ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DO PORTO

INSTITUTO POLITÉCNICO DO PORTO

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EXPANDING OUR THERAPEUTIC OPTIONS:

β-BLOCKERS FOR COLON CANCER?

Dissertação submetida à Escola Superior de Tecnologia e Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação científica da Professora Doutora Laura Ribeiro e co-orientação institucional do Professor Doutor Rúben Fernandes.

setembro de 2013

À minha família

"Se as raízes estão bem, todo o resto está bem."

Peter Sellers

AGRADECIMENTOS

À Professora Doutora Laura Ribeiro, toda a orientação, incentivo e dedicação. Por todo o tempo disponibilizado, pela simpatia e por todo o conhecimento que me transmitiu. Por todo a apoio e confiança que depositou no meu trabalho. Por me incentivar a prosseguir quando os contratempos pareciam não acabar. Pela possibilidade de investigar este tema.

Ao Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto e a todas as pessoas que dele fazem parte. Por me terem dado todas as condições técnicas e humanas necessárias para a conclusão de todo o trabalho que desenvolvi.

Ao Departamento de Farmacologia e Terapêutica da Faculdade de Medicina da Universidade do Porto, em especial à Engª. Paula Serrão e à Engª Joana Afonso por toda a simpatia e disponibilidade sempre que precisei.

À Reitoria da Universidade do Porto e ao Banco Santander Totta pela valorização da investigação jovem universitária e pelo financiamento deste projeto PP-IJUP2011-320.

A todos os meus bons amigos agradeço a partilha de experiências, o ânimo, o alento, as boas conversas e a amizade sincera. Àqueles que estão cá desde sempre. Aos que fiz ao longo deste percurso, em especial ao André Cabral e ao Pedro Coelho pela preciosa ajuda e amizade durante este ano. Ao Carlos, toda a paciência, motivação e partilha.

E por último mas mais importante que tudo, a toda a minha Família, a quem devo tudo o que sou. Às minhas princesinhas Nónó e Inês pela responsabilidade das minhas melhores gargalhadas. À minha cunhada Ana. À minha mãe e ao meu irmão por serem os melhores seres humanos que conheço.

FINANCIAMENTO

Supported by U. Porto/Santander Totta (IJUP) (PP-IJUP2011-320)

PUBLICATIONS UNDER THE SCOPE OF THIS THESIS

Communications to scientific meetings

Marisa Coelho, Gustavo Correia, Miguel Moz, Ana Luísa Teixeira, Rui Medeiros, Laura Ribeiro. ***Expanding our therapeutic options: adrenergic beta-blockers for colon cancer?*** IJUP- 6th Meeting of Young Researchers at UP. Porto, February 13-15, 2013.

M. Coelho, M. Moz, G. Correia, A. Sarmento-Cabral, A. Teixeira, R. Medeiros, L. Ribeiro. "***Adrenergic receptor agonists and inverse agonists: role in the HT-29 human colon cancer cell line.***" Neuroimmune Pharmacology: Challenging Paradigms Beyond Boundaries - Integrating pharmacology, immunology, and neurosciences in a therapeutic perspective. November 15-16, 2013, Center of Research in Medical Pharmacology, University of Insubria, Italy. (***BEST POSTER AWARD)***

M. Coelho, M. Moz, G. Correia, A., A. Teixeira, R. Medeiros, L. Ribeiro. "***Role of beta-adrenergic receptor in a human colon cancer cell line***." ASCB Annual Meeting, December 14-18, 2013, New Orleans, LA, USA.

Conference Proceedings

Coelho M, Moz M, Correia G. Teixeira AL, Medeiros R, Ribeiro L. Role of beta-adrenergic receptor in a human colon cancer cell line. *Mol Biol Cell* (accepted for publication).

Coelho M, Moz M, Correia G, Sarmento-Cabral A, Teixeira AL, Medeiros R, Ribeiro L. Adrenergic receptor agonists and inverse agonists: role in the HT-29 human colon cancer cell line. *Journal of Neuroimmune Pharmacology* (accepted for publication).

ABSTRACT

Colon cancer is the fourth and third most common cancer, respectively in men and women worldwide and its incidence is increasing. Stress response has been associated to the incidence and development of cancer. The catecholamines (CA), adrenaline (AD) and noradrenaline (NA), are crucial mediators of stress response, exerting their effects through interaction with α- and β- adrenergic receptors (AR). Colon cancer cells express β-AR and their activation has been implicated in carcinogenesis and tumor progression. Recently, interest in the efficacy of β-AR blockers as possible additions to cancer treatment paradigms has been gaining strength.

The aim of this work was to investigate the effect of several AR agonists and β-blockers, upon cellular proliferation and viability of HT-29 cells, a human colon adenocarcinoma cell line. For this purpose, in the first phase of this work, we determined the EC50 and IC50 values for proliferative and antiproliferative effects, respectively of AR agonists and antagonists. Afterwards, HT-29 cells were incubated in the absence (control) or in the presence of the AR-agonists, AD, NA and isoprenaline (ISO) (0.1-100 μM) for 12 hours or 24 hours. All tested AR agonists revealed proliferative effects upon HT-29 cells. In order to study the effect of several β-blockers upon both proliferation and viability induced by AR activation, cells were treated with propranolol (PRO; 50 μM), carvedilol (CAR; 5μM), atenolol (ATE; 50 μM), or ICI 118,551 (ICI; 5 μM) for 45 minutes prior, and simultaneously, to the incubation with each of the AR agonists, AD and ISO, both at 1 and 10 μM.

Our results suggest that adrenergic activation play an important role in colon cancer cells proliferation most probably through β-AR. All the β-blockers under study were able to revert the proliferation induced by AD and ISO, and some of them, per se, significantly decreased the proliferation of HT-29 cells. The elucidation of the intracellular pathways involved in CA-induced proliferation of colon cancer cells, and also in the reversion of this effect by β-blockers, might contribute to reveal promising strategies in cancer treatment.

Key words: Stress, catecholamines, adrenergic receptors, β-blockers, cell proliferation.

RESUMO

O cancro do cólon é o quarto e terceiro cancro mais comum, respetivamente nos homens e nas mulheres em todo o mundo, e a sua incidência está a aumentar. A resposta ao stresse tem sido associada a um aumento da incidência e desenvolvimento do cancro. As catecolaminas (CA), adrenalina (AD) e noradrenalina (NA) são os principais mediadores da resposta ao stresse, exercendo os seus efeitos através da interação com os recetores adrenérgicos (RA), α e β. As células do cancro do colon expressam predominantemente os RA do tipo β, e a sua ativação está implicada na carcinogénese e na progressão dos tumores. Recentemente, tem sido notório o interesse na eficácia dos RA do tipo β como possíveis adjuvantes para o tratamento do cancro.

O objetivo deste trabalho foi investigar o efeito de vários agonistas para os RA, e de β-bloqueadores, na proliferação e viabilidade de uma linha celular de adenocarcinoma de colon humano, as células HT-29. Para este efeito, na primeira fase deste trabalho, determinámos os valores dos EC50 e IC50, respetivamente para o efeito proliferativo e antiproliferativo dos agonistas e antagonistas dos RA em estudo. Posteriormente, as células foram incubadas na ausência (controlo) e na presença dos agonistas, AD, NA e isoprenalina (ISO) (1-100 μM) durante 12 ou 24 horas. Todos os agonistas em estudo aumentaram significativamente a proliferação das células HT-29. Para estudar os efeitos de vários β-bloqueadores na proliferação e na viabilidade induzida pela ativação dos RA, as células foram tratadas com propranolol (50 μM), carvedilol (5 μM), atenolol (50 μM) ou ICI 118,551 (5 μM) 45 minutos antes, e em simultâneo, do tratamento com cada um dos agonistas AD e ISO a 1 e 10 μM.

Os nossos resultados sugerem que a ativação adrenérgica desempenha um papel importante na proliferação das células do cancro do cólon, muito provavelmente através dos recetores β. Todos os β-bloqueadores testados foram capazes de reverter a proliferação das células HT-29 induzida pela AD e pela ISO, sendo que alguns deles por si só diminuíram significativamente a proliferação destas células.

A elucidação das vias envolvidas na proliferação de células de cancro do cólon induzida pelas CA, e também na reversão deste efeito pelos β-bloqueadores, pode contribuir para revelar estratégias promissoras no tratamento do cancro.

Palavras-chave: Stresse, catecolaminas, recetores adrenérgicos, β-bloqueadores, proliferação celular.

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ABBREVIATIONS

5-LOX	5-lipoxygenase
AAAD	Aromatic L-amino acid decarboxylase
cAMP	Cellular cyclic adenosine-3,5-monophosphate
AC	Adenylyl cyclase
ACTH	Adrenocorticotropin hormone
AD	Adrenaline
ANS	Autonomic nervous system
APC	Adenomatous polyposis coli
AR	Adrenergic Receptor
ATE	Atenolol
BARK	β-adrenergic receptor kinase
BH4	Tetrahydrobiopterin
BrdU	5-bromo-2-deoxyuridine
CA	Catecholamines
CAR	Carvedilol
CNS	Central Nervous System
COX2	Cyclooxygenase-2
CRC	Colorectal Cancer
CREB	cAMP response element-binding
DA	Dopamine
DAG	Diacylglycerol
DDC	Dopa decarboxylase
DMSO	Dimethyl sulfoxide
DOPA	Dihydroxyphenylalanine
DβH	Dopamine-β-hydroxylase
EGFR	Epidermal Growth Factor Receptor
EPAC	Exchange protein activated by adenylyl cyclase

FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
GPCRs	G-protein-couple receptors
HMG-CoA	3-Hydroxil.3methylglutaril-CoA Reductase
HPA	Hypothalamic-Pituitary-Adrenal Axis
ICI	ICI 118,551
IL-6	Interleukin-6
IH	Infantile hemangioma
IP3	Inositol trisphosphate
ISO	Isoprenaline
MAPK	Mitogen-activated protein kinase
MMPs	Metalloproteinases
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-di- methyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA	Noradrenaline
NF-κB	Activate nuclear factor κ-B
NNK	Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PBS	Phosphate buffer saline
PI3K	Phosphatylinositol-3 kinase
PKA	Protein kinase A
PNMT	Phenylethanolamine N-methyltransferase
PRO	Propranolol
SAMS	Sympathetic Adrenomedullary System
SNS	Sympathetic Nervous System
STAT-3	Signal transducer and activator of transcripton-3
TGF-α/β	Transforming growth factor α and β

TH	tyrosine hydroxylase
Tyr	L-Tyrosine
VEGF	Vascular endothelium growth factor

I. CHAPTER

INTRODUCTION

1. INTRODUCTION

1.1. Cancer

Cancer is defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells. This pathology can be caused by both external and internal factors that may act together or in sequence to initiate or promote cancer (1). Despite the genetic and molecular events that accelerate or inhibit cancer induction is known best ever, cancer continues to kill millions of people worldwide (1).

