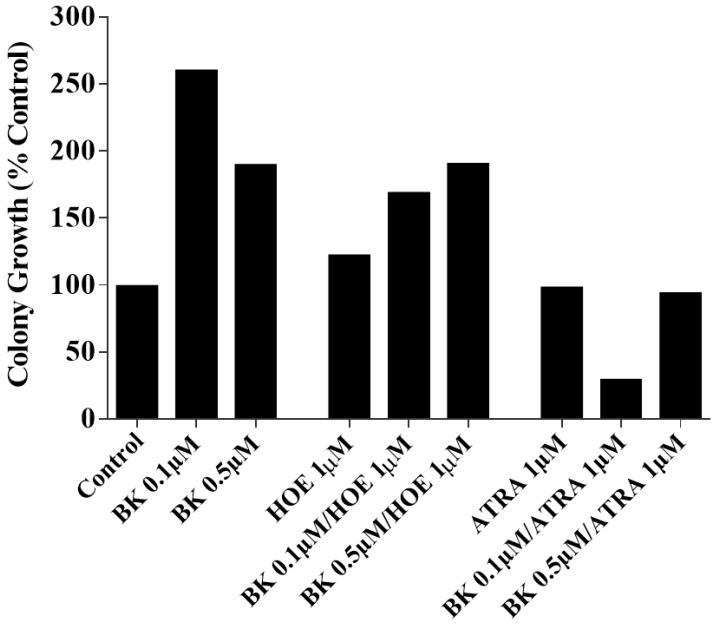


Figure 14. Effect of bradykinin, ATRA, and Hoe 140 on anchorage-independent growth of human colorectal cancer cells with *p53* null status. HCT116 $p53^{-/-}$ were suspended in triplicates in soft agar with the corresponding vehicles or with the indicated concentrations of bradykinin (BK), ATRA, and Hoe 140. Treatment and fresh media were replenished every other day. Colony growth was quantified after 8 days of seeding. Results are expressed in triplicate wells as percentage of control cells and are derived from preliminary data from one experiment.

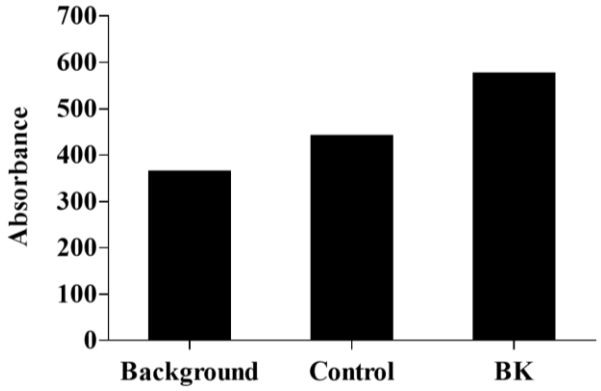


Figure 15. Normal colon cells do not grow in soft agar. NCM460 cells were resuspended in triplicates in soft agar with or without 0.1 μM bradykinin (BK). Treatment and fresh media were replenished every other day. Colony growth was quantified after 8 days of seeding. Colony growth is expressed as the average of absolute absorbance values of the background, control cells, and BK-treated cells. Values represent the average of triplicate wells.

E. Regulation of Bradykinin 2 Receptor Protein Levels by Bradykinin and ATRA in Human Colorectal Cells

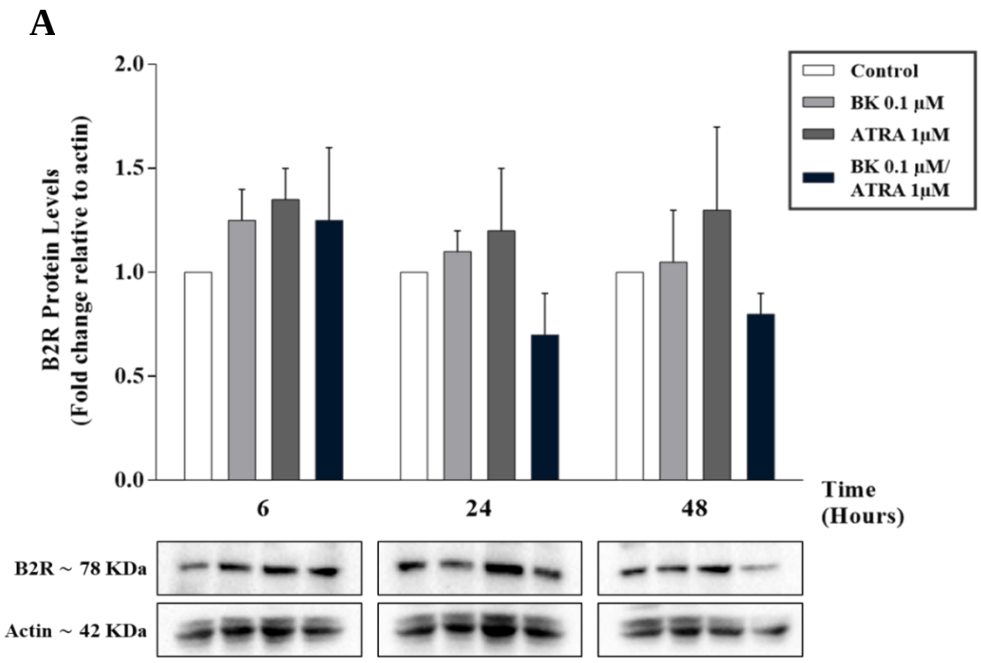
After assessment of the basal levels of bradykinin and retinoic acid receptors in colorectal cells, we investigated by western blot analysis the effects of BK, ATRA, and their combination treatments on B2R protein levels. NCM460, HCT116, and HCT116 $p53^{-/-}$ cells were treated with 0.1 μM BK, 1 μM ATRA, and their combination for 6, 24, and 48 hours. Then, B2R protein levels were measured relative to actin and treated cells were compared to the control.

In NCM460 cells, B2R levels were slightly increased at 6 hours due to BK treatment (1.3 folds ± 0.2) (Figure 16A). ATRA also increased slightly B2R levels at all three-time points, mostly at 6 hours (1.4 folds ± 0.2). As for the combination treatment at 6 hours B2R was slightly increased having the same level as when treated with BK (1.3 folds ± 0.5). However, B2R levels decreased at 24 (0.7 folds ± 0.3) and 48 hours (0.8 folds ± 0.1) upon treatment with the combination of BK and ATRA (Figure 16A).

In HCT116, BK increased B2R levels at 6 hours (1.7 folds ± 0.5, BK *versus* control), then B2R levels decreased gradually reaching below basal levels at 48 hours (0.7 folds ± 0.2) (Figure 16B). ATRA treatment gave the same trend as BK on B2R profile. At 6 hours, ATRA significantly induced B2R levels (2.2 folds ± 0.7) which returned to basal levels at 48 hours. Co-treatment with BK and ATRA resulted in lower levels of B2R than each treatment alone (1.4 folds ± 0.2, BK/ATRA *versus* control with no statistical significance). And at 24 and 48 hours post-treatment, B2R levels were below basal (0.8 folds ± 0.1 and 0.8 folds ± 0.2 respectively) upon BK and ATRA combination treatment (Figure 16B).

In HCT116 $p53^{-/-}$ cells, BK increased slightly B2R levels at 24 hours and 48 hours (1.3 folds $\pm$ 0.3 and 1.2 folds $\pm$ 0.2, respectively) (Figure 16C). ATRA resulted in similar effects to BK at 24 and 48 hours (1.2 folds $\pm$ 0.2 and 0.1, respectively). On the other hand, co-treatment of BK and ATRA had no effect on B2R levels at all time points (Figure 16C).

In summary, BK, ATRA, and their combination treatments did not significantly modulate B2R protein levels in NCM 460, HCT116, and HCT116 $p53^{-/-}$ cells.