Cancer is a leading cause of death in economically developed countries and the second leading cause of death in developing countries. The incidence of cancer is increasing in economically developing countries, not only as result of population aging and growth but also by the adoption of lifestyles associated with cancer like physical inactivity, smoking, and consumption of "westernized" diets (2). In 2008, worldwide is estimated to have occurred about 12.7 million cancer cases and 7.6 cancer deaths, of which 56% of the cases and 64% of the deaths in the economically developing world (2) (Figure 1). It is estimated that 1 of each 3 persons is affected by cancer all over the world, ten million new cases are diagnosed each year, and it is predictable that this number will double in the next 20 years (3).

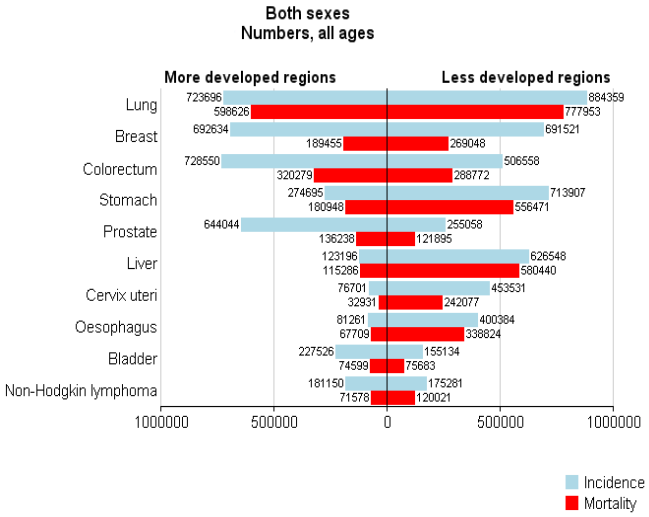

Figure 1. Incidence and mortality of the ten most common cancers in the worldwide (more and less developed regions) for both sexes. (GLOBOCAN 2008, IACR)

Carcinogenesis is a multistep process that comprises genetic alterations that ultimately lead to progressive transformation of normal into malignant cells. This transformation requires alterations in cell physiology to promote the development of tumor cells. Hanahan & Weinberg (2000) (4) defined the following six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion from programmed cell death (cellular apoptosis), limitless replicative potential, sustained angiogenesis, and invasion and metastasis (4). In 2011, the same authors proposed two new emerging hallmarks: deregulation cellular energetics and avoidance of immune destruction, and two enabling characteristics: genome instability and mutation and tumor-promoting inflammation (5).

1.2. Coloretal Cancer

Colorectal cancer (CRC) is the third most common cancer in men representing 10% of the total, and the second most common cancer in women, about 9,4% of total worldwide (6). Significant variations have been observed in the distribution of CRC (7), but nearly 60% of the cases occur in developed regions (6). The highest incidence rates of colorectal cancer are found in Europe, North America and Oceania whereas the lowest rates of CRC are found in Asia and South America (7) as we can see in Figure 2. Almost 608,000 deaths from CRC are predictable worldwide, which represents about 8% of all of deaths from cancer making it the fourth most common cause of death from cancer (6). The highest mortality rates in both sexes are estimated in Central and Eastern Europe and the lowest in Middle Africa (6). The majority of CRC are thus nonhereditary and sporadic, which makes early detection important. Most of the cases are identified at advanced stages, rendering curative treatment impossible (8).

The main modifiable risk factors for CRC include overweight and obesity, red and processed meat consumption, excessive alcohol consumption, smoking, physical inactivity (2), and non-modifiable risk factors, are age, family history inflammatory bowel diseases (e.g. ulcerative colitis and Crohn’s disease) (9).

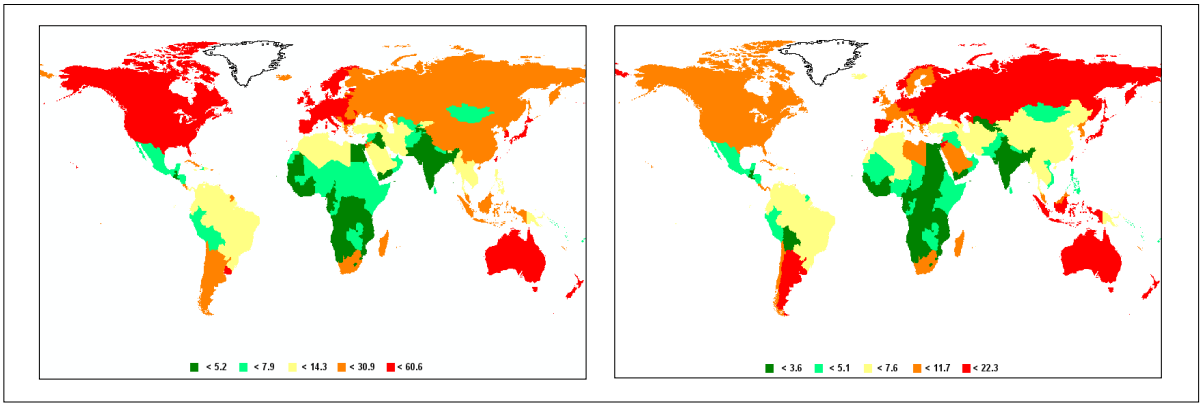

Figure 2. a) Incidence and b) distribution of colorectal cancer among both sexes for all ages (per 100.000) in 2008 (GLOBOCAN 2008, IACR)

The progression of CRC includes several genetic and molecular alterations in cell proliferation, cell survival, differentiation, resistance to apoptosis, metastasis and angiogenesis (9). The progression cascades involve the accumulation of mutations of genes as well as the alteration of morphological and cellular events. These processes are commonly characterized by histologically distinct steps, this is, colonic crypt hyperplasia, dysplasia, adenoma, adenocarcinoma, and distant metastasis. In tumororigenesis of CRC several genetic alterations can be involved, an example is the oncogenic mutation of K-ras which results in Ras and its downstream effectors, such as Raf/MEK/ mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways (9,10). Another important singnalling pathway involved in tumorogenesis of CRC is the stimulation of Wnt/β-catenin/Tcf4 pathway which is triggered by the loss of Adenomatous polyposis coli (APC) function, mutation and constitutive activation of β-Catenin and *K-ras.* This pathway, in turn, causes the transcription of downststream genes such as ciclin D1, myc, vascular endothelium growth factor (VEGF) and matrix metalloproteinases (MMPs), all important factors involved in carcinogenesis (9). Epidermal growth factor receptor (EGFR) signalling and transforming growth factor-β pathway also involved in regulating colonocyte growth and differentiation, and are upregulated in hiperproliferative aberrant crypt foci as well as contribute to malignant growth of colon cancer (9).

In recent years new therapeutic targets have emerged namely inhibition of iNOS, COX-2, 3-hidroxi-3-metilglutaril-coenzima A (HMG-CoA), retinoid X receptor-α, estrogen receptor-β, β-catenin, 5-lipoxygenase (5-LOX), signal transducer and activator of transcripton-3 (STAT-3), and metalloproteinase (MMPs) (8,9). These therapeutic strategies have shown protective effects against CRC development in different animal models suggesting that they are crucial targets for mucosa inflammation and colon carcinogenesis (8,9). Prognosis in CRC patients is highly dependent on disease stage at diagnosis. Screening has become a compelling strategy for prevention of colorectal tumors and was shown to reduce CRC mortality, although only 40% of cases are diagnosed at early stages (11).

Human colon is particularly sensitive to stress because of the close distribution of noradrenergic fibers to its basement lamina (12). The expression of β-adrenergic receptors (AR) has been identified on colon cancer cells, and several studies have shown that their activation has been implicated in carcinogenesis and tumor progression in various types of colon cancer cells *in vitro* (12–15). Consequently the interest in the efficacy of β-blockers as possible additions to cancer treatment paradigms has been gaining strength recently (16–18). However, it is not yet clear which of these drugs can be useful for these purposes.

1.1. Stress and Allostatic Systems

Stress is an inevitable element of our lives. Hans Selye, a pioneer in the approach of principles of physiology and pathophysiological of stress, defined *stress* as "the nonspecific response of the body to any demand" and *Distress* as "stress that is unpleasant or harmful to the body" (19–21).The physiologist Walter Cannon, introduced for the first time the terms *Homeostasis* and *fight-or-flight* response (21,22). Cannon also proposed that both adrenal medulla and sympathetic nervous system (SNS) operate as a unit and that adrenaline (AD) is not only the active mediator of the adrenal medulla but also a neurotransmitter of the SNS (21,22).

Recently, Goldstein *et al*.(1990) proposed a new definition of stress: "Stress is a condition in which expectations, where genetically programmed, established by prior learning, or deduced from circumstances, do not match the current or anticipated perceptions of the internal or external environment, and this discrepancy between what is observed or sensed and what is expected or programed elicits patterned, compensatory responses ", and of Distress: "is a form of stress with additional defining features-consciousness, aversiveness, observable signs, and pituitary-adrenocortical and adrenomedullary activation" (21,23).

The maintenance of homeostasis, a complex dynamic equilibrium, is constantly challenged by intrinsic or extrinsic, real or perceived adverse forces, the stressors, and it is this control that allows life to exist (24). The term *Allostasis* was introduced by Sterling and Eyer (25) to refer the process of adaptation of the body upon the exposure to various stressors (21), this is, the process whereby an organism maintains physiological stability by changing parameters of its internal milieu by appropriately matching them with environmental demands (25,26). Adaptations involving allostasis are determined by genetic, developmental and experiential factors (23). *Allostatic load* represents the "wear and tear" the body experiences when repeated allostatic responses are activated during stressful events (23,26). Changes in homeostasis often produce not only neuroendocrine and physiological effects but also behavioral responses (27).

Stress can be either acute or chronic, respectively when is short-lived, or when is repetitive, or occurring over an extended period of time (28). Stress is an intricate process involving environment and psychosocial factors that trigger multiple information processing pathways in central nervous system (CNS) and periphery. The activation of these physiological stress coordinated responses prepares an individual to survive to a threat were thus deemed adaptive (29), but if these responses are inadequate or excessive and/or prolonged, may affect personality development and behavior, and may have adverse consequences on physiologic functions, such as growth, metabolism, circulation, reproduction and inflammatory/immune responses (24). These pathways trigger fight-or-

flight stress responses in the autonomic nervous system (ANS), or defeat/withdrawal responses induced by the hypothalamic-pituitary-adrenal axis (HPA) (30).

Despite psychology has elaborated sharp distinctions between concepts such as stress, distress, depression and social isolation, the biological responses have not been differentiated in the framework of biobehavioral oncology research (31). Thus, Lutgendorf *et al.* (2011) (31) described all of these terms as "biobehavioral risk factors" to convey the general phenomenon that biobehavioral process appear to systematically impact a variety of important hallmarks of cancer biology (31).

1.2. Stress Responses

Stress responses are mediated by the system of stress which includes both central and peripheral components. In the hypothalamus and brainstem are located the central components of the stress system and the peripheral limbs of the HPA axis, together with the efferent sympathetic/adrenomedullary system, represent the peripheral components of this complex system (24). Stress exposure involves differentiated responses of the SNS and adrenomedullary hormonal systems depending on the type and intensity of the stressor, how it is sensed by the organism and interpreted in light of the previous experience (27) Currently, stress is not recognized as a disease but as a triggering factor for the majority of diseases when allostatic overload is generated (32).

In response to stress, glucocorticoids and CA are the main mediators released after activation of the hypothalamus-pituitary-adrenal cortex axis and SNS, respectively. Currently it is accepted that each stressor triggers its own neurochemical prolife, contrary the general notion described by Cannon and Selye that response to stress is primitive and unspecific. SNS activation predominates in response to orthostasis, moderate exercise, and exposure to cold, whereas adrenomedullary hormonal system predominates in response to glucoprivation and emotional distress (27).

Thus, NA levels, and thereby overall sympathetical nervous "activity" would play key roles in the appropriate distribution of blood volume and in the homeostasis of blood pressure (or blood delivery to the brain), during orthostasis, cold exposure, mild blood loss, locomotion, exercise, altered salt intake, and water immersion. On the other hand, adrenomedullary system activation and AD release occur in response to global or metabolic threats, such as hypoglycemia, hemorrhagic hypotension, heavy exercise, asphyxiation, emotional distress and shock (24,27).

1.2.1. The Hypothalamic-Pituitary-Adrenal Axis (HPA)

The principal hypothalamic regulator of the HPA axis is corticotropin release hormone (CRH) a 41-amino acid peptide. This peptide stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. A synergistic factor of CRH is arginine vasopressin, also produced by the Hypothalamus. ACTH regulates the secretion of glucocorticoids, such as cortisol, by adrenal cortex namely by zona fasciculata, and also adrenal androgen by the zona glomerulosa (24,29). As previously mentioned, the final effectors of HPA axis are glucocorticoids that can influence metabolism, growth, immune function and also play an important role in regulation of basal function and stress reactivity in different organs. Depending on the stressor, other factors may be secreted, which potentiate HPA axis activity, such as angiotensin II, cytokines and lipid mediators of inflammation that may act on hypothalamic, pituitary, and/or adrenal components (24).

1.2.2. The Sympathetic Adrenomedullary System (SAMS)

The ANS responses to stress are fast and control a wide range of functions being mediated primarily by the activation of the SNS from sympathetic neurons and the adrenal medulla (24,33). The sympathetic adrenomedullary system (SAMS) include the SNS and the adrenal medulla. The SNS is organized into a central component, the pre-ganglionic neuron located in spinal cord. The peripheral component is a postganglionic neuron, situated into sympathetic ganglion, being activated by pre-ganglionic neurons and acts directly on structures and target organs through the release of noradrenaline (NA). This

central component also enervates the adrenal medulla to release mainly adrenaline (AD), into the bloodstream, but also NA, both having systemic effects (24,34,35). The SNS innervates widely the smooth muscle of the vasculature, the heart, skeletal muscles, kidney, gut, fat and many other organs (24).

A fast response evoked by adrenergic system is critical to survival; however, prolonged activation of these pathways have deleterious consequences on several systems ultimately leading to the development of several pathologies.

1.3. Stress and Cancer

The association between stress and cancer has a long history. In 200 AD, Galen suggested that women with a "melancholic" disposition were more susceptible to cancer than women with a more "sanguine" disposition (36). In the recent years, clinical and epidemiological studies have recognized that biobehavioral factors such as stress is a risk factor for the development and progression of several types of cancer (28,31,36).

1.3.1. Epidemiological Studies

In recent years, the relationship between stress and cancer has been widely studied. There are a large number of epidemiological studies that investigated the association between stress related psychosocial factors and cancer outcomes, however many of them are inconclusive and contradictory. Some studies show that stress increases the risk of cancer and mortality (37), contrary to others that do not find this association (38).

A recent meta-analysis (39) that studied the relationship between stress and various types of cancer, show that studies available in the literature are very heterogeneous and have many and important methodological limitations. This systematic review confirmed using meta-analytic methods that stress and related psychosocial factors are associated with adverse effects on cancer incidence, survival and mortality. These associations differed depending on study characteristics such as sample size, follow-up period, quality score cancer type of psychosocial factor and cancer location. The authors found that

studies with longer follow-up periods had more expressive associations (39). The variability of studies associating stress with cancer are mainly due the difficulty in evaluating the response generated by a negative event. Indeed, most of the instruments like structured questionnaires to assess the number and intensity of stressors are subjective. Therefore, results obtained with these instruments should be complemented with biological parameters such as plasma levels of the stress hormones, AD, NA and cortisol to enable objectively determine the relationship of stress and cancer (39). This meta-analysis suggests by its results that stress-related psychosocial factors have in fact an adverse effect on cancer outcomes, although effects vary by type of psychosocial factor, cancer site and cancer outcome (39).

1.4. Catecholamines

CA actions influences many functions practically in all tissues of the organism. CA are biogenic amines that possess a catechol group with an attached amino group (27,40,41). The non-essential amino acid L-Tyrosine (Tyr) is the precursor of CA synthesis. The two primary sources of Tyr, are diet and hydroxylation of the amino acid phenylalanine in liver (21). Upon entry into an adrenal chromaffin cell or sympathetic nerve terminals, Tyr is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), a soluble cytoplasmic enzyme which catalyzes the rate-limiting step of CA synthesis. TH use tetrahydrobiopterin (BH4) as cofactor and molecular oxygen to generate DOPA, dihydrobiopterin, and water (42). DOPA is converted into dopamine (DA) by a nonspecific enzyme, aromatic L-amino acid decarboxylase (AAAD), also known as Dopa decarboxylase (DDC), which uses pyridoxal phosphate as cofactor. After, DA is taken up from the cytoplasm into storage vesicles and converted into NA by dopamine-β-hydroxylase (DβH). A percentage of this enzyme is released simultaneously with NA and AD by exocytosis.NA is converted into AD by phenylethanolamine N-methyltransferase (PNMT) a soluble cytoplasmic enzyme that uses S-adenosyl-methionine as cofactor. The main location of this enzyme is the adrenal medulla, but is also present in sympathetical innervated organs and some brain areas that are able to synthetize small amounts of AD

(21,43) (Figure 3). The biologic effects of the CA, AD and NA, are mediated by the AR α and β, which show different patterns of tissue distribution (41).

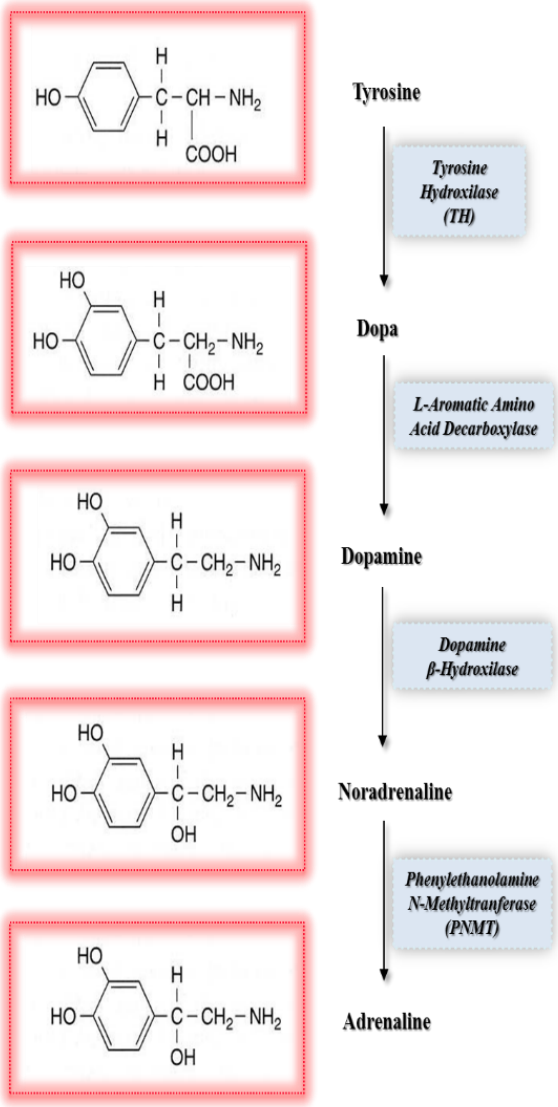

Figure 3. Pathway for catecholamine biosynthesis. In catecholamine synthesis, tyrosine hydroxylase catalyzes the conversion of the amino acid tyrosine to DOPA. Then, DOPA is converted to dopamine by dopa decarboxylase. In chromaffin granule, dopamine is converted to noradrenaline by dopamine-β-hydroxylase. Finally, in the cytoplasm, phenylethanolamine N-methyltransferase catalyzes the conversion of noradrenaline to adrenaline. Adapted from Richard Kvetnansky et al.(21).

1.4.1. The role of catecholamines in the pathogenesis of cancer

As previously referred, cellular and molecular events that promote cancer growth can be affected by stress, being CA important mediators in this process (33). AR, more specifically the β-AR, have been identified in several tumor cell lines (33).

A solid tumor, as colon cancer, is innervated by sympathetic nerve fibers like an integrated organ. Adrenergic nerve cells and nerve fibers exist and branch profusely into tumor tissues. However, the functions of these fibers in the tumor microenvironment have not been extensively studied. Neurotransmitters as CA are released upon the stimulation from sympathetic nerve fibers and are spread into tumor microenvironment. Tumor microenvironment and multiple physiologic processes of tumor development may be controlled by SNS. Biological behaviors of tumor and of stroma cells can be modulated by signalling of neurotransmitters by binding to respective receptors present in these cells (44).