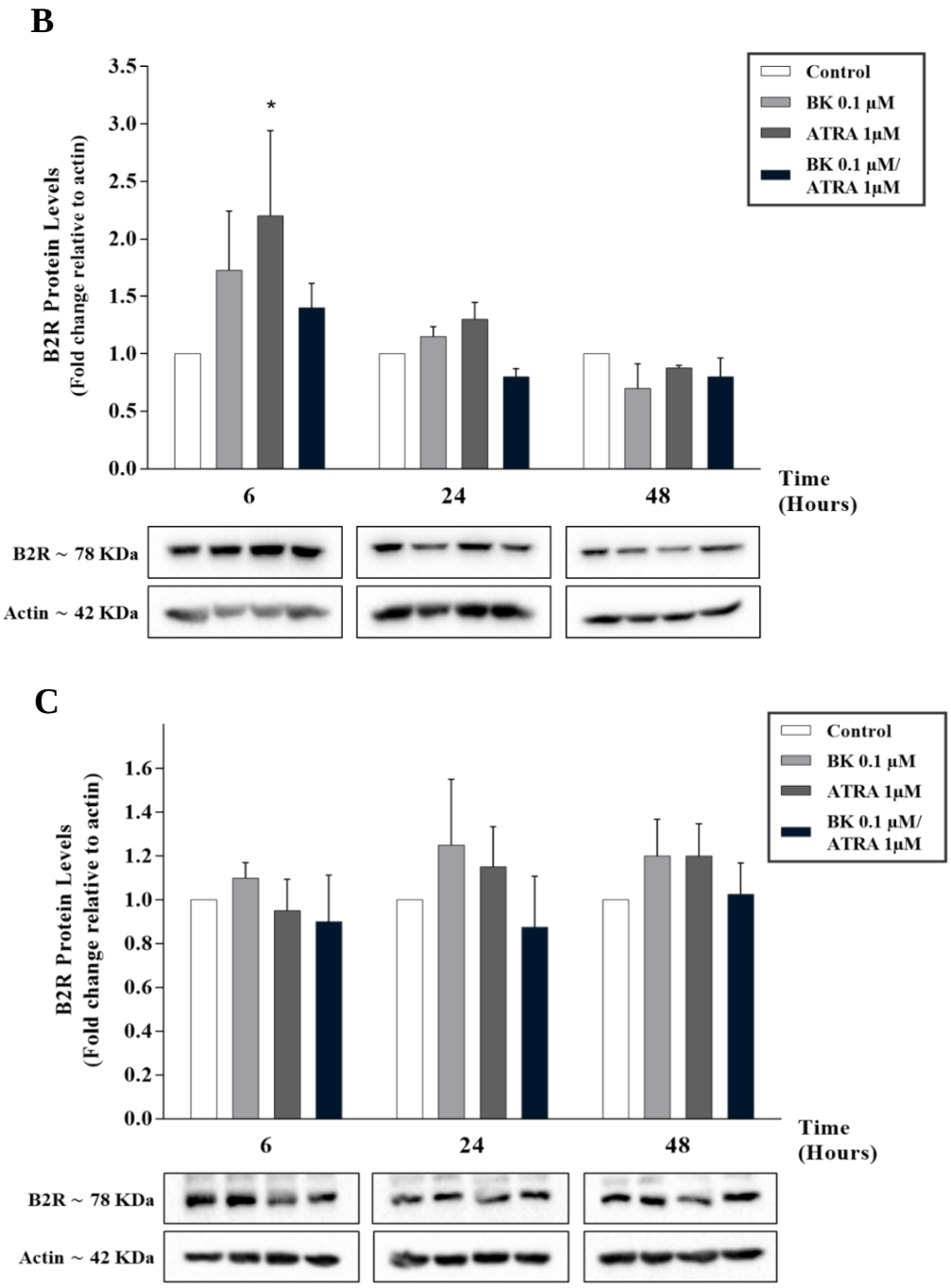


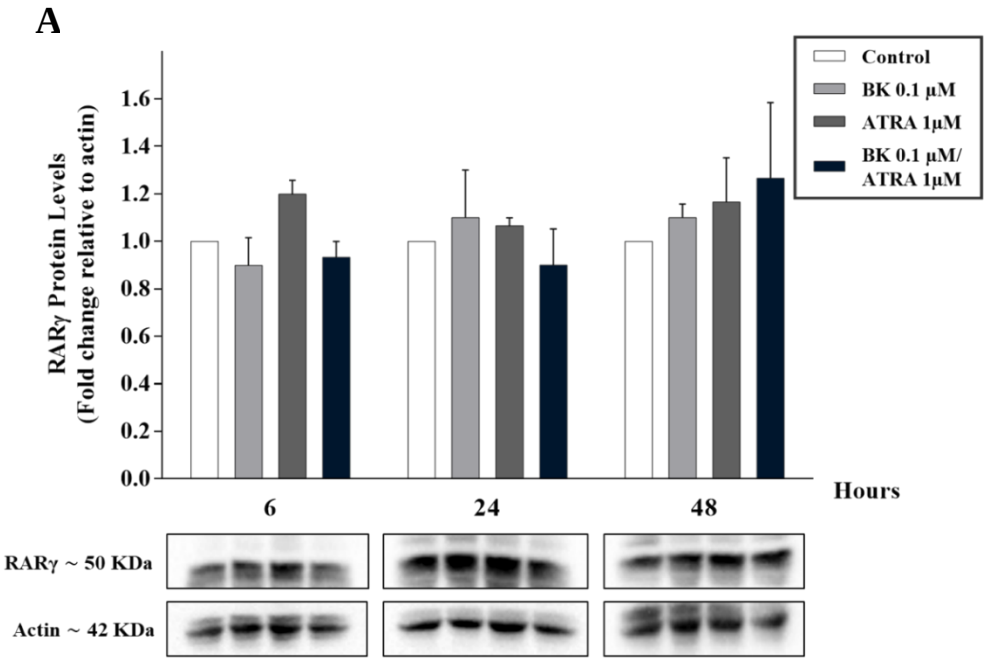
Figure 16. Regulation of B2R protein levels by bradykinin and ATRA in human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 µM bradykinin (BK), 1 µM ATRA, and their combination for 6, 24, and 48 hours. Total proteins were extracted from these cells and immunoblotted against B2R and actin antibodies. The bar graph represents the fold change of B2R protein levels relative to actin; expressed as fold of control cells. Results are representative of the mean of 2 independent experiments ± SD for NCM460 and 4 independent experiments ± SEM for HCT116 and HCT116 $p53^{-/-}$. Statistical significance is reported by two-way ANOVA post hoc Dunnett in HCT116 indicating differences between treated cells and control at various time points (*, $P < 0.05$).

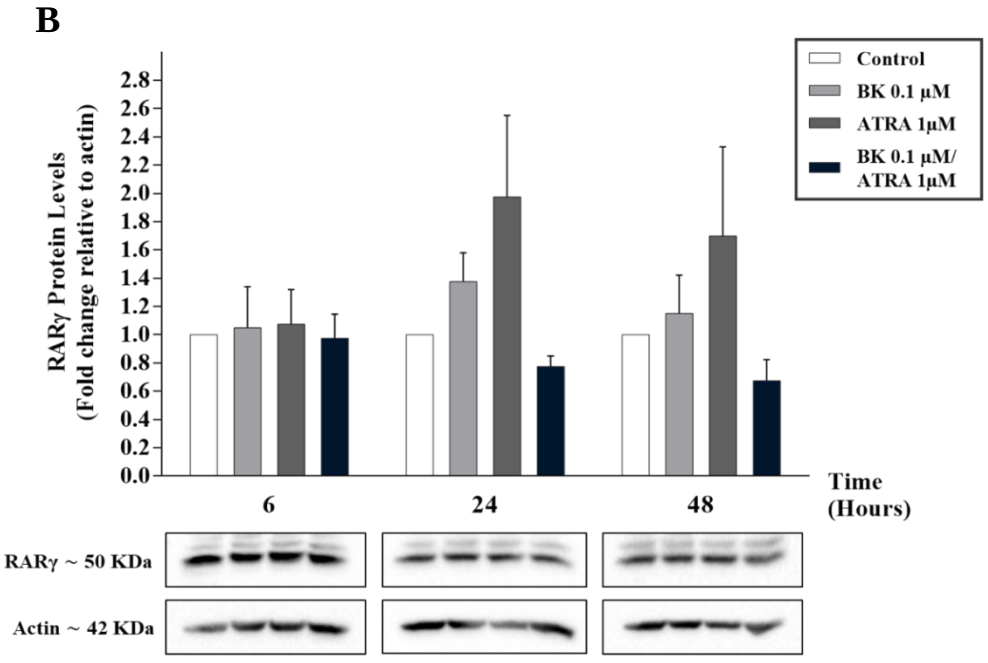
F. Regulation of Retinoic Acid Receptor Gamma Protein Levels by Bradykinin and ATRA in Human Colorectal Cells

The effect of BK, ATRA, and their combination on RARγ protein levels was also evaluated. NCM460, HCT116, and HCT116 $p53^{-/-}$ were treated with 0.1 μM BK, 1 μM ATRA, and their combination for 6, 24, and 48 hours. Then, RARγ protein levels were measured relative to actin by western blot analysis and its levels in treated conditions were compared to control.

In NCM460, cells BK treatment had no effect on RARγ protein levels while ATRA increased slightly the levels of this protein at 6 and 48 hours (1.2 folds ± 0.1 and 1.2 folds ± 0.2, respectively) and co-treatment with BK and ATRA increased slightly RARγ levels at 48 hours (1.3 folds ± 0.3) (Figure 17A). In HCT116 cells, BK treatment induced an increase in RARγ at 24 and 48 hours (1.4 folds ± 0.2 and 1.2 folds ± 0.3, respectively). ATRA also increased RARγ levels but to a higher extent than BK treatment at 24 and 48 hours, however, this increase was not significant (2 folds ± 0.6 and 1.7 folds ± 0.6, respectively). In contrast, BK and ATRA combination treatment reduced slightly RARγ levels at these two-time points (0.8 folds ± 0.1 and 0.7 folds ± 0.1, respectively) (Figure 17B). In HCT116 $p53^{-/-}$ cells, BK increased the levels of RARγ at 6 hours and significantly at 48 hours (1.4 folds ± 0.2 and 1.5 folds ± 0.1, respectively) (Figure 17C). ATRA induced an increase in this receptor protein levels at all three-time points, which was significant at 24 and 48 hours (1.9 folds ± 0.4 and 1.5 folds ± 0.1, respectively). Co-treatment with BK and ATRA also increased RARγ protein levels, which was significant at 6 hours (1.8 folds ± 0.2) (Figure 17C).

In summary, BK, ATRA, and their combination treatments enhanced RARγ protein levels in HCT116 $p53^{-/-}$ but not in NCM460 and HCT116 cells.


A
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
RARγ Protein Levels
(Fold change relative to actin)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6
24
48
Hours
RARγ ~ 50 KDa
Actin ~ 42 KDa


B
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
RARγ Protein Levels
(Fold change relative to actin)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6
24
48
Time
(Hours)
RARγ ~ 50 KDa
Actin ~ 42 KDa

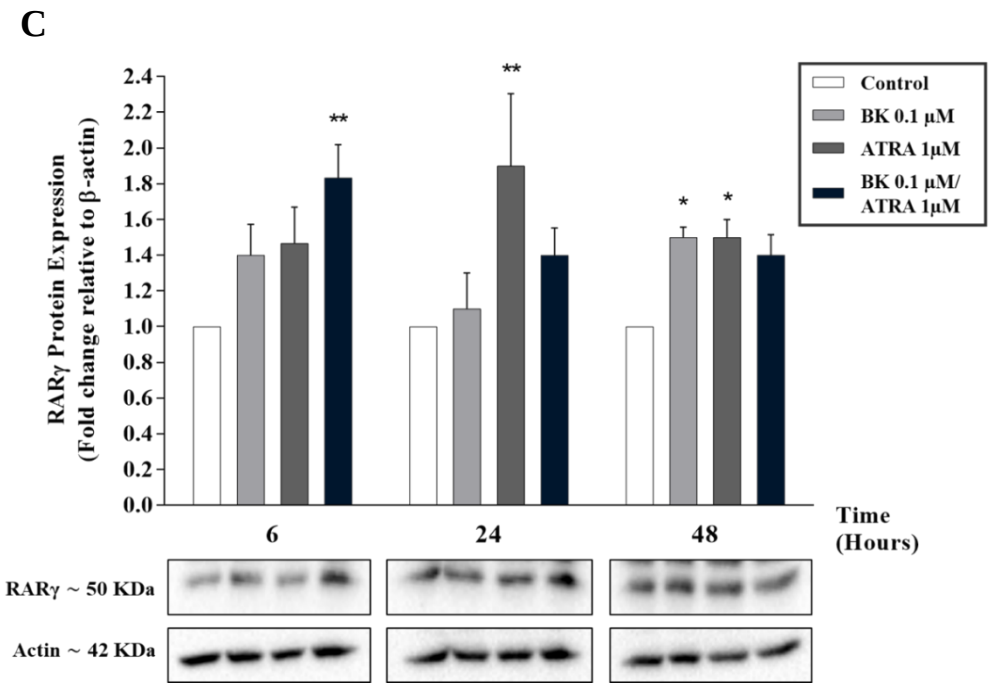


Figure 17. Regulation of RARγ protein levels by bradykinin and ATRA in human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 μM bradykinin (BK), 1 μM ATRA, and their combination for 6, 24, and 48 hours. Total proteins were extracted from these cells and immunoblotted against RARγ and actin antibodies. The bar graph represents the fold change of RARγ protein levels relative to actin; expressed as fold of control cells. Results are representative of the mean of at least 3 independent experiments ± SEM. Statistical significance is reported by two-way ANOVA post hoc Dunnett indicating differences between treated cells and control at various time points (*, $P < 0.05$; **; $P < 0.01$).

G. Effect of Bradykinin on the Subcellular Localization of Bradykinin 2 Receptor and Retinoic Acid Receptor Gamma in Human Colorectal Cells

To study the subcellular localization of B2R and RARγ upon BK treatment, a subcellular fractionation protocol was optimized and performed on HCT116 cells treated with 0.1 μM BK for 30 minutes.

We chose to use and optimize a modification of the fractionation protocol described by (Heckler et al., 2016) for the following reasons. First, this protocol does not require complex buffer preparation nor many reagents. Also, it is straightforward,

fast, and simple to perform. Finally, this subcellular localization protocol does not require lots of cells to be extracted. In summary, it is easier to achieve with lower cost.

Several optimization steps were performed till obtaining the best separation of nuclear and cytoplasmic fractions shown by Ponceau red and immunoblot analysis where β-tubulin was used as a marker for the cytoplasmic and NUP98 for the nuclear fractions (Figure 18A, 18B). β-tubulin prominently appeared in the cytoplasmic extracts while NUP98 signal showed in the nuclear extracts (Figure 18B).

Once optimized, nuclear and cytoplasmic fractions were extracted from HCT116 cells treated with 0.1 μM BK for 30 minutes and were immunoblotted against B2R and RARγ to check for their subcellular localization. Preliminary data shows that the B2R was exclusively localized in the cytoplasm of these cells whether unstimulated or treated with BK (Figure 19). RARγ was localized in both cellular fractions but was predominantly cytoplasmic before and after BK treatment. Although mainly cytoplasmic, RARγ protein levels slightly increased in the nuclear compartment upon BK treatment for 30 minutes (Figure 19).