Over the last years the biological effects of stress pathways on cancer progression have been focused on studying the effects of stress hormones on tumor cell proliferation and apoptosis, tumor invasion and metastasis, tumor angiogenesis, stroma cells in the microenvironment and cellular immune responses (Figure 4) (28). The regulation of cancer cell development by neurobiological signals expands the opportunities for pharmacological interventions in cancer therapy.

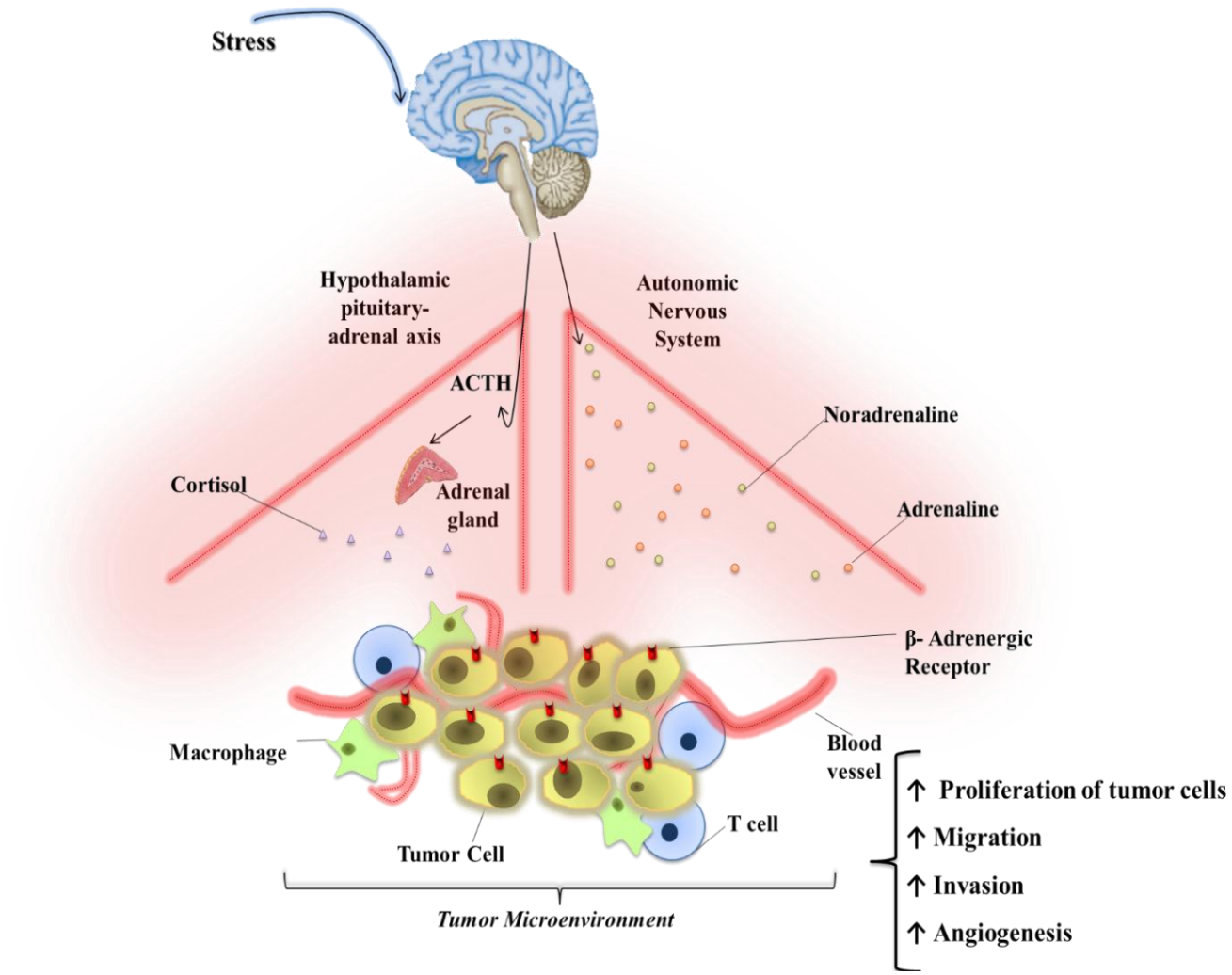

Figure 4. Stress responses on tumor microenvironment. Stress response results in the activation of autonomic nervous system (ANS) and the hypothalamic-pituitary–adrenal axis causing the release of AD, NA and cortisol, respectively. The tumoral microenvironment can be altered by these mediators enhancing cellular proliferation, migration, invasion, and angiogenesis contributing to tumor progression. Adapted from Lutgendorf et al (28).

1.4.1.1. Proliferation and apoptosis

Human cells to acquire tumorigenicity must to overcome two barriers, replicative senescence and cellular crisis in order to achieve immortalization (22). Essential characteristic of cancer cells are the ability to sustain chronic proliferation and resistance to apoptosis (5). In normal tissues, the cell number control is exerted by production and

release of growth signals that regulate the cell growth and cellular division cycle maintaining the normal functions of tissues and architecture (5). By deregulating these signals cancer cells become under an uncontrolled cell division state. These signals, typically growth factors that bind cell-surface receptors, emit signals through several signalling pathways. These pathways regulate not only cell cycle and growth and lastly progression, but also survival and energy metabolism (5). An effective treatment strategy for tumors may be the control of cellular proliferation (17).

In 1989, Schuler and Cole (45) provided the first evidence that the β-AR agonist isoprenaline (ISO) promotes a significant increase in the proliferation of lung adenocarcinoma cells. In addition, these researchers also demonstrated that this effect was inhibited by propranolol (PRO), a β-adrenergic antagonist, commonly used in clinical setting (45). After this study, other reports have shown that the CA, AD and NA, and the β-AR synthetic agonist ISO, through the activation of AR trigger signalling pathways that induce cell proliferation. Indeed, several *in vitro* and *in vivo* studies have shown that AD and NA can induce cell proliferation in different types of cancer such as non-small cell lung carcinoma (46) , ovarian cancer (47) , colon cancer (12) and oral squamous carcinoma (48).

The actions of CA upon tumor cells appear to be mediated primarily by β_2-AR activation involving the cellular cyclic adenosine-3,5-monophosphate (cAMP) - protein kinase A (PKA) signalling pathway (49). Nevertheless, Thaker et al (2006) (47) also reported in an elegant paper that AD stimulate directly the proliferation of an esophageal cancer cell line through the β_1/β_2-AR/Extracellular signal-regulated kinase (ERK)/ Cyclooxygenase-2 (COX-2) pathways, β_1 dependent kinases, and also AR-dependent upregulation of cyclins and cyclin-dependent kinases (50). In breast cancer cells, Carie and Sebti (51) showed *in vitro* and *in vivo* studies that ARA-211, a β_2-selective AR agonist, leads to a reduction of cell proliferation and tumor growth by blockade of the Raf-1/Mek-1/Erk1/2 pathway (51).

In human gastric cancer cells was shown that nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) enhance cell proliferation and that PRO was able to block this effect. In addition, atenolol (ATE) and ICI 118,551 blocked the regulatory action of nicotine on the PKC/ERK1/2/COX-2 pathway suggesting that β-AR are involved through this pathway in the promotion of gastric cancer development (49,52).

Prostate tissue has a high amount of β-AR, and several studies have demonstrated that β-AR agonists can stimulate adenylyl cyclase (AC) and increase cAMP levels in rat and human prostate cancer cell lines. These results show that β-AR, particularly β_2-AR, through these pathways play an important role in carcinogenesis (49,53).

In ovarian carcinomas, in patients with biobehavioral risk factors, an increase activity of several β-adrenergically-linked transcription control pathways such as cAMP response element-binding (CREB)/AKT, activate nuclear factor κ-B (NF-κB) and STAT family transcription factors was observed (54). Furthermore, in these patients was also seen an increase of intra-tumor levels of NA, indicating that these biobehavioral risk factors might be linked with higher levels of CA and consequently with a more invasive into pattern of ovarian cancer (55). These effects in ovarian cancer cells are primarily mediated by β_2-AR, via cAMP-PKA signalling pathway (55).

In rat C6 glioma cells, ISO stimulate cell proliferation, whereas PRO cause a significant decrease of proliferation. Moreover, when these cells were treated with TNF-α, the gene expression and protein levels of β_1 and β_2 increased (56).

Some studies also have studied the effect of β-AR agonists on colon cancer. In one of these studies was shown that NA and ISO stimulate the proliferation on HT-29 (human colorectal adenocarcinoma) cells and that cAMP levels were increased by β-activation with ISO treatment, and this effect was significantly decreased with ICI 118,551 (14). In a more recent paper, the same authors demonstrate that AD also affect HT-29 cells proliferation in a concentration-dependent manner, and both β_1 and β_2- AR selective antagonists, ATE and ICI respectively, revert this effect (12).

Apoptosis (programmed cell death) is often mediated by anoikis, occurring when anchorange-dependent cells become separated from matrix extra cellular (57). Anoikis can be mediated by both intrinsic and extrinsic apoptotic pathways. Cancer cells acquire the ability to resist anoikis, and this resistance is one of most important hallmarks of malignant transformation (5,58). Tumor cells become able to survive in absence of matrix attachment and facilitating migration, reattachment and colonization of secondary sites (31,58). Indeed, i*n vitro* and *in vivo* studies have shown that CA can protect ovarian cancer cells from anoikis being this effect mediated by focal adhesion kinase (FAK). FAK is a tyrosine kinase that stimulates cell adhesion and it is activated by NA through phosphorylation of $pFAK^{Y397}$. Tumor tissue from depressive patients with ovarian cancer present higher levels of NA and $pFAK^{Y397}$(31).

1.4.1.2. Angiogenesis

Tumors stimulate the growth of host blood vessel, through a process called angiogenesis (59). This process, i.e. the growing of new blood vessels, is essential for supplying nutrients to tumor, for its growth, but also for metastization (59,60). Many factors are able to promote angiogenesis, including vascular endothelial factor (VEGF), interleukin-6 (IL-6) transforming growth factor α and β (TGF-α/β) and tumor necrosis factor α (TNF-α) (28). In normal physiology, angiogenesis is a process intricately balanced between the proangiogenic and antiangiogenic factors, however in pathological conditions such as cancer, this balance is lost, leading to the formation of abnormal vessels with increased permeability (61).

Recently, it has been shown that stress hormones may promote angiogenic mechanisms (29,61). In fact, the synthesis of many proangiogenic factors in tumor cells is up-regulated by AD and NA by acting through β-AR. Thaker *et al.* in 2006 (47) demonstrated that NA stimulates angiogenesis in human ovarian tumors (Hey-A8, SNOV3ip1) growing orthotopically in nude mice by acting through β_2-AR expressed in these tumor cells (47,61). In these malignant ovarian tumors, the signalling pathway through angiogenesis occurs is described as β-AR→cAMP→PKA→VEGF (47,61). Also

in these tumors, it was found that NA and isoproterenol induced the expression of VEGF (28,62). In human pharyngeal carcinoma cells line (HONE 1) and in several human multiple myeloma cell lines (NCI-H929, MM-MI and FLAM-76) similar results were observed (63,64). Another important proangiogenic molecule, IL-6, is also involved in NA- and AD-mediated ovarian cancer angiogenesis (48). Recently, in human ovarian tumor cells (Hey-A8, SNOV3ip1 and EG) an increase of the synthesis and release of IL-6 was shown after treatment with NA. The authors confirmed the involvement of NA and β-AR on this effect through the use of β-AR antagonists, which were able to abolished NA effect (61,65). In this case, the proposed pathway in human ovarian tumor cells is NA→ β-AR → Src kinase→ IL-6. Src kinase is over expressed in human cancer ovarian tissues with increased tumor neovascularization. Furthermore, in human ovarian tumor cells the VPF / VGEF and IL-8, proangiogenic molecules are induced by Src, suggesting that CA are responsible for regulating the synthesis of the proangiogenic molecules in this model (48,61).

Recently, other studies also have shown that the signal transducer and activator of transcription factor-3 (STAT-3) that is activated by growth factors and cytokines, such as VEGF and IL-6, can be involved in angiogenesis in tumor stress-mediated. AD and NA, induce the expression, cellular localization and activation of STAT3 (29,66).

Interestingly enough, among the endogenous molecules recently identified as potential targets for future therapeutics, CA have been of recent interest due to their actions in the regulation of the angiogenic process (40).

1.4.1.3. Migration and Invasion

The ability of cancer cells to separate from the original tumor, invade through the basement membrane and enter the bloodstream is a key step in the metastatic cascade (28). The process by which tumor cells intrude the host basement membrane consists of multiple steps that involve attachment, matrix dissolution, motility and then intrusion (29).

There are several lines of evidence suggesting that stress hormones can be involved in this important step of cancer progression, contributing to tumor cell movement and invasion (29). CA can influence these processes by increasing the production of MMP by cancer cells and by acting as chemoattractants to induce cell migration (28,67).

MMP are enzymes secreted by tumor cells but also by stromal cells that facilitate the breakdown and remodeling of the extracellular matrix by degrading components of the extracellular matrix, such as elastins, fibronectines, laminins, collagen and the protein core of proteoglycans enabling the tumor to spread locally or distantly (68). It has been shown that the expression of MMP is modulated by stress hormones. For instance, in an ovarian carcinoma, *in vitro* model, the use of NA mimicking stress conditions, increased the production of MMP-2 and MMP-9 and the potential invasion of cancer cells, being this effect inhibited by the β-adrenergic antagonist, PRO, suggesting the involvement of β-AR. Furthermore, ISO used *in vivo* simultaneously with PRO, was able to substantially reduce tumors size (68).

In colon carcinoma cells (SW 480 cells), NA was capable to induce migration that was reverted with the β-adrenergic antagonist PRO but not with a β_1-selective adrenergic antagonist, ATE. This finding suggest that the use of β_2-blockers might inhibit metastasis and the progression of colon cancer (15). Other studies have reported similar findings in several other tumor types such as nasopharyngeal carcinoma model (63) prostate cancer (69) and breast cancer (70). Stress hormones might directly regulate the growth and metastatic potential of tumor cells, and this effect might be independent of the immune system (47).

All of these findings provide important evidences that stress hormones can promote cell invasion in different types of cancer and that β-blockers can revert this effect.

1.5. Adrenergic Receptors, β-blockers and cancer

As already mentioned throughout this introduction, CA exert their effects by interacting with receptors α and β-AR.

The activation of these AR trigger several pro-angiogenic signalling pathways encouraging cancer growth by cell proliferation, evasion of apoptosis, invasion, angiogenesis and metastasis (35).

1.5.1. Adrenergic Receptors

AR are targets for many therapeutically important drugs such as that used for cardiovascular diseases, asthma, prostatic hypertrophy, nasal congestion obesity and pain (71). These receptors are divided in two main classes: α and β. Each group is further subdivided in two main α-receptor subtypes, α_1 and α_2, and three β-receptor subtypes, β_1, β_2 and β_3. In total, there are nine subtypes of α-AR: α_{1A}, α_{1B}, α_{1D}, $\alpha_{2\ A/D}$, α_{2B}, α_{2C}. Other two AR candidates were recently described (α_{1L} and β_4), that may be conformational states of α_{1A} and β_1-AR, respectively (72). (72)

AR are coupled to second-messenger systems via G proteins (71,72). G-protein-couple receptors (GPCRs) like the AR are characterized by the presence of seven membrane spanning α-helical segments (73). These segments are separated by alternating intracellular an extracellular loop regions (Figure 5). Despite these similarities, individual GPCRs have unique combinations of signal-transduction activities involving multiple G-protein subtypes, as well as G-protein-independent signalling pathways and complex regulatory process (73).

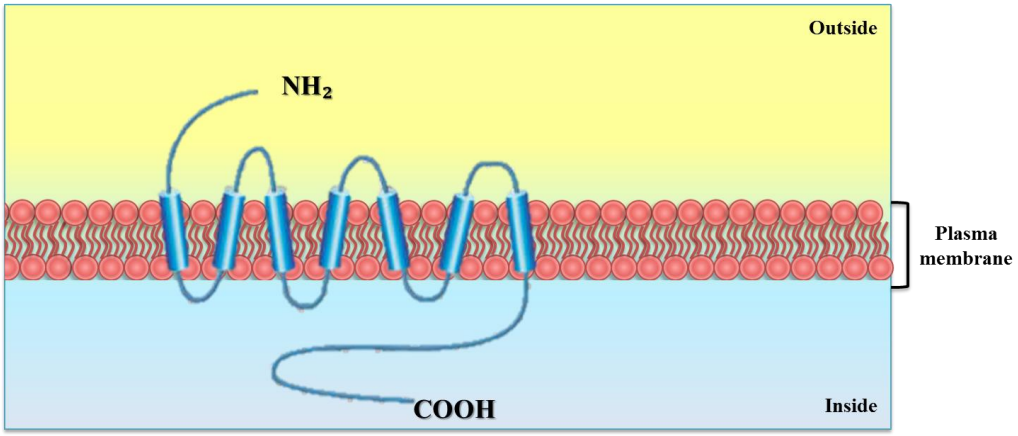

Figure 5. Structure of adrenergic receptors (AR). AR have extracellular amino terminals with sites for N-linked glycosylation, seven α-helical domains that are each thought to span membrane and intracellular carboxy terminals containing aminoacids sequences creating three intracellular and three extracellular loop domains. Adapted from Insel (1996) (107).

1.5.1.1. α-AR

As mentioned before, the α-AR are subdivided into two main classes α_1 and α_2 (Table 1). α_1-AR are found in bladder, uterus, gastrointestinal tract, bronchi, and smooth muscle of the blood vessels. α_1-AR are mainly coupled to Gq/11 protein and lead to the stimulation of phospholipase C activity (71,74). Phospholipase C promotes the hydrolysis of phosphatidylinositol bisphosphate producing inositol trisphosphate (IP3) and diacylglycerol (DAG) which act as second messengers (71). α_1-AR is also involved in the activation of MAPK (74).

α_2-AR are predominantly coupled to the inhibitory G proteins, G_i and $G_{(o)}$, inhibiting the activity of AC, and consequently decreasing intracellular cAMP levels leading to membrane hyperpolarization(71). α_2-AR when located presynaptically have a role as modulator of neurotransmission inhibiting the release of NA and acetylcholine. They also cause platelet aggregation, contraction of vascular smooth muscle and inhibition of insulin release (74). α_2-AR are found at post-junctional or non junctional sites in several tissues, and thus their activation may also lead to the trigger of other intracellular pathways such as the activation of phospholipase A_2, C, and D, with arachidonic acid mobilization,

increasing in phosphoinositide hydrolysis, and increase in the intracellular availability of Ca^{2+}. In addition, α_2-AR can activate MAPKs (71). However, some of the effects of α_2-AR are independent of their ability to inhibit AC (71).

Table 1. α-AR subtypes: α_1, α_2, α_3 and main transduction signals and rank of order of potency of agonists and selective antagonists. Adapted from (Alexander SPH *et al*., 2011) (72)

Nomenclature	**α_1**			**α_2**		
	α_{1A}	α_{1B}	α_{1D}	α_{2A}	α_{2B}	α_{2C}
Other names	α_{1a}, α_{1c}	α_{1b}	$\alpha_{1A/D}$, $\alpha_{1a/d}$	α_{2D}	-	-
Principal transduction	$G_{q/11}$			$G_{i/o}$		
Rank order of potency	AD=NA			AD>NA		
Selective antagonists	Tamsulosin, silodosin, (+)niguldipine, SNAP5089	-	BMY7378	BRL44408	Imiloxan	JP1302 Oxymetazoline

1.5.1.2. β-AR

β-AR are constitutively expressed in most mammalian cells and are related with several regulatory pathways operating under conditions of stress (49).

The three β-AR subtypes are encoded by three different genes located on human chromosomes 10 (β_1), 5 (β_2), and 8 (β_3) (75) (Table 2). β-AR show distinct patterns of distribution and signal through distinct biochemical pathways (76,77). They bind both to AD and NA, as well as to exogenously drugs, including synthetic β-agonists like ISO and also to several antagonists, commonly termed β-blockers (75).

β_1-AR are predominantly found in the heart where their activation results in as increased rate and force of contraction whereas in the sphincter muscle of the gut their activation leads to relaxation (78).

β_2-AR when activated in smooth muscle leads to vasodilation, whereas activated in bronchial smooth muscle leads to bronchodilatation. Activation of β_2-AR also promotes relaxation of visceral smooth muscle, hepatic glycogenolysis and muscle tremor (71,78).

β_3-AR are found in the adipose tissue where they initiate lipolysis in white adipose tissue, with the release of fatty acids and glycerol to the circulation. These AR are also involved in thermogenesis process in the brown adipose tissue. Although also present in heart, their functions in this organ are not fully known (71,79).