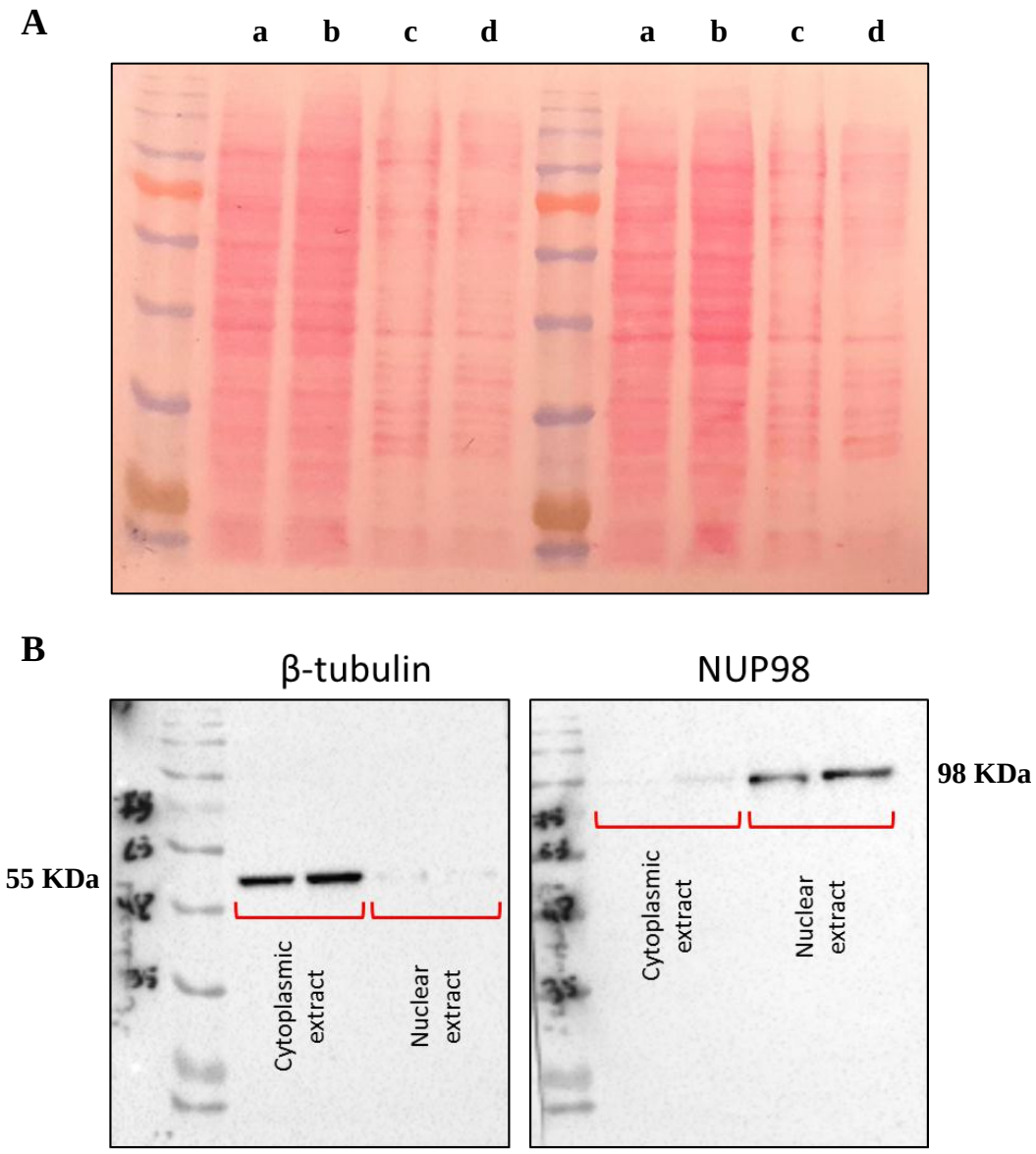


Figure 18. Subcellular fractionation of HCT116. Nuclear and cytoplasmic fractions were extracted from these cells and evaluated by western blot assay. (A) Ponceau red representative of a blot containing cytoplasmic (a, b) and nuclear (c, d) fractions of HCT116 protein extracts ran on SDS-page. (B) Proteins were immunoblotted against β-tubulin and NUP98 antibodies.

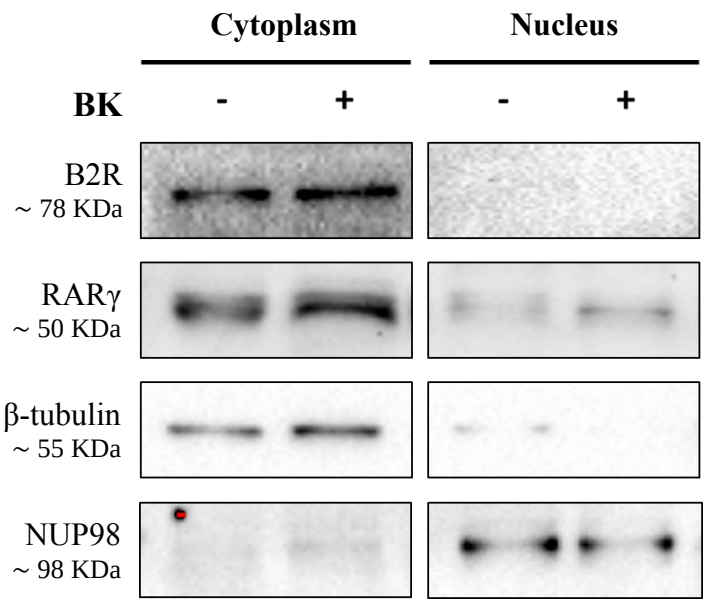


Figure 19. Regulation of intracellular localization of B2R and RARγ by bradykinin in colorectal cancer cells. HCT116 cells were treated with 0.1 μM bradykinin (BK) for 30 minutes. Cytoplasmic and nuclear extracts were immunoblotted against B2R, RARγ, β-tubulin, and NUP98 antibodies.

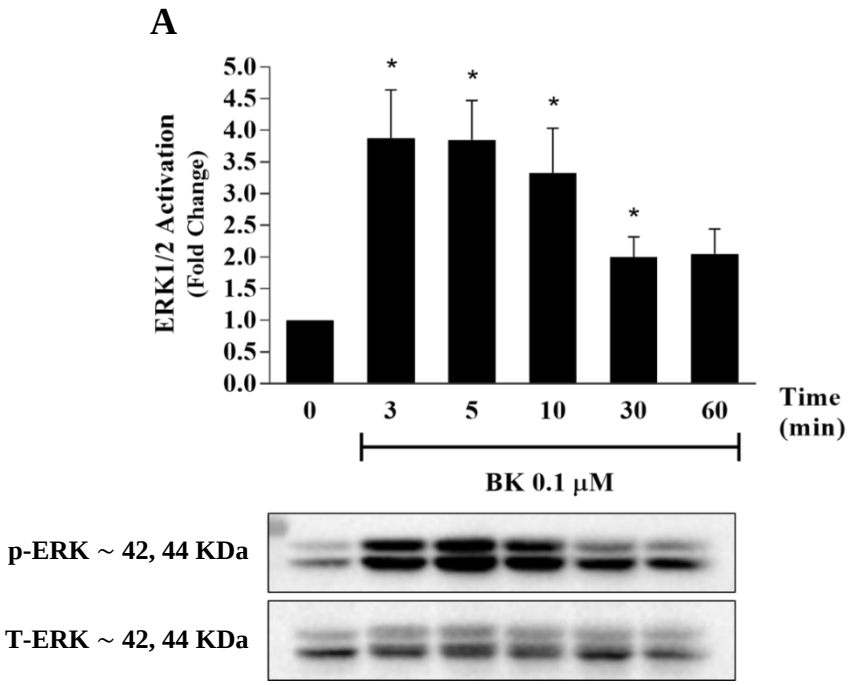
H. Regulation of Extracellular-Signal-Regulated Kinase by Bradykinin in Human Colorectal Cells

ERK1/2 signaling is the most studied MAP kinase pathway. It is involved in the regulation of a wide range of cellular processes such as cell cycle progression, proliferation, apoptosis, angiogenesis, and differentiation. Deregulation of ERK1/2 signaling pathway is commonly encountered in one-third of human cancers affecting tumor development and progression (Dhillon et al., 2007, Meloche and Pouyssegur, 2007).

To evaluate whether BK regulates the activity of ERK1/2 in colorectal cancer cells with different *p53* status and whether BK effects differ in their normal colon counterparts, NCM460, HCT116, and HCT116 $p53^{-/-}$ cells were treated with 0.1 μM BK

for up to 60 minutes and the phosphorylation levels of ERK1/2 relative to its total ERK1/2 protein levels were assessed by western blot analysis.

In NCM460, BK induced the rapid phosphorylation of ERK1/2 up to 30 minutes where it peaked significantly at 3 and 5 minutes (3.9 folds $\pm$ 0.8 and 3.9 folds $\pm$ 0.6, respectively), then phospho-ERK (p-ERK) levels decreased at 60 minutes (Figure 20A). ERK1/2 was also activated in HCT116 yet to a lower extent than in NCM460 cells. p-ERK levels peaked significantly at 3 minutes (1.8 folds $\pm$ 0.2) and decreased gradually afterward (Figure 20B). In sharp contrast, BK had no significant effect on the activation of ERK1/2 in HCT116 $p53^{-/-}$ as shown in Figure 20C.



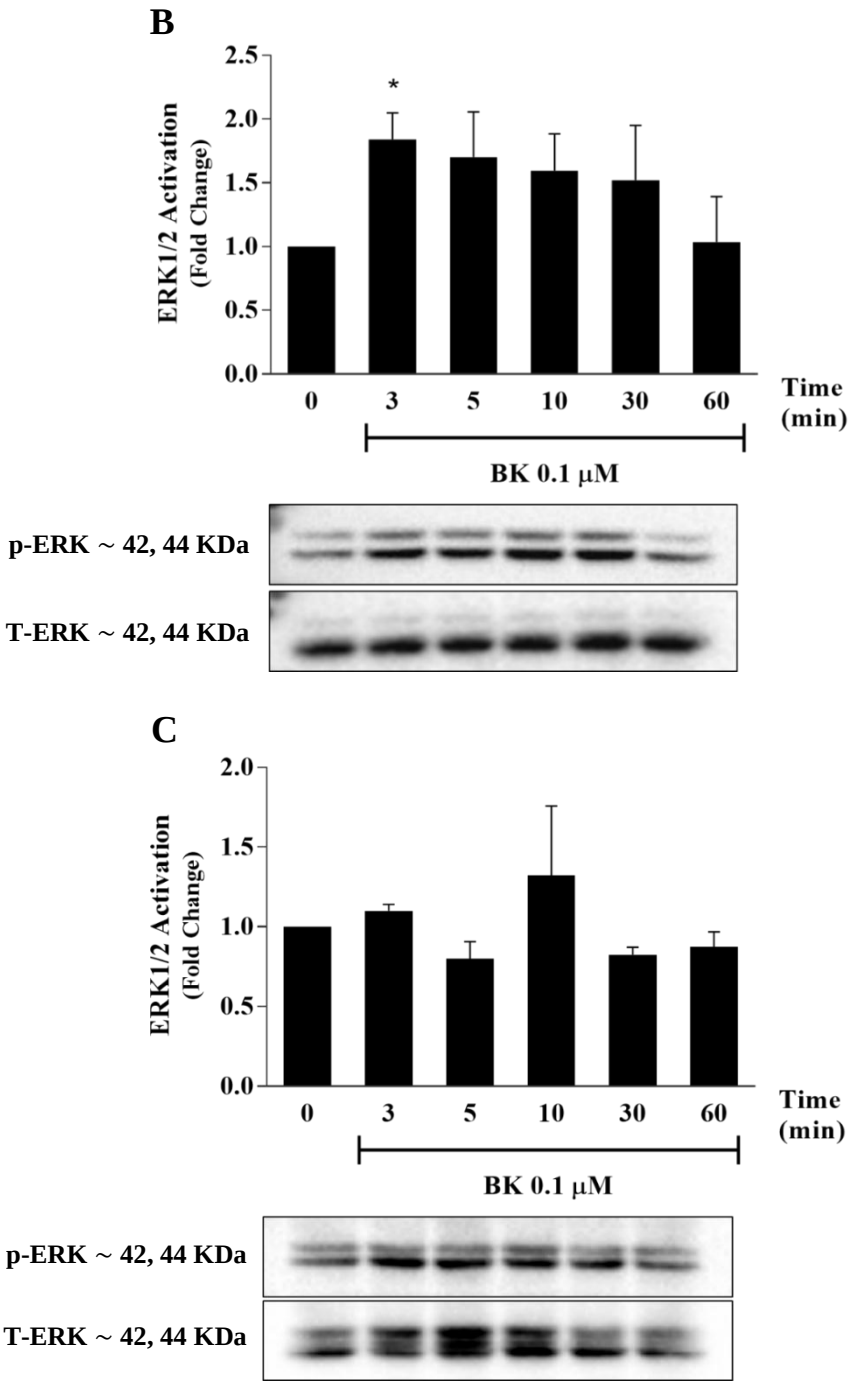


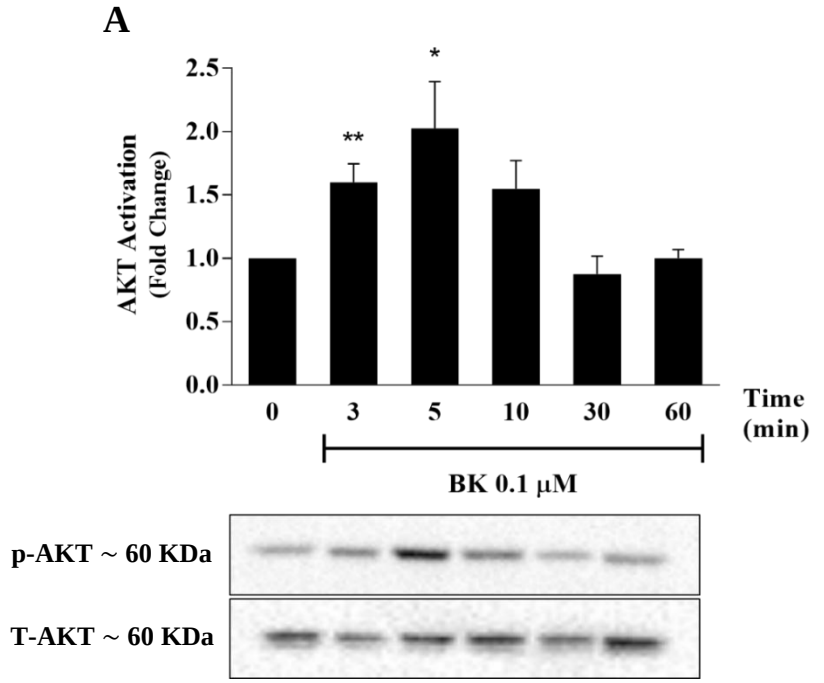
Figure 20. Regulation of ERK1/2 activity by bradykinin in human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 μM bradykinin (BK) for 3, 5, 10, 30, and 60 minutes. Total proteins were extracted from these cells and immunoblotted against phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (T-ERK1/2) antibodies. The bar graph represents the fold change of p-ERK1/2 protein levels relative T-ERK1/2; expressed as fold of control cells. Results are representative of the mean of 4 independent experiments ± SEM. Statistical significance is reported by Student's t-test for NCM460 and HCT116 and by Median test for HCT116 $p53^{-/-}$, indicating differences between BK-treated and control cell (*, $P < 0.05$).

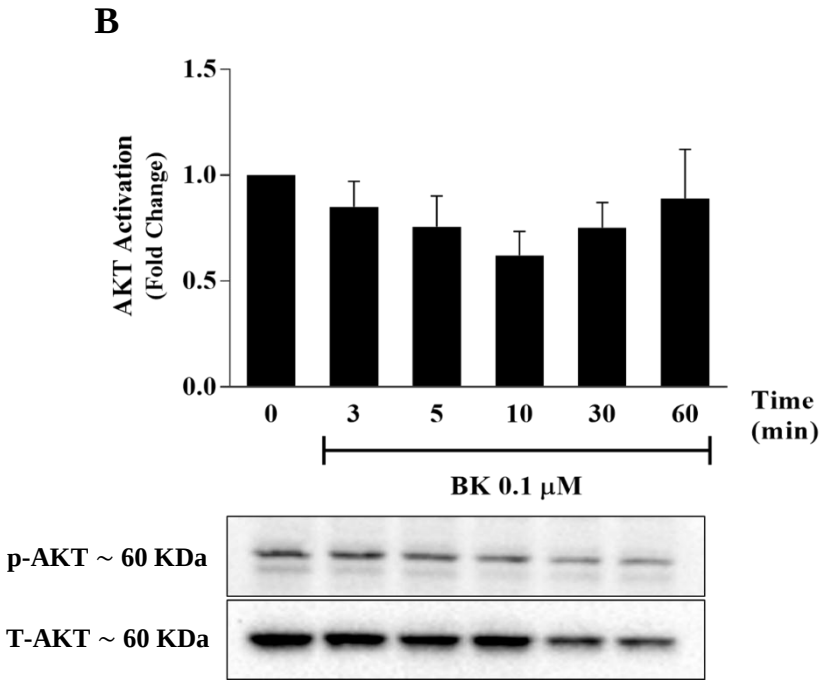
I. Regulation of Protein Kinase B by Bradykinin in Human Colorectal Cells

Protein kinase B or AKT is a prime intermediate of various signaling pathways that regulate divergent cellular processes such as cell proliferation, growth, differentiation, glucose metabolism, and angiogenesis (Testa and Tsichlis, 2005). Abnormal regulation of AKT signaling is reported in many types of cancers which seems to be involved in tumor progression (Nicholson and Anderson, 2002).

Thus, we tested the effect of BK on AKT regulation in normal colon and colorectal cancer cells with different *p53* status. NCM460, HCT116, and HCT116 $p53^{-/-}$ cells were treated with 0.1 μM BK for 3, 5, 10, 30, and 60 minutes and the phosphorylation levels of AKT (p-AKT) relative to total AKT (T-AKT) levels were verified by western blot analysis.

BK significantly induced the activation of AKT in NCM460 cells as early as 3 minutes, where p-AKT levels peaked at 5 minutes (2 folds $\pm$ 0.4) then decreased gradually back to basal levels at 60 minutes (1 fold $\pm$ 0.1) (Figure 21A). As for HCT116 and HCT116 $p53^{-/-}$ cells, there was no significant effect of BK on the phosphorylation of AKT in the treated *versus* control conditions (Figure 21B and 21C).


A
AKT Activation
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
0
3
5
10
30
60
Time
(min)
BK 0.1 μM
p-AKT ~ 60 KDa
T-AKT ~ 60 KDa


B
AKT Activation
(Fold Change)
1.5
1.0
0.5
0.0
0
3
5
10
30
60
Time
(min)
BK 0.1 μM
p-AKT ~ 60 KDa
T-AKT ~ 60 KDa

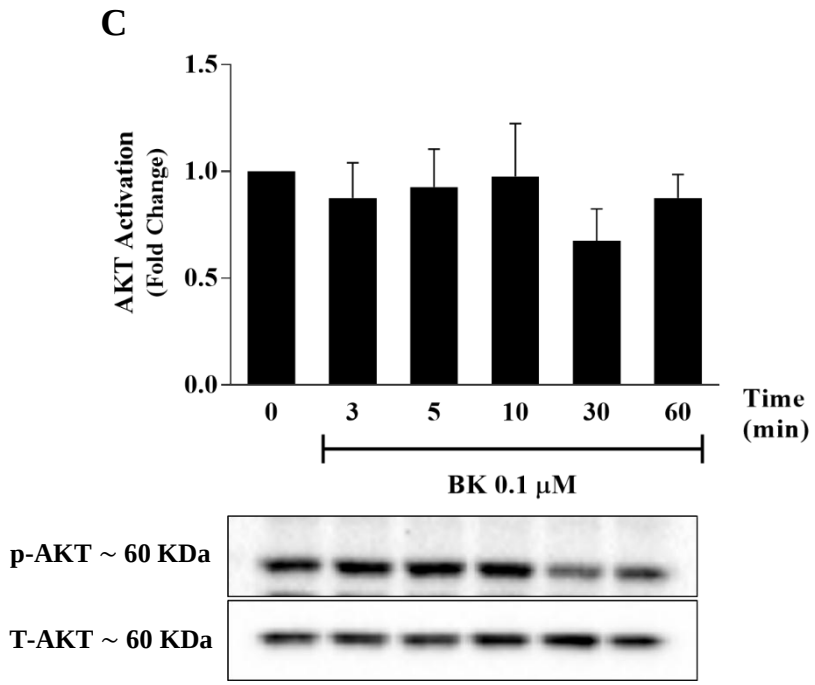


Figure 21. Regulation of AKT activity by bradykinin in human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 μM bradykinin (BK) for 3, 5, 10, 30, and 60 minutes. Total proteins were extracted from these cells and immunoblotted against phosphorylated AKT (p-AKT) and total AKT (T-AKT) antibodies. The bar graph represents the fold change of p-AKT protein levels relative T-AKT; expressed as fold of control cells. Results are representative of the mean of 4 independent experiments ± SEM. Statistical significance is reported by Student's t-test indicating differences between BK-treated and control cells (*, $P < 0.05$; **, $P < 0.01$).

J. Regulation of Epidermal Growth Factor Receptor by Bradykinin in HCT116

EGFR is involved in signaling pathways regulating many cellular processes including cell growth, proliferation, differentiation, cytoskeleton reorganization, and motility (Seshacharyulu et al., 2012, Wang, 2016). Besides ligand binding to the extracellular domain of EGFR, this receptor is induced through transactivation in various cell types by numerous agonists including BK. Once activated, EGFR can stimulate different signaling cascades such as the ERK1/2 and AKT. EGFR transactivation is involved in many diseases including many types of cancers (Wang, 2016).

To evaluate whether BK is involved in EGFR transactivation in colorectal cancer, HCT116 cells were treated with 0.1 μM BK for up to 60 minutes. Western blot analysis was used to assess the levels of the phosphorylated form of EGFR (p-EGFR) relative to its total levels (T-EGFR). BK had no significant effect on the activation of EGFR, rather p-EGFR levels were reduced in BK-treated *versus* control cells and reaching significantly its lower levels at 60 minutes (0.7 folds ± 0.1) as shown in Figure 22.

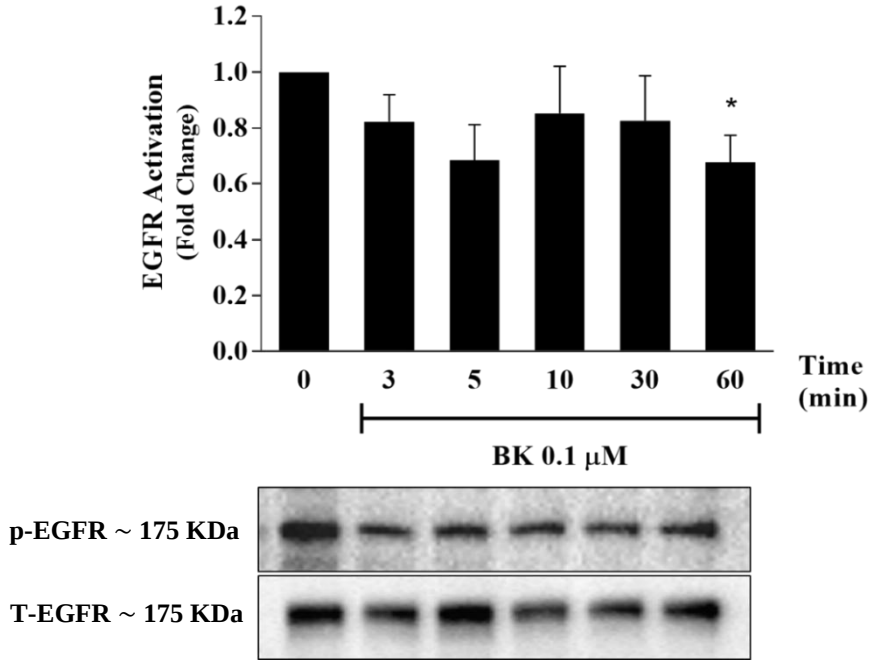


Figure 22. Regulation of EGFR activity by bradykinin in colorectal cancer cells. HCT116 cells were treated with 0.1 μM bradykinin (BK) for 3, 5, 10, 30, and 60 minutes. Total proteins were extracted from these cells and immunoblotted against phosphorylated EGFR (p-EGFR) and total EGFR (T-EGFR) antibodies. The bar graph represents the fold change of p-EGFR protein levels relative T-EGFR; expressed as fold of control cells. Results are representative of the mean of 4 independent experiments ± SEM. Statistical significance is reported by Student's t-test indicating differences between BK-treated and control cells (*, $P < 0.05$).