Table 2. β-AR subtypes: β_1, β_2, β_3, respective main transduction signals and rank of order of potency of agonists and selective antagonists. Adapted from (Alexander *et al*., 2011) (72)

Nomenclature	**β_1**	**β_2**	**β_3**
Other names	-	-	atypical β
Principal transduction	G_s	G_s	G_s
Rank order of potency	ISO> NA> A	ISO > A>>NA	ISO ~ NA > A
Selective antagonists	Atenolol	ICI 118,551	

In the context of cancer, the three β-AR subtypes are present at many sites related with tumor growth and metastasis process namely in brain, lung, liver kidney, colon, adrenal gland, breast, ovary, prostate, lymphoid tissues, bone marrow and vasculature. β-AR signalling also regulates the function of several cancer-relevant cell types such as epithelial cells, vascular myocytes and pericytes, fibroblasts, immune cells, adipocytes and neuronal and glial cells (76,77).

The first evidence of β-ARs as regulators of cancer cells was provide by Schuller *et al* in 1989 (80). This study showed a significant increase in the proliferation of human lung adenocarcinoma cells in response to ISO, and that this response was inhibited by PRO (80).

In 2005, William *et al.* (14) characterized the expression of both β_1 and β_2 in several colon cancer cells (Figure 6) and showed that mRNA expression of both receptors was detected in HT-29, SW1116 and SW480. Indeed, these authors confirmed that HT-29 cells strongly expressed β_2-AR mRNA and that β_1-adrenoceptor expression is just barely detectable (14).

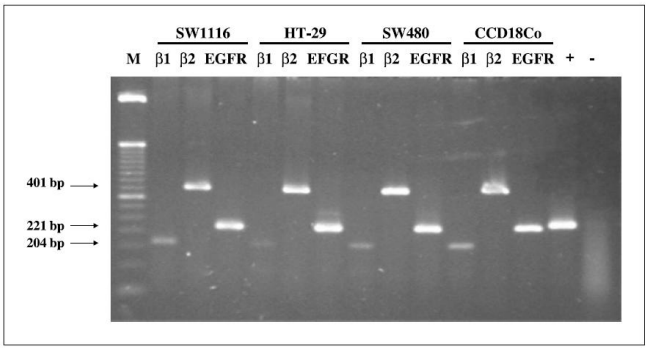

Figure 6. RT-PCR analysis revealed the expression of β_1 and β_2-AR in three colon cancer cell lines including HT-29. 50bp marker; +, β-actin; -, negative control (PCR product without reverse transcription (14)).

More recently, another study confirmed the detection of β_1 and β_2 mRNA levels, as well as protein levels by Western blot in several CRC cell lines, including HT-29 whereas β_3-AR was not detected in these cells (Figure 7) (81).

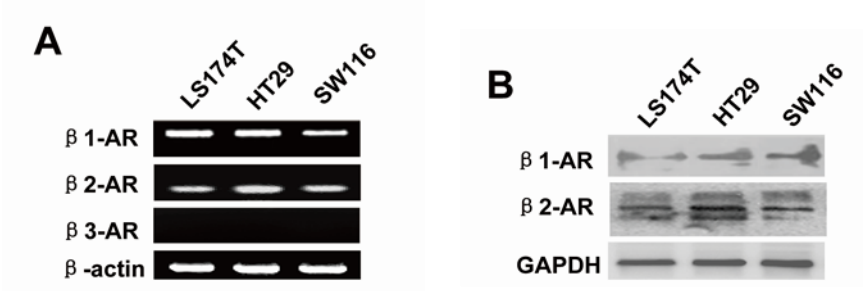

Figure 7. (A) Expression of mRNA levels by RT-PCR assay of β1, β2 and β3 - AR mRNA levels in CRC HT29, SW116 and LS174T cells. (B) Expression of protein levels by Western blot analysis of β1 and β2 - AR in CRC HT29, SW116 and LS174T cells T29, SW116 and LS174T cell lines by western blot analysis (82).

Several cellular evidences show that β-AR are important mediators of growth, invasiveness and differentiation, making these receptors a promising target for treatment and prevention of various types of cancers. Several reports show the expression of β-AR in normal and malignant colon tissues; however, their modulatory action on cancer growth and their relationship to stress are still elusive.

When CA binds to β-AR, activate the $G_{\alpha s}$ guanine nucleotide-binding protein to stimulate AC and the synthesis of cAMP. cAMP can regulate a diverse array of cellular process via two major downstream effector systems (76) (Figure 8).

The first one, consist of the activation of PKA by cAMP, which consequently phosphorylates serine or threonine residues on target proteins. Through this pathway several process are affected such as metabolism, growth, differentiation, morphology, motility, secretion, neurotransmission, and gene transcription for instance, the cAMP-responsive element binding protein/activating transcription factor (CREB/ATF) family. Transcriptional alterations induced by PKA promotes cell differentiation and proliferation. Furthermore, PKA is also able to activate the β-adrenergic receptor kinase (BARK). BARK induces β-arrestin which desensitize β-AR signalling and activate the Src/Ras/MAPK pathway (76).

The second effector system includes the guanine nucleotide exchange protein activated by AC (EPAC). EPAC activates the Ras-like guanine triphosphatase Rap1A. Rap1A stimulates the downstream effectors B-Raf, MAP/extracellular signal-regulated kinase (ERK) 1/2, and ERK1/2. β-adrenergic influences on inflammation, angiogenesis, invasion and proliferation appear to be mediated by PKA induction of genes encoding cytokines and growth factors. EPAC induces complementary but distinct effects on cell morphology and motility (76).

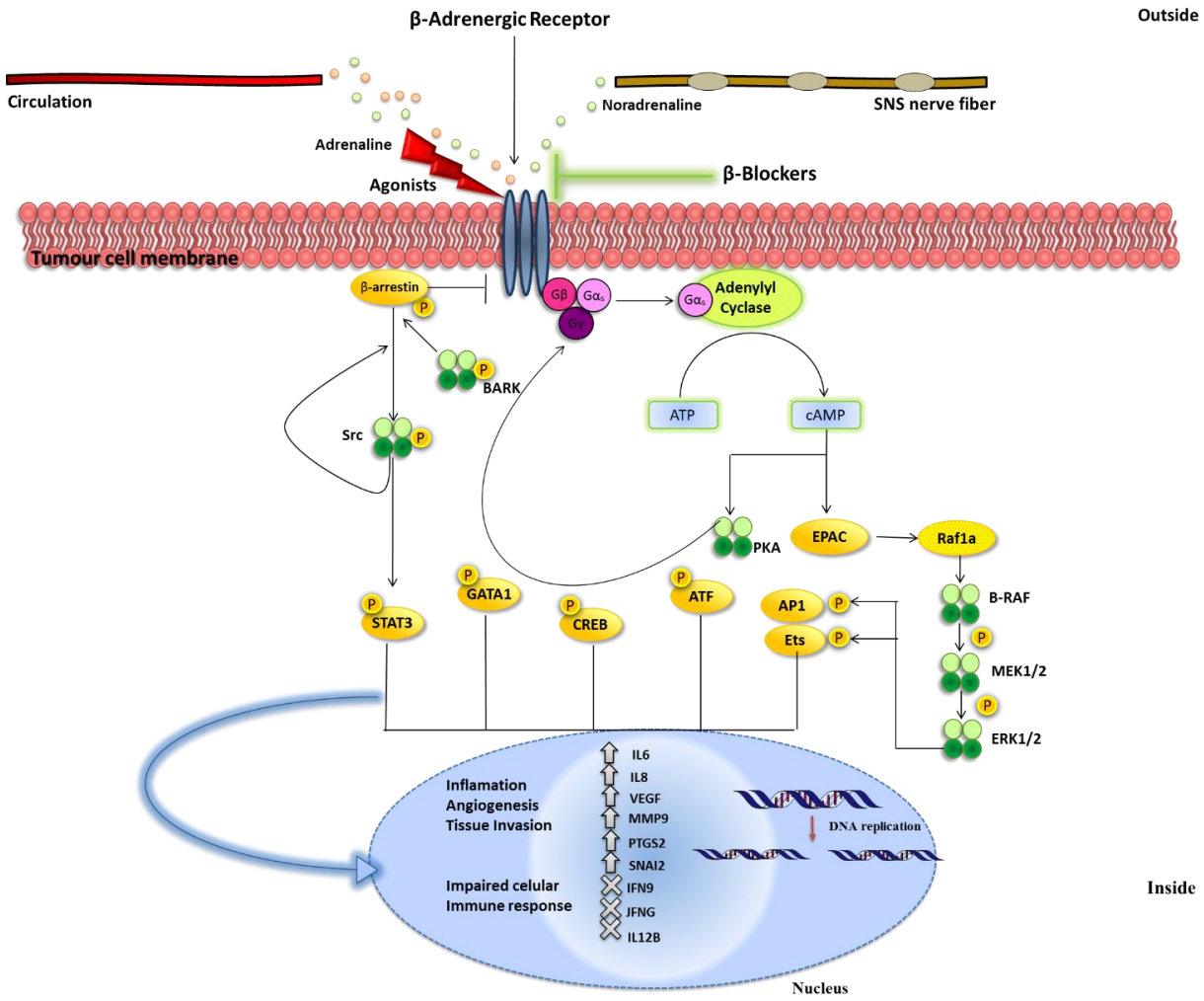

Figure 8. Main β-adrenergic signaling pathways in cancer. The stress hormones, adrenaline (AD) and noradrenaline (NA), bind to β-ARs, resulting in Gαs-mediated activation of adenylyl cyclase and subsequent cAMP synthesis. cAMP effectors involve the activation of both PKA and exchange protein activated by adenylyl cyclase (EPAC). In the first one, PKA phosphorylates multiple target proteins, including transcription factors of the CREB/ATF and GATA families, as well as β-AR kinase (BARK). BARK recruitment of β-arrestin inhibits β-AR signalling and activates Src kinase, resulting in activation of transcription factors, such as STAT3. In the second via, the activation of EPAC leads to Rap1A-mediated activation of the B-Raf/mitogen-activated protein kinase signalling pathway and downstream effects on diverse cellular processes, including gene transcription mediated by AP-1 and Ets family transcription factors. The general pattern of transcriptional responses induced by β-adrenergic signalling include the upregulation of expression of metastasis-associated genes involved in inflammation, angiogenesis, tissue invasion, and the downregulation of expression of genes facilitating antitumor immune responses. Besides the direct effects on β-receptor-bearing tumor cells, β-adrenergic effects on stromal cells in the tumor microenvironment generally synergize with direct effects on tumor cells in promoting cancer survival, growth and metastatic dissemination. Adapted from Steven W. Cole and Anil K. Sood (2011) (76).