K. Effect of Bradykinin and ATRA on IL-6 Secretion in Human Colorectal Cancer and Macrophage-Like Cells

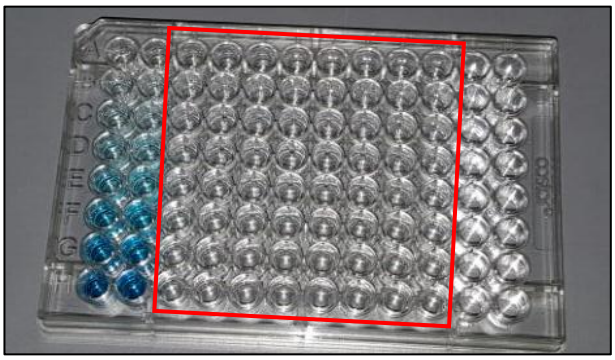
Reports in the literature regarding the expression and secretion of IL-6 in human colorectal cancer cell lines are contradictory. Some studies demonstrate that HCT116 and SW480 cells do not express IL-6, while other studies detected IL-6 transcripts and secretion in the supernatant of HCT116 cells (Patel et al., 2014, Pathak et al., 2015).

First, we aimed to validate whether our colorectal cancer cell lines express and secrete IL-6 and whether BK and ATRA treatments modulate IL-6 levels. HCT116 cells were treated with 0.1 μM BK, 1 μM ATRA, co-treatment of 0.1 μM BK and 1 μM ATRA, and 1 μg/ml LPS for 3, 6, 12, 24, and 48 hours, and IL-6 mRNA levels were assessed by RT-qPCR relative to β-actin mRNA levels and IL-6 secreted levels were evaluated by ELISA assay. LPS was used as a positive control to check for the presence of IL-6 on the mRNA or on the protein levels in the tested cells.

At the level of mRNA, IL-6 was not detectable in HCT116 cells in three independent experiments at 6 hour time point, where the lowest Ct values for all treatment conditions was 37.6 out of 40 cycles. Moreover, the Ct values of LPS treatment had a mean of 37.9 ± 0.5, which is still considered high implicating the absence of detectable mRNA levels in these samples (data not shown). Using ELISA assay, IL-6 secreted protein levels were also not detectable for all tested conditions in two independent experiments at 6, 12, and 24 hours as shown in Figure 23, where O.D. values of the samples were approximately similar to the values of the blank and did not reach the O.D. of the lowest concentration of the standard curve (3.125 pg/ml).

Altogether our results show that HCT116 cells do not express IL-6 as previously demonstrated (Patel et al. 2014).

Before stop solution



After stop solution

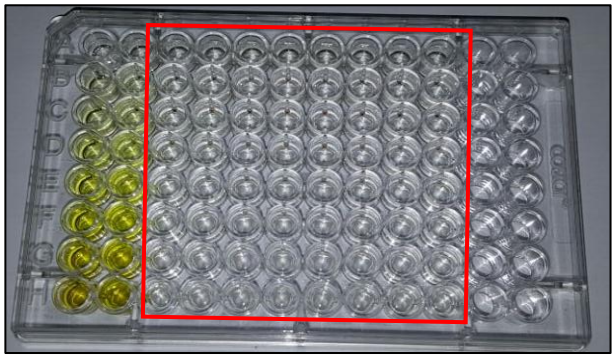


Figure 23. Human IL-6 ELISA plate for HCT116 cells before and after adding the stop solution. The Blue and yellow colors demonstrate the standard curve ranging from 3.125 pg/ml to 200 pg/ml. HCT116 control and treated samples (colorless) are shown in the red square. Results are representative of two independent experiments.

In addition to the malignant cells in the cancer tissue, immune cells are found in the tumor microenvironment that can either promote tumor development or affect its progression (Whiteside, 2006). We hypothesized that BK in the cancer tissue can induce IL-6 expression in immune cells which in turn can affect the tumor cells and that ATRA and co-treatment with BK and ATRA modulate IL-6 expression. We tested this hypothesis on THP-1 human monocyte-like cell line and on THP-1 cells that were differentiated into macrophage-like cells using 50 ng/ml PMA. These cells were treated with 0.1 μM BK, 1 μM ATRA, 0.1 μM BK/1 μM ATRA, and 1 μg/ml LPS for 6 hours and IL-6 mRNA levels were measured by RT-qPCR relative to β-actin mRNA levels.

In THP-1 cells, preliminary data shows that IL-6 mRNA levels were very low (based on the high values of the Ct). LPS treatment, although had a slightly lower Ct, IL-6 induction was still negligible for a sample used as a positive control (data not shown). However, LPS treatment on macrophage-like cells induced elevated expression of IL-6 mRNA (98 folds relative to control) (Figure 24). This result shows that this type of cells expresses IL-6 on the transcript levels. Moreover, BK treatment increased the expression of IL-6 mRNA by 2.7 folds in these macrophage-like cells. Interestingly, ATRA abrogated completely IL-6 mRNA expression (0.1 folds), and abolished BK-induced IL-6 expression to below basal levels (0.7 folds) (Figure 24).

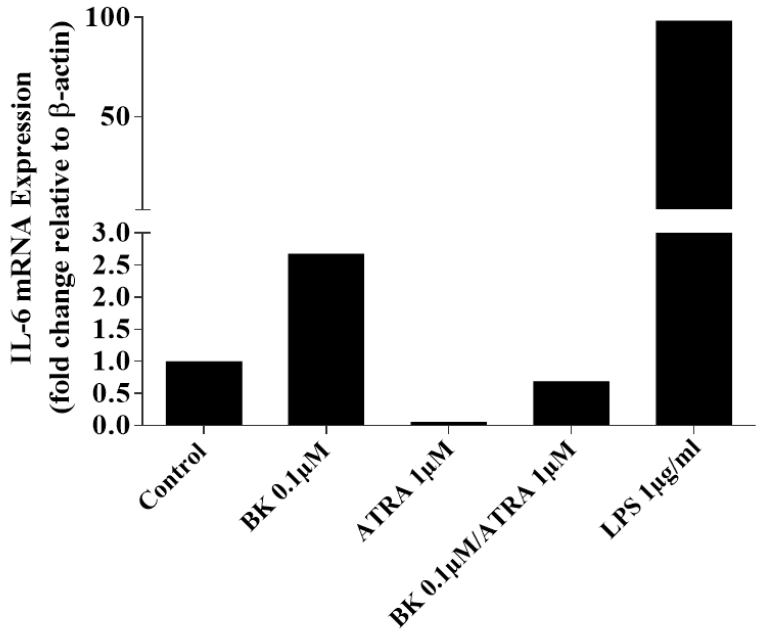


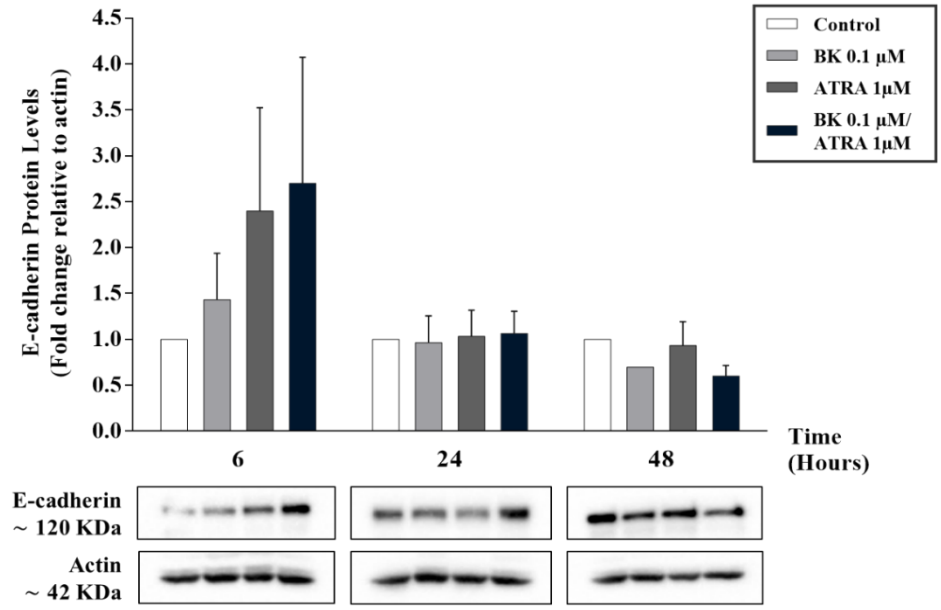
Figure 24. Regulation of IL-6 expression by bradykinin, ATRA, and LPS in macrophage-like cells. Differentiated THP-1 macrophages cells were treated with 0.1 μM bradykinin (BK), 1 μM ATRA, their combination, and 1 μg/ml lipo polysaccharides (LPS) for 6 hours. Total RNA was extracted from these cells and IL-6 and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents data from one experiment and results are plotted as fold change of IL-6 mRNA levels relative to β-actin; expressed as fold of control cell.

L. Regulation of E-cadherin Protein Levels by Bradykinin and ATRA in Human Colorectal Cells

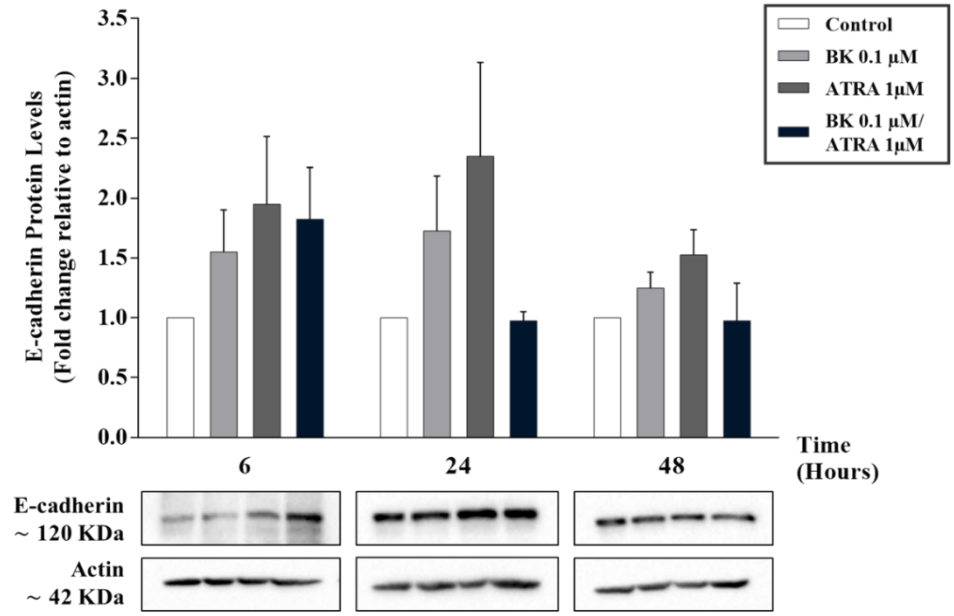
E-cadherin, a cell-cell adhesion glycoprotein, regulates various cellular functions including migration, proliferation, and differentiation (Graziano, 2013). Therefore, downregulation of E-cadherin expression or loss of its function may correlate with tumor development and progression. In fact, loss of E-cadherin has been linked to poor cell-cell adhesion, increase in cell movement, and acquirement of epithelial to mesenchymal transition (EMT) properties (Graziano, 2013, Roger et al., 2010).