1.5.2. β-blockers

β-blockers are used for various therapeutic indications such as hypertension, tremors, management of cardiac arrhythmia, migraines and cardioprotection after myocardial infarction (17,77). In fact, β-blockers have a long history of use in humans for the treatment of hypertension and cardiovascular disorders (49).

Classically, drugs that modulate β-AR are through to stabilize the proportion of receptors that are in an active signaling conformation relatively to those in an inactive, non-signaling conformation. Thus, whereas an agonist induces a certain receptor conformation, which leads to its activation, an antagonist stabilizes the inactive conformation. This binary categorization underestimates the true complexity of receptor ligand behavior (82).Actually, now it is recognized that there are more than two possible conformations for β -AR and that their activation can trigger more than one signaling pathways, namely heterotrimeric G-proteins and β-arrestin (82,83). In addition, AR ligands preferentially signal through either the G protein- or β-arrestin-mediated pathway (i.e. display bias towards one pathway over the other) (84,85). This behavior gave rise to the new concept of biased agonism (82). In fact, β-blockers are not merely antagonists for G-protein pathway, but they may indeed independently modulate the two pathways and behave as partial agonist, inverse agonists or pure antagonists in each pathway(84). Biased agonism might have outstanding implications for β-AR blockers therapeutic use in cancer, since distinct signaling through these parallel pathways is thought to have distinct functional consequences (84). In fact, β-AR blockers could act as antagonists of G protein-mediated functions, but evoke different effects upon β-arrestin-mediated signaling (84). This could result in markedly different effects *in vivo*, and may even explain the distinct efficacy profiles of β-AR blockers in the treatment of various cardiovascular diseases (86). For example, clinical trials assessing the efficacy of these drugs in the treatment of congestive heart failure revealed that carvedilol, metoprolol and bisopropol, but not bucindolol, decreased mortality, and the reason for the lack of beneficial action of

bucindolol remains elusive (84). In a similar way, in cancer, it is crucial to investigate which β-AR blockers are more effective in antagonizing the effects promoted by CA.

As already mentioned, there are several studies showing that β-blockers have significant antitumorigenic effects at various stages of carcinogenesis such as in control of cell proliferation, angiogenesis and metastasis in some cancers such as breast (87), lung (46), pancreas (88), prostate (89), colon (12,15), stomach (52) and ovarian cancer (68).

1.5.2.1. Epidemiological Studies

A number of retrospective population studies suggested that β-blockers may have a protective role in reducing the incidence and progression of some cancer types (17).

The first clinical evidence that reported in humans the protective effect of β-blockers in the treatment of breast cancer was provided by Powe *et al.* (2010) (90). These authors studied patients with breast cancer, who were treated for hypertension with or without β-blockers (90). The group under β-blocker medication demonstrated a significant reduction in distant metastasis and tumor recurrence, and a 71% reduction in cancer-specific mortality. After this study, Ganz *et al.*(2011) (91), analyzed the association between β-blockers and breast cancer recurrence, breast cancer-specific mortality and overall mortality, concluding that woman taking β-blockers show a nonsignificant 14 % reduction in the risk of breast cancer recurrence, a nonsignificant 24% reduction in the risk of cancer specific mortality, and no reduction in all-cause mortality (91). A population study of Barron *et al.* (2012) (92) compared women diagnosed with stages I and IV invasive breast cancer, taking either PRO, ATE or not taking β-blockers. The findings of this study reveal that PRO users were significantly less likely to present a T4, node-positive or metastatic disease, whereas ATE users had no significant differences in T4, node-positive, and breast cancer specific mortality. In other study, Melhem-Bertrandt *et al.*(1999) (60) reviewed patients with breast cancer who received neoadjuvant chemotherapy, concluding that among β-blockers metoprolol and ATE, were the most commonly prescribed. When these authors compared β-blockers use with pathological

complete response, relapse-free survival and overall survival, they concluded that β-blocker use was not associated with pathological complete response, but was associated with a significantly better relapse-free survival, mainly among patients with triple-negative breast cancer (60).

β-blockers also seem to have an important role on infantile hemangioma (IH). This tumor, characterized by the abnormal proliferation of endothelial cells and angiogenesis is the most common form of vascular tumor, affecting 5% to 10% of all infants and up to 30% of premature infants (93). In 2008, Léauté-Labrèze *et al* (2008) (94) showed that PRO had an anti-proliferative effect in severe IH. After this study, others researchers verified that other β-blockers were effective at controlling IH growth. In a randomized, double-blind, placebo-controlled, parallel-group trial conducted by Hogeling *et al* (2011) (95) PRO users were more likely to show a significant reduction in both tumor volume and redness. Another blinded cohort study by Pope *et al* (2012) (96), studied the efficacy and safety of nadolol, a nonselective β-blocker with no intrinsic sympathomimetic activity, in patients with IH and concluded that patients treated with nadolol had a more favorable response and fewer parental reports of minor adverse events, than patients who were treated with PRO (96).

Two retrospective studies, by De Giorgi *et al*.(2011) (97) and Lemeshow *et al*.(2011) (98), studied the association between exposure of melanoma patients to β-blockers and survival. In the study of De Giorgi *et al*.(2011) (97) authors concluded that β-blocker treatment was inversely associated with recurrence, and that there was a significant reduction in the risk of relapse. Regarding the study of Lemeshow *et al*.(2011) (98), the authors showed that in melanoma patients receiving β-blockers (including metoprolol, PRO and ATE) a significant reduction of mortality due to melanoma and all-cause mortality was observed.

With regard to colon cancer, in a recent population-based case control study, Jansen L. *et al* (99) did not find association between CRC risk and the use of β-blockers. However, they found in β-blockers a lower risk of stage IV CRC.

II. CHAPTER

AIM

2. AIM

This work aimed to investigate the effects of several adrenergic agonists and antagonists upon cellular dynamics, namely proliferation and viability, of colon cancer cells. For that purpose, the effect of these drugs was investigated, using the colon cancer cell line, HT-29.

III. CHAPTER

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Compounds and Treatments

The adrenergic agonists, AD (Adrenaline - L-Adrenaline (+)-bitartrate salt), NA (Noradrenaline - L-(−)-Noradrenaline (+)-bitartrate salt monohydrate) and ISO (Isoprenaline - (−)-Isoprenaline (+)-bitartrate salt) and the adrenergic antagonists, PRO (Propranolol - DL-Propranolol hydrochloride), ICI (ICI118,551-(±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride) and ATE (Atenolol-(±)-4-[2-Hydroxy-3 [(1methylethyl)amino] propoxy] benzeneacetamide were purchased from Sigma (St. Louis, MO, USA) and CAR (Carvedilol-1-(9H-Carbazol-4-yloxy)-3-((2-(2-methoxyphenoxy)ethyl)amino)-2-propanol) from Enzo Life Sciences, Inc., Farmingdale, NY, USA.

For the treatment of cells with the several drugs under study, stock solutions of each compound were prepared (1 or 10 mM) with the corresponding solvents (AD and ISO: H_2O; PRO and ATE: ethanol (0.1% (v/v); CAR and ICI: DMSO (dimethylsulfoxide) (0.1% (v/v)). Corresponding controls were prepared with the solvent for each drug dissolved in FBS-free medium, and FBS-medium was the positive control. AD and NA concentrations used in the experiments reflect the levels described in tumors. To determine the effects of the adrenergic agonists AD, NA and ISO on HT-29 cells proliferation and viability, cells were incubated for 12 or 24 hours with these drugs at 0.1 μM, 1 μM, 10 μM and 100 μM. In order to study the effects of β-blockers over AD and ISO actions, cells were pretreated with PRO, CAR, ATE and ICI 45min prior, and simultaneously with, to treatment with the adrenergic agonists. The β-blockers to be tested were selected based not only on their prevalent use in pharmacological studies and clinical settings, but also by their distinct affinity for β-AR and efficacy in modulating several signalling pathways.

3.2. Materials

RPMI 1640 medium (Gibco, Portugal), Fetal Bovine Serum (FBS) (St. Louis, MO, USA), Penicillin, Streptomycin, (St. Louis, MO, USA); trypsin-EDTA. Cell Titer 96 Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], colorimetric assay (Promega, Madison, EUA); Cell Proliferation ELISA BrdU kit (Roche Diagnostics, Portugal); MTT ((3-(4,5-di-methyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO, USA). Ethanol and DMSO (Invitrogen Corporation, CA, USA.).

3.3. Cell Culture

Human colon adenocarcinoma HT-29 cells were kindly provided by Prof. Bruno Sarmento (INEB) and Prof. Fernando Magro (FMUP). HT-29 cells were cultured in RPMI 1640 medium supplemented with 10% of FBS, 100U/mL penicillin and 100µg streptomicyn. Cells were grown at 37ºC in a humidified 5% CO^2 atmosphere. Culture medium was changed every 2–3 days. Once the cells reached 90–100% confluence, medium was removed, and cells monolayer was washed with PBS. The cell monolayer was treated with 1 mL of 0.25% (w/v) trypsin-EDTA and incubated 2 minutes to ensure complete cell detachment. For sub-culturing, cells were sub-cultured in plastic culture dishes (21cm^2; Ø 60mm; Corning Costar, Corning, NY). For the experiments, HT-29 cells were seeded into 96-well (0.37 cm2, Ø 6.9 mm, TPP) or 24-well plastic cell culture clusters (2cm^2 Ø 16mm; TPP), depending on the experimental conditions. The experiments were performed 4-5 days after the initial seeding (90–100% confluence).

3.4. Viability experimental studies

3.4.1. Trypan blue exclusion assay

Cellular viability was determined by the Trypan Blue exclusion assay, and experiments were only performed when viability was higher than 90%. In brief, cells were

trypsinized and stained with 0.4% trypan blue and viable cells counted with a hemocytometer.

3.4.2. MTS assay

HT-29 cells were seeded at 1×10^5 cells/mL in 96-well plates for 24 hours, and afterwards incubated with each drug for 12 or 24 hours, depending on the experimental conditions. Cell viability was assessed using the Cell Titer 96 Aqueous ONE Solution Reagent (MTS colorimetric assay, according to the instructions provided by the manufacturer). Briefly, culture medium was removed and the cells were preincubated with the test compounds dissolved in culture medium at 37°C for 12 or 24 hours. After removing this medium, cells were incubated for 3 h with 100 µL of FBS-free culture medium and MTS.