Based on this fact, we checked for the regulation of BK on E-cadherin protein levels and whether ATRA can regulate this effect in colorectal cells. NCM460, HCT116, and HCT116 $p53^{-/-}$ cells were treated with 0.1 μM BK, 1 μM ATRA, and 0.1 μM BK/1 μM ATRA for 6, 24, and 48 hours and E-cadherin protein levels were measured by western blot analysis relative to actin. In NCM460 cells, a high level of variability was noted at the different tested conditions at 6 hours (Figure 25A). All three treatments increased E-cadherin levels at 6 hours. BK had the lowest increase in E-cadherin levels (1.4 folds $\pm$ 0.5), followed by ATRA (2.4 folds $\pm$ 1.1), and combination treatment induced an increase in E-cadherin levels by 2.7 folds $\pm$ 1.4, yet without reaching any significance for all treatments relative to control. No changes were observed in E-cadherin levels at 24 and 48 hours of treatment (Figure 25A). Similarly, a high level of variability in E-cadherin protein expression levels was observed in the different tested conditions in HCT116-treated cells (Figure 25B) and HCT116 $p53^{-/-}$ cells (Figure 25C) resulting in non-significant elevated levels of E-cadherin at the different tested time points.

A


E-cadherin Protein Levels
(Fold change relative to actin)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6
24
48
Time
(Hours)
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
E-cadherin
~ 120 KDa
Actin
~ 42 KDa

B


E-cadherin Protein Levels
(Fold change relative to actin)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6
24
48
Time
(Hours)
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
E-cadherin
~ 120 KDa
Actin
~ 42 KDa

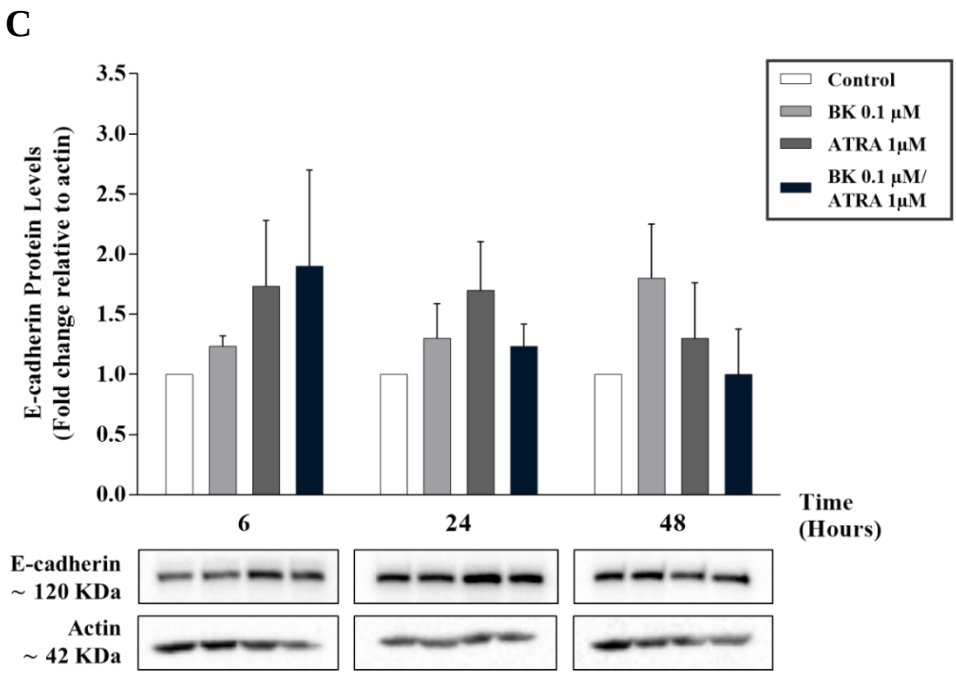


Figure 25. Regulation of E-cadherin protein levels by bradykinin and ATRA in human colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 µM bradykinin (BK), 1 µM ATRA, and their combination for 6, 24, and 48 hours. Total proteins were extracted from these cells and immunoblotted against E-cadherin and actin antibodies. The bar graph represents the fold change of E-cadherin protein levels relative to actin; expressed as fold of control cells. Results are representative of the mean of at least 3 independent experiments ± SEM.

M. Regulation of Fibronectin Protein Levels by Bradykinin and ATRA in Human Colorectal Cells

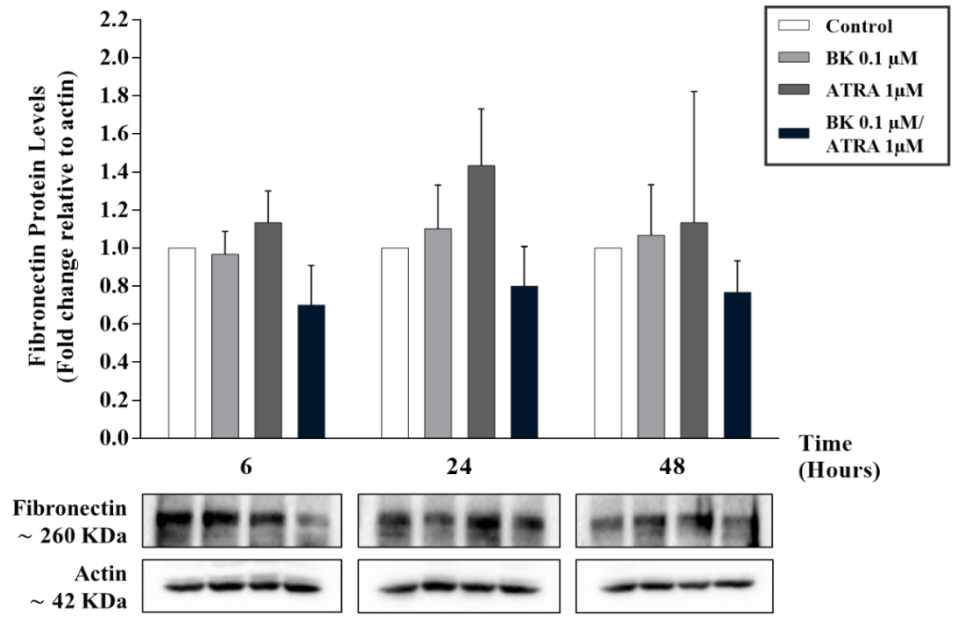
Fibronectin (FN) is an extracellular protein involved in different cellular and physiological processes such as cell adhesion, migration, and wound healing. On the other hand, this protein was found to be increased in many types of cancers and to be implicated in several stages of tumor development and progression (Niknami et al., 2017, Wang and Hielscher, 2017).

Therefore, we checked for the regulation of BK on FN protein levels and whether this effect may be modulated by ATRA in colorectal cells. NCM460, HCT116, and HCT116 $p53^{-/-}$ cells were treated with 0.1 µM BK, 1 µM ATRA, and 0.1 µM BK/1

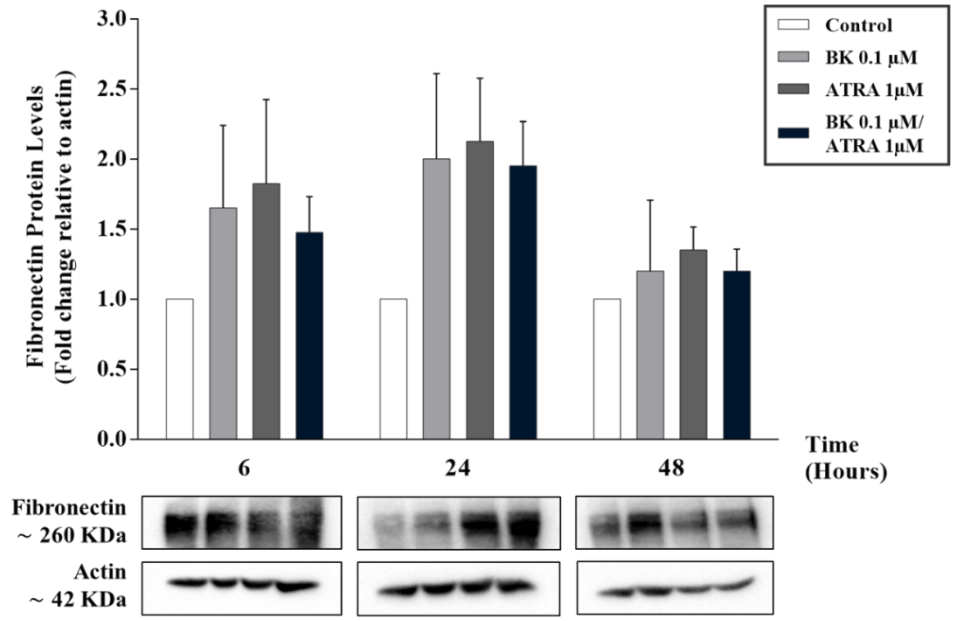
μM ATRA for 6, 24, and 48 hours, then western blot analysis was used to check for FN levels relative to actin. In NCM460 cells, BK did not exert an effect on FN protein levels (Figure 26A). While ATRA, increased FN levels at 24 hours yet this effect was not significant (1.4 folds ± 0.3). Co-treatment with BK and ATRA decreased FN levels at all three-time points having its lowest levels at 6 hours (0.7 folds ± 0.2, with no statistical significance) (Figure 26A). As for HCT116 cells, BK induced an increase in FN levels relative to control-treated cells for a maximum of 2 folds ± 0.6 at 24 hours without statistical significance. ATRA displayed the same pattern as BK treatment and FN protein levels increased to its maximum at 24 hours (2.1 folds ± 0.5). Similarly, co-treatment with BK and ATRA, FN protein levels increased upon treatment reaching 2 folds ± 0.3 relative to control at 24 hours (Figure 26B). In HCT116 $p53^{-/-}$ cells, BK increased FN levels (1.5 folds ± 0.1, 0.5, and 0.3 at 6, 24, and 48 hours respectively) relative to control at all three-time points while ATRA increased FN levels at 48 hours (1.7 folds ± 0.8). Finally, co-treatment with BK and ATRA slightly increased FN levels at all time points reaching a maximum of 1.3 folds ± 0.1 relative to control at 6 hours (Figure 26C).

In summary, these results indicate that BK and ATRA do not consistently regulate FN protein levels in colorectal-treated cells.

A


Fibronectin Protein Levels
(Fold change relative to actin)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
6
24
48
Time
(Hours)
Fibronectin
~ 260 KDa
Actin
~ 42 KDa

B


Fibronectin Protein Levels
(Fold change relative to actin)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
BK 0.1 µM
ATRA 1µM
BK 0.1 µM/
ATRA 1µM
6
24
48
Time
(Hours)
Fibronectin
~ 260 KDa
Actin
~ 42 KDa

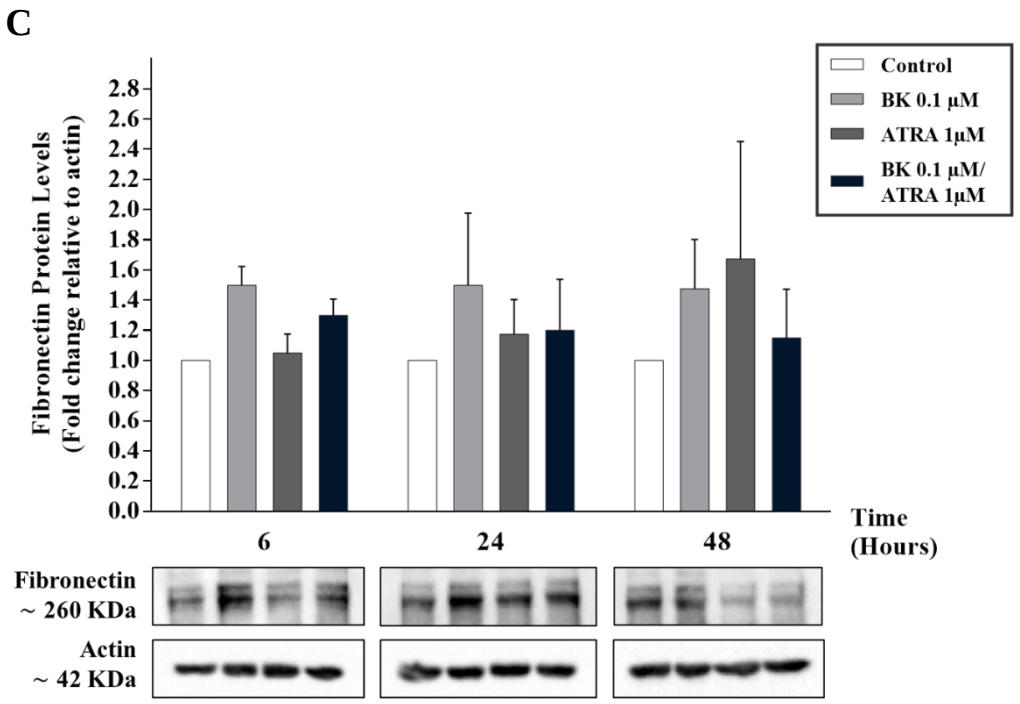


Figure 26. Regulation of fibronectin protein levels by bradykinin and ATRA in colorectal cells. (A) NCM460, (B) HCT116, and (C) HCT116 $p53^{-/-}$ cells were treated with 0.1 µM bradykinin (BK), 1 µM ATRA, and their combination for 6, 24, and 48 hours. Total proteins were extracted from these cells and immunoblotted against fibronectin and actin antibodies. The bar graph represents the fold change of fibronectin protein levels relative to actin; expressed as fold of control cells. Results are representative of the mean of at least 3 independent experiments ± SEM.

CHAPTER V

DISCUSSION

Colorectal cancer is the third most common type of cancer worldwide (Arnold et al., 2017). Despite the introduction of new screening tools and improved treatments, almost half of colorectal cancer patients advance to the metastatic stage with a survival rate of five years (Arvelo et al., 2015). Thus, there is a pressing need for novel diagnostic and therapeutic approaches for colorectal cancer. Chronic inflammation is a major contributor to colorectal tumorigenesis, yet the molecular mechanisms by which inflammation affects tumor progression and development remain elusive and require further investigations.

The pro-inflammatory properties of KKS players and specifically BK are well-established and considerable evidence exists on the role of kinins and their receptors in tumorigenesis, whether by increasing cell growth, cell migration, and inducing angiogenesis (da Costa et al., 2014, Kashuba et al., 2013). Therefore, targeting these players for potential use in cancer therapy should be investigated.

Retinoids are well known for their apoptotic, anti-proliferative, differentiating effects, which made them candidates as chemotherapeutic or chemopreventive agents and anti-cancer drugs to be used in the clinic (Das et al., 2014). Delivery of retinoids to patients can be problematic due to their rapid metabolism, deregulated signaling, and to epigenetic changes that can make neoplastic cells resistant to their effects. Consequently, ideal cancer therapy involving retinoids probably requires combination with other drugs (Tang and Gudas, 2011).

Based on these facts, investigating the possible interactions between the KKS and retinoid system can offer new insights in colorectal cancer which can ultimately be translated to novel biomarkers or therapeutic opportunities using this novel crosstalk.

First, we were interested in identifying the basal levels of the kinin and retinoid receptors in normal and colorectal cancer cells. Our findings show higher levels of B2R in colorectal cancer cells when compared to their normal counterparts. This increase was general among a variety of colorectal cancer cell lines, except for LoVo cells. This cell line is derived from a metastatic nodule of a grade 4 colorectal adenocarcinoma, unlike the others. Further studies are required to explain the fact that they have reduced B2R levels as compared to a variety of colorectal cancer cells. Interestingly, loss of *p53* led to a decrease in B1R levels in comparison with the colorectal cancer cells or normal ones with wild-type *p53*. This may implicate p53 in the regulation of of B1R in these colorectal cancer cells. In fact, p53 and other transcription factors were shown to modulate B1R and B2R gene expression. Moreover, p53 was shown to repress B1R but to activate B2R gene transcription (Bulut et al., 2009). Further studies are required to the check whether B1R is a p53 target gene. Regarding the retinoid receptors profiling in colorectal *in vitro* model, RARα was found to be increased in the colorectal cancer cells when compared to their normal counterparts, while RARγ was decreased in a variety of human colorectal cancer cells. Moreover, loss of *p53* seems to further reduce RARγ expression in colorectal cancer. In fact, RARγ expression was shown to be downregulated in colorectal tumor progression (Perraud et. al 2011). Furthermore, RARα expression in tumor tissues was lower than in adjacent non-tumor specimens and its expression correlated with remission. Thus, our result regarding RARα levels in

these specific cell lines is not in agreement to what was reported in colorectal cancer tissues and may due to tumor heterogeneity *in vivo*.

In summary, kinins and retinoid receptors are deregulated in colorectal cancer cell lines which may be of potential interest to be used as biomarkers, especially when *p53* expression is deregulated which is a common event (Li et al., 2015).

We investigated whether the crosstalk between BK and ATRA regulate colorectal cancer cell growth and transformation. BK induced cell growth of HCT116 cells that was not major, yet it had an opposite effect on their normal-like counterparts. This demonstrates that BK elucidates different mechanisms between normal and cancerous cells and that BK, although it is not a growth factor by itself, is mitogenic in colorectal cancer. On the other hand, soft agar colony formation assay is more relevant to the *in vivo* setting than the conventional 2D assays and colony growth in soft agar is an index of cell transformation. Our tested HCT116 cells were shown to be malignant and to grow in suspension in soft agar whereas the normal-like NCM460 did not grow. BK-induced HCT116 colony formation was B2R-mediated since pre-treatment with the selective B2R antagonist Hoe 140 abolished BK-induced effect on colony growth. Hoe 140 alone induced slightly the colony growth of HCT116; this antagonist at 1 μM concentration might be acting as a biased agonist. However, this effect should be validated with more experiments to rule out if Hoe 140 is acting as a biased agonist. Hoe 140 was shown to can act as a mitogenic agonist where it increased the DNA synthesis rate in various cell lines including SW480 at 1 μM concentration (Drube and Liebmann, 2000). This effect on cell growth is partially understood and whether it is mediated by biased agonist action on B2R receptor still needs verification (Reversi et al., 2005). Interestingly, while ATRA on its own had no effect on colony growth, it

completely abolished BK-induced HCT116 anchorage-independent growth. To our knowledge, these findings are reported for the first time in colorectal cancer. Moreover, these results confirm the possibility of targeting the crosstalk between retinoids and bradykinin signaling pathways for anti-cancer therapy. Using RAR inhibitors, we can identify whether ATRA effect on BK-induced colony growth is RAR-dependent. HCT116 $p53^{-/-}$ cells showed higher induction of colony growth than its parental cell line at lower BK concentrations. This result may be anticipated since in general *p53* null cells are more aggressive than their wild-type counterparts. Hoe 140 reduced BK-induced colony growth in HCT116 $p53^{-/-}$ at this concentration, which may indicate that BK effects may be mediated by other receptors besides B2R. Interestingly, although ATRA had no effect on HCT116 $p53^{-/-}$ colony growth, it abolished BK-induced anchorage-independent growth to below control levels. Hence, ATRA effect in abrogating BK-induced colony growth was shown to be more effective than the receptor's competitive inhibitor in HCT116 $p53^{-/-}$. Altogether, these results indicate that BK augments anchorage-independent growth of colorectal cancer cells in a B2R-dependent manner and ATRA treatment abolishes this effect independently of p53. We have also preliminary evidence showing that BK induces colorectal cell migration and that ATRA reduces this effect (data not shown).

Then, we investigated the potential crosstalk between BK and ATRA regarding B2R and RARγ in colorectal cancer in comparison with the normal colon cells. BK induced its own receptor in the various cancer cell lines which was mostly pronounced in HCT116 and RARγ protein levels in the colorectal cancer cells. ATRA also induced its own receptor, RARγ, and B2R protein levels in the colorectal cancer cells. Further investigations are needed to validate whether ATRA effect on B2R is mediated through

its own receptors. A pan-RAR antagonist may be first used then followed by specific inhibitors for each RAR subtype to identify which RAR partner is mediating ATRA response. Interestingly, ATRA and BK combination treatment had opposite effects on B2R levels than when they were used as single treatment, resulting in reduced levels in the various cell lines. This same observation was noted regarding RARγ levels in HCT116. This implicates a potential crosstalk between those two signaling pathways upon BK and ATRA treatment in HCT116 cells. However, in HCT116 $p53^{-/-}$ cells, BK and ATRA combination treatment, in the early stage, resulted in a synergistic effect and thus elevated levels of RARγ.

Next, we investigated whether BK may influence the subcellular localization of B2R and RARγ and to check whether there is a potential special crosstalk between these two receptors. Preliminary data shows that B2R was cytoplasmic before and after BK treatment. This result is generally anticipated since B2R present on the plasma membrane undergoes endocytosis into the cytoplasm and gets recycled upon treatment. On the other hand, RARγ was predominantly accumulated in the cytoplasm of HCT116 cells. This implicates that RARγ in this colorectal cancer cell line has extranuclear activities and it remains to be determined whether this localization contributes to tumorigenesis. Earlier studies have shown that RARγ resides mainly in the cytoplasm when lung cancer cells were cultured at high-density, released from serum deprivation, and treated with various growth factors (Han et al., 2009). Thus, further investigations are needed to verify if any of those factors affect the cytoplasmic localization of RARγ in our cancer cells. The cytoplasmic localization of the nuclear receptors might regulate transcription of their target genes by controlling the availability of those nuclear receptors in the nucleus. Moreover, when retinoid receptors resided in the cytoplasm of

some cell types, they were shown to be involved in several processes such as growth, apoptosis, differentiation, and inflammation (Han et al., 2009). Interestingly, it seems that upon BK stimulation, the levels of RARγ increased in the nucleus of the treated HCT116 cells. This may implicate BK influencing RARγ localization and subsequently its activity. However, we should take into consideration that this experiment must be repeated several times to validate our findings regarding RARγ localization and if it is regulated by BK. Moreover, this experiment has to be performed on the normal-like colon cells to check for the subcellular localization of RARγ in normal conditions and whether this extranuclear localization of RARγ is specific to colorectal cancer. Using an antagonist for B2R, such as Hoe 140, we can determine if BK mediated-effect on the subcellular localization of RARγ is through the membrane coupled receptor B2R. Furthermore, the localization of other RAR subtypes can be investigated. Importantly, the subcellular localization of RXRα, the heterodimer for the RARs, can be evaluated, since this receptor was shown to retain RARγ in the nucleus after they heterodimerize (Han et al. 2009). Finally, ATRA effect alone and combined with BK should be assessed on the receptors subcellular localization in the context of investigating the spatial crosstalk between the kinin and retinoid systems signaling pathways. Using immunofluorescence, RARγ localization was shown to be influenced by BK treatment in HCT116 (Khati, 2015). When unstimulated, RARγ displayed both cytoplasmic and nuclear localization. 30 minutes stimulation with BK resulted in cytoplasmic distribution while 24 hours treatment showed predominant nuclear localization of RARγ. Interestingly, in HCT116, ERK1/2 was shown to be involved in RARγ subcellular localization before and after treatment with BK (Khati, 2015).

Experimental evidence shows that AKT and ERK1/2 pathways are constitutively active in several types of cancer, and BK in several studies was shown to mediate its action through these effectors (Dhillon et al., 2007, Nicholson and Anderson, 2002). In normal-like colon cells, ERK1/2 and AKT were both activated after short stimuli with BK. However, in HCT116 cells only ERK1/2 was activated upon BK stimulation while AKT was not activated. Interestingly, neither ERK1/2 nor AKT were activated in HCT116 $p53^{-/-}$. These findings demonstrate that there is a difference in the response to BK treatment between normal and colorectal cancer cells. Furthermore, the fact that BK was unable to activate ERK1/2 in HCT116 $p53^{-/-}$ unlike its wild-type counterpart suggests that p53 is involved in the activation of this kinase. In cervical cancer, p53 was proposed to act as an upstream regulator of ERK activation which presented an apoptotic function in response to DNA damage stress instead of its usual role in inducing survival (Singh et al., 2007). p53 protein might regulate MAPK signaling through the transcriptional activation of the phosphatases that modulate the activity of those kinases (Singh et al., 2007). To our knowledge, our findings regarding differential ERK1/2 and AKT activation by BK in these colorectal cancer cells with different *p53* status are the first to be reported. Further investigations are required to better understand the mechanism underlying this differential activation of AKT and ERK1/2 between normal and colorectal cancer cells and specifically p53 modulation of ERK1/2 signaling in colorectal cancer. Moreover, ATRA effect can be studied on these same signaling transduction proteins; and assessing the effect of BK and ATRA on the activation of p38 MAPK would be of interest since this kinase was shown to regulate RARγ localization and subsequently its activity by phosphorylating this receptor (Han et al., 2009). By identifying the specific activated downstream effectors of BK

signaling, we can assess whether BK modulation of cell growth, transformation, and IL-6 secretion is mediated via those effectors. Using PD98059, an ERK1/2 inhibitor, we can unravel whether this kinase is involved in BK-mediated effects in cancer cells, and subsequently target it, as a therapeutic approach, to abrogate BK detrimental effects on colorectal cancer. In addition to the above, basal activity of ERK1/2 and AKT between colorectal cancer and normal colon cells should have been evaluated to check whether these mediators are further activated in colorectal cancer or not in comparison with the normal state

As for EGFR, BK did not induce its transactivation in HCT116 cells but rather it decreased its phosphorylation to below basal levels. It remains to be determined whether BK is activating phosphatases that regulate EGFR activity such as protein tyrosine phosphatases (PTPs). Worth noting, that p-EGFR exhibited high basal levels due to autonomous constitutive EGFR activation in our tested colorectal cancer cells. Colorectal cancer cells with low autonomous EGFR phosphorylation had highest increase in the activation of the receptor upon stimulation with its own ligand, while cells with high basal p-EGFR showed no response upon EGF stimulation (Keese et al., 2005). Therefore, it is conceivable that our tested HCT116 cells should be starved for a longer period or pretreated with inhibitors of metalloproteinases to reduce the basal activity of EGFR before treating with BK, and EGF should be used as a positive control to validate if upon stimulation EGFR is being activated beyond basal levels.

In this study, we demonstrated that our tested colorectal cancer cell lines do not express IL-6 and that stimulation with this molecule cannot be autocrine. The interaction between tumor cells and stromal components in the tumor microenvironment can induce several cellular processes favoring cancer development and progression. We

were able to demonstrate that macrophage-like cells responded to BK treatment by inducing IL-6 expression. Interestingly, ATRA completely abrogated IL-6 expression in THP-1 macrophages when used alone, and reduced BK-induced effect on IL-6 expression. These findings provide evidence of the potential pro-inflammatory role of BK in the tumor microenvironment. However, whether colorectal cancer cells express IL-6 receptors and can be stimulated by this cytokine in a paracrine manner still needs to be validated. Of interest is that ATRA is secreted in response to cytokine production (Kim, 2011). ATRA effect on IL-6 production opens promising area on the use of this metabolite to target inflammation-induced tumor development and progression. Worth mentioning that screening of the expression and secretion of other cytokines besides IL-6 in these colorectal cancer cell lines should be investigated in order to confirm if BK and/or ATRA effect is linked to inflammation and that IL-6 on its own is not enough to prove this hypothesis.

Finally, during tumorigenesis and metastasis, E-cadherin expression is lost in many types of tumors specifically in association with EMT (Petrova et al., 2016). ATRA treatment increased E-cadherin levels in the normal-like and colorectal cancer cells. E-cadherin levels were also found to be increased upon ATRA treatment reaching it maximum at 5 μM in HCT116 cells (Woo and Jang, 2012). Our results show that BK was also able to increase E-cadherin levels in the normal and colorectal cancer cells. More studies need to be performed to understand the implication of BK effect and the mechanism underlying this increase as it is unexpected in light of our general findings. In MCF-7 breast cancer cells, BK had no effect on E-cadherin levels, while in SGC-7901 and HGC-27, gastric carcinoma cells, BK decreased E-cadherin levels (Searovic et al., 2009, Wang et al., 2017). On the other hand, FN is a mesenchymal marker that is

found to be increased in many types of cancer (Wang and Hielscher, 2017). Our results show that BK increased its levels in the colorectal cancer cells. Surprisingly, ATRA also augmented FN levels in the normal and the colorectal cancer cell lines but this unexpected finding was nonconsistent among the different cell cultures. ATRA was found to decrease FN and vimentin levels in HCT116 at 5 μM after 48 hours treatment with no starvation conditions (Woo and Jang, 2012). Perhaps this may be due to the use of higher 5 μM ATRA concentrations than our used 1 μM treatment. BK and ATRA combination treatments resulted in lower levels of FN than when each treatment was used alone in all tested cells, specifically in NCM460 where it was below basal levels at all time points.

In summary, our findings suggest a potential crosstalk between bradykinin and retinoic acid receptors signaling that was translated into various functional biological aspects of colorectal cancer. Moreover, our study sheds light on the differences in the underlying mechanisms between normal colon state and colorectal cancer with different *p53* status.

a. Limitations

Several limitations in this study need to be addressed such as experimental variabilities in some western blot analysis, some experiments need to be performed for several other independent ones in order to validate our results, and studies on the mRNA levels should be performed to give a more thorough understanding of the crosstalk between BK and ATRA signaling. Other limitations include the use of the pharmacological inhibitor Hoe 140 to turn off BK signaling through B2R which led to a biased agonist effect; therefore, targeting B2R by siRNA or shRNA or using cell lines with knockout B2R gene represent another approach to block this receptor activity.

b. Future Perspectives

In future studies, studying other functional aspects of BK, ATRA, and their combination treatments is worth completing such as their effect on cell migration and invasion. Also, the mRNA and secreted levels of VEGF, the key mediator of angiogenesis in cancer, may be assessed, along MMP-2 and MMP-9 Levels. Furthermore, the differentiation marker HOXA5 can be evaluated, especially since HOXA5 was shown to counteract cancer stem cell phenotype in colorectal cancer (Ordóñez-Morán et al.). Moreover, other signaling transduction proteins involved in this crosstalk or in inflammation-induced tumorigenesis can be investigated such as the nuclear factor κB (NF-κB). This latter is a transcriptional factor known for its main role in the initiation and propagation of colorectal cancer (Vaiopoulos et al., 2013). The effect of B2R and RARγ on colorectal cancer can be assessed *in vitro* and as well as *in vivo* using knockout cell lines for those two receptors. Cell growth, transformation, migration, and invasion assays *in vitro* can be assessed using those knockout cells and tumor development and progression in mouse xenografts can be monitored. Moreover, the effect of single and combination treatments using ATRA and Hoe140, in order to counteract BK malignant effect, can be evaluated as potential anti-cancer therapeutic agents in HCT116-xenografted tumor cells or APC colorectal cancer model (Moser et al., 1990, Oshima et al., 1995).

Finally, the crosstalk between bradykinin and ATRA in addition to their downstream signaling pathways seem to be promising targets to ultimately decrease the inflammatory conditions surrounding colorectal cancer and to reach more efficient anti-cancer effect of retinoids in colorectal cancer therapy.

Receptor	HCT116 *versus* NCM460	HCT116 p53$^{-/-}$ *versus* NCM460
B2R	↑↑	↑↑
B1R	↑	↓↓
RARα	↑↑	↑↑
RARβ	↑	↑
RARγ	↓↓↓	↓↓↓

Table 2. Kinin and retinoic acid receptors protein profile in colorectal cancer cells compared to normal colon cells.

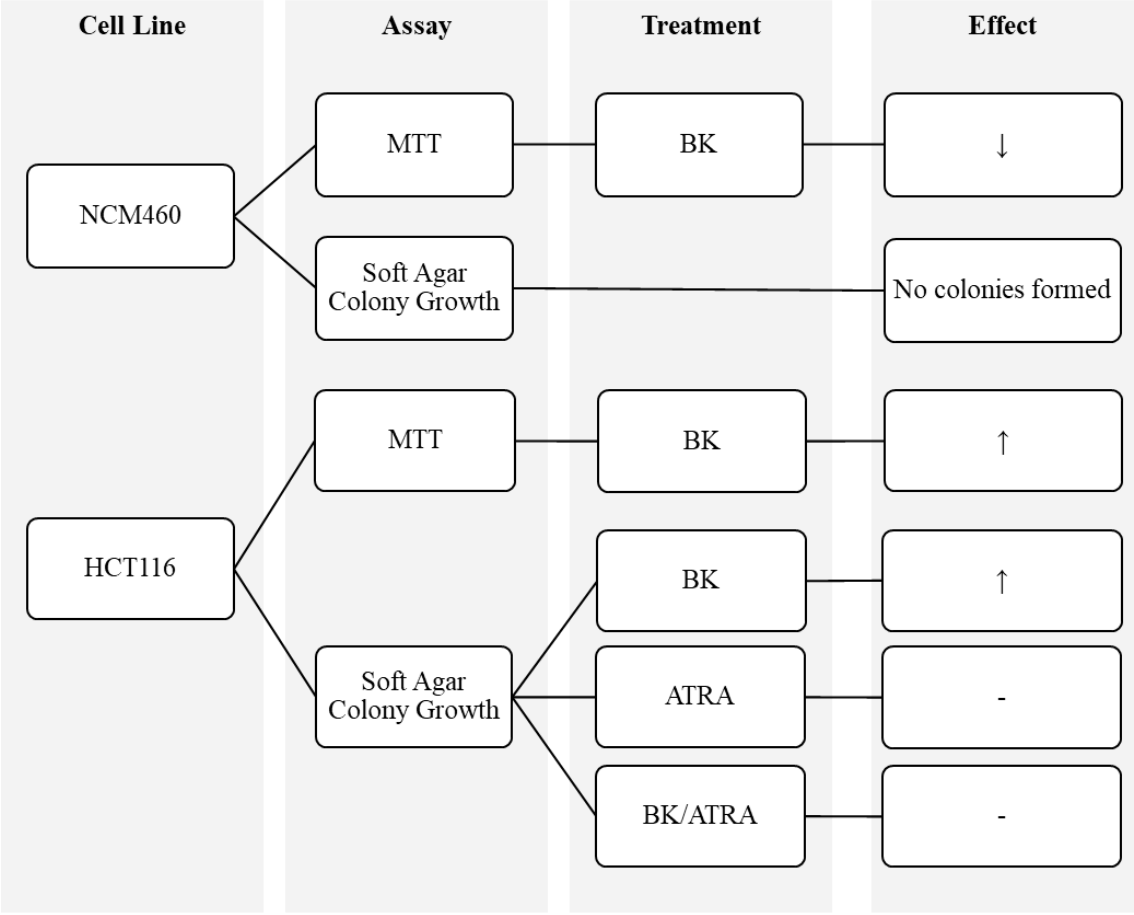


Figure 27. Representative scheme of the effect of BK and/or ATRA on cell growth in MTT assay and colony growth in soft agar assay.

Transduction protein	NCM460	HCT116	HCT116 $p53^{-/-}$
p-ERK1/2	↑	↑	-
p-AKT	↑↑	-	-

Table 3. Effect of bradykinin on ERK1/2 and AKT activation in normal colon and colorectal cancer cells.

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