Absorbance was measured at 492 nm. Results were expressed as percentage of control.

3.4.3. MTT assay

The effect of β-blockers on cellular viability was assessed by the MTT method. The MTT assay is based on mitochondrial dehydrogenase activity. Indeed, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals, which are insoluble in aqueous solutions. The amount of these formed products was determined spectrophotometrically. Briefly, after the incubation periods with the several drugs, cells were washed once with PBS and exposed to a MTT solution at a final concentration of 5mg/ml for 3h and then lysed with DMSO. Absorbance was measured at 540nm. All samples were assayed in triplicated and at least in three independent experiments, and the mean value for each experiments was calculated. The results are given as mean (± SEM) and are expressed as percentage of control.

For determination of the half maximal inhibitory concentration (IC50), the concentration that reduces the effect by 50%, of the several drugs under study, and the half maximal effective concentration (EC50) values, the concentration that gives half maximal response, cells were seeded into 96-well plates at a density of 1×10^4 cells/well for 24 hours, and then incubated for 24 hours with increasing concentrations of the various compounds (0.1, 1, 5, 10, 20, 50 and 100 µM). Cells were then washed twice with warm PBS and incubated with MTT (0.5 mg/mL) for 2 h at 37 °C. Blue formazan crystals were solubilized by adding 100 µl DMSO/well, and the colored solution was subsequently read at 550 nm. Results are expressed as % of MTT reduction compared to untreated control conditions (adapted from Juranic *et al.* (100)).

3.5. Proliferation experimental studies

Cell proliferation was assessed as DNA synthesis. To evaluate DNA synthesis, the incorporation of either ^{3}H-thymidine or of 5' bromodeoxyuridine (BrdU) into DNA was determined.

3.5.1. Incorporation of ^{3}H-Thymidine

HT-29 cells were seeded for attachment at 5×10^4 cells/well into 24-well (1.65 cm^2; Ø 14.5 mm; Orange Scientific, Belgium) plastic cell culture clusters in a final volume of 0.5 mL culture medium containing 10% FBS. After 24 hours in culture, cells were treated with different concentrations of the adrenergic agonists in culture medium (controls were made in the presence of culture media). After 24 hours, cells were incubated with 0.2 mL of methyl-[3H]-thymidine (0.5µCi/well) for 4 hours. The medium was removed and cells fixed by incubation in 0.3 mL of 10% TCA for 1 h at 4°C. The cells were then washed twice with 0.3 mL of 10% TCA to remove unbound radioactivity. The plates were air-dried and cells lysed with 0.28 mL/well of 1 M NaOH. A 0.25 mL aliquot of the lysate was neutralized with 0.050 mL of HCl prior to the addition of scintillation fluid. The radioactivity of the samples was quantified by a liquid scintillation counter. The counts

(disintegrations per min) of each treatment were averaged and expressed as the percentage of controls (adapted from (Miranda *et al*., 1999) (101).

3.5.2. Incorporation of 5' bromodeoxyuridine (BrdU)

The incorporation of BrdU is a method based on the incorporation of the pyrimidine analogue BrdU, a thymidine analogue, instead of thymidine into DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by immunoassay.

HT-29 cells were grown at 1×10^5 cells/ml in 96-well plates for 24 hours, and proliferation measured using the Cell Proliferation ELISA BrdU kit, according to the manufacturer's instructions. Briefly, cells were labeled with BrdU (final concentration 10 µM/well) for 12 hours at 37º C. Afterwards, cells were denatured with FixDenat solution, and then incubated for 120 minutes with 1:100 diluted mouse anti-BrdU conjugated to peroxidase. After removing the antibody conjugate, and washed twice with washing solution (PBS 1X), substrate solution was added for 25 minutes and the reaction stopped with 1 M H_2SO_4 solution. Absorbance was measured within 5 minutes at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader. The blank corresponded to 100 µL of culture medium without BrdU, and the control was made in the presence of culture media without any treatment. The results are given as mean (± SEM) and are expressed as percentage of control.

For treatments during 24 hours, another protocol for BrdU assay was tested. Briefly, $5x10^3$ cells per well were seeded in 96-well plate. The medium was supplemented with antibiotics plus 1% FBS for cell attachment, and then, cells were starved in serum-free medium for another 12 hours to synchronize cell cycle. HT-29 cells were incubated with AD, ISO or NA (0, 1, 10 µM) for 24 hours to study the growth-promoting effect of these adrenergic agonists. To examine the effects of various β-blockers, cells were pretreated with or without the several adrenergic antagonists, PRO (50 µM), CAR (50 µM), ATE (5 µM) and ICI (5 µM) for 45 minutes prior to, and also simultaneously with,

AD or ISO treatment. Cell proliferation was indicated by the amount of DNA synthesis measured with the BrdU incorporation assay kit, according to the manufacturer's instructions. Briefly, cells were labeled with 10 ml/well BrdU and incubated at 37°C for 4 hours. After removal of the labeling medium, cells were fixed and probed with the anti-BrdU monoclonal antibody at 25°C for 2 hours and its substrate tetramethyl-benzidine (TMB) at 25°C for 30 min. After removal of the unconjugated antibody, cells were rinsed 3 times with the washing solution and treated with 200 µl/well substrate solution. After color development, 1 M H_2SO_4 was added (25 µl/well) to stop the substrate reaction, and the absorbance of each sample was measured in an enzyme-linked immunosorbent assay (ELISA) microplate reader at 450 nm (with a reference wavelength at 690 for blank to disccount the nonspecific binding to the anti-BrdU antibody). The value from the nonspecific binding was subtracted from all the other values. The results are given as mean (± SEM) and are expressed as percentage of control.

3.6. Statistical Analysis

The results are expressed as arithmetic mean ± SEM. Differences in proliferation or viability between cells treated and corresponding untreated controls were tested using a Student's t-test. For the calculation of EC50 and IC50 values, the parameters of the Hill equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (101). n represents the number of replicates of at least three different experiments. Comparisons between three or more groups were performed with one-way analysis of variance (ANOVA) followed by Tamhane or Bonferroni test. Differences were considered to be significant when $p < 0.05$.

Given the variability of the results on different days, each experimental data was adjusted to respective controls.

IV. CHAPTER

RESULTS

4. RESULTS

In the present study, we intended to clarify the role of the stress hormones, AD and NA, and β-blockers in colon cancer cells proliferation by using a human colon adenocarcinoma cell line (HT-29 cells). Thus, using selective agonists and antagonists for β-AR, we investigated the differential involvement of these receptors, namely of β_1 and β_2, in colon cancer cells growth.

To assess the effect of the adrenergic agonists, AD, NA and ISO on HT-29 cells proliferation as DNA synthesis we performed two assays: ^{3}H-Thymidine incorporation (for treatments with these drugs during 12 hours) and BrdU incorporation (for treatments during 12 and 24 hours with these drugs and also for experiments with the several β-blockers).

To evaluate cell viability in cultured HT-29 cells in response to agonists and β-blockers we used both MTS (for treatments during 12 and 24 hours with the AR agonists) and MTT assays (for the simultaneous exposure to agonists and β-blockers).

4.1. Determination of EC50 values for the adrenergic agonists, adrenaline and isoprenaline, in HT-29 cell line

In order to determine the EC50 values of AR agonists in this cellular model, HT-29 cells were treated in control conditions (without drugs) and with increasing concentrations (0.1, 1, 5, 10, 20, 50 and 100 μM) of AD and ISO for 24 hours to determine the effects of these drugs on cellular growth. The above experiments generated concentration–response curves for both agonists (Figure 9) allowing to calculate the EC50 values (Table 3). Based on the concentration-response curves for both agonists, EC50 values were calculated, being 9.98 (0.51-197.2) and 29.27 (0.72-1194.0) μM, respectively for AD and ISO.

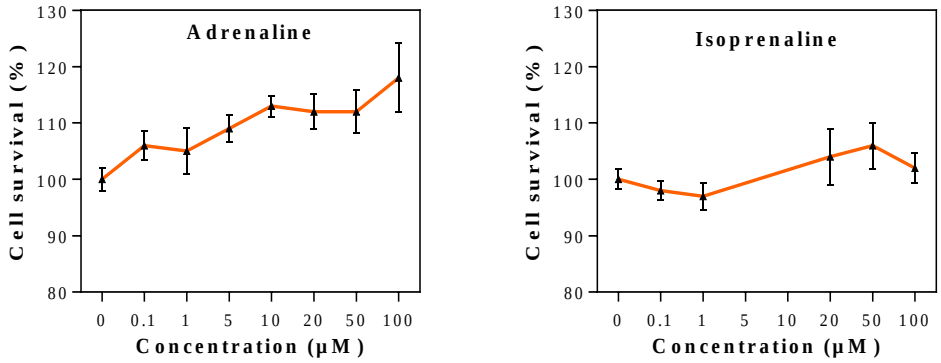

Figure 9. Effect of adrenaline and isoprenaline on cell growth of HT-29 cells assessed by MTT assay after 24 hours of treatment. Results are expressed as mean ± SEM and are normalized to 100% of control groups (without the drugs).

Table 3. EC50 values for adrenaline and isoprenaline on HT-29 proliferation.

Cell type	Agonist	EC_{50} µM	95%	n
HT-29	**Adrenaline**	9.98	0.51 – 197.2	10-11
	Isoprenaline	29.27	0.72-1194.0	8-12

Shown are the EC50 values with the corresponding 95% confidence intervals.

4.2. Effect of chronic treatment (12 hours) with adrenergic agonists on HT-29 cells proliferation and viability

The exposure of HT-29 cells to the adrenergic agonists AD, NA and ISO (at 0.1µM-100µM) markedly increased the proliferation (Figures 10A and 11) and viability (Figure 10B) of these cells. After 12 hours, AD had its maximum effect on proliferation at 10 µM (131.0 ± 8.7%, n=10), NA at 100 µM (146.3±17.1%, n=8) and ISO at 100 µM (150.9 ± 25.5%, n=7), relatively to controls (Figure 10A). In relation to the viability assessed by MTS (Figure 10B), only the positive control, with 10% FBS (CT+), and NA at 100 µM significantly increased cellular viability comparing to controls.

When the ^{3}H-Thymidine incorporation assay was used (Figure 11), AD was able to increase proliferation by 18% (n=8) and 36% (n=6) respectively at 0.1 and 1.0 μM. NA at 10 μM led to a significant increase of proliferation by 33% (n=6) and ISO increased proliferation at all concentrations tested (to 138%, 123% and 140%, respectively at 0.1, 1.0 and 10 μM (n=5-7)).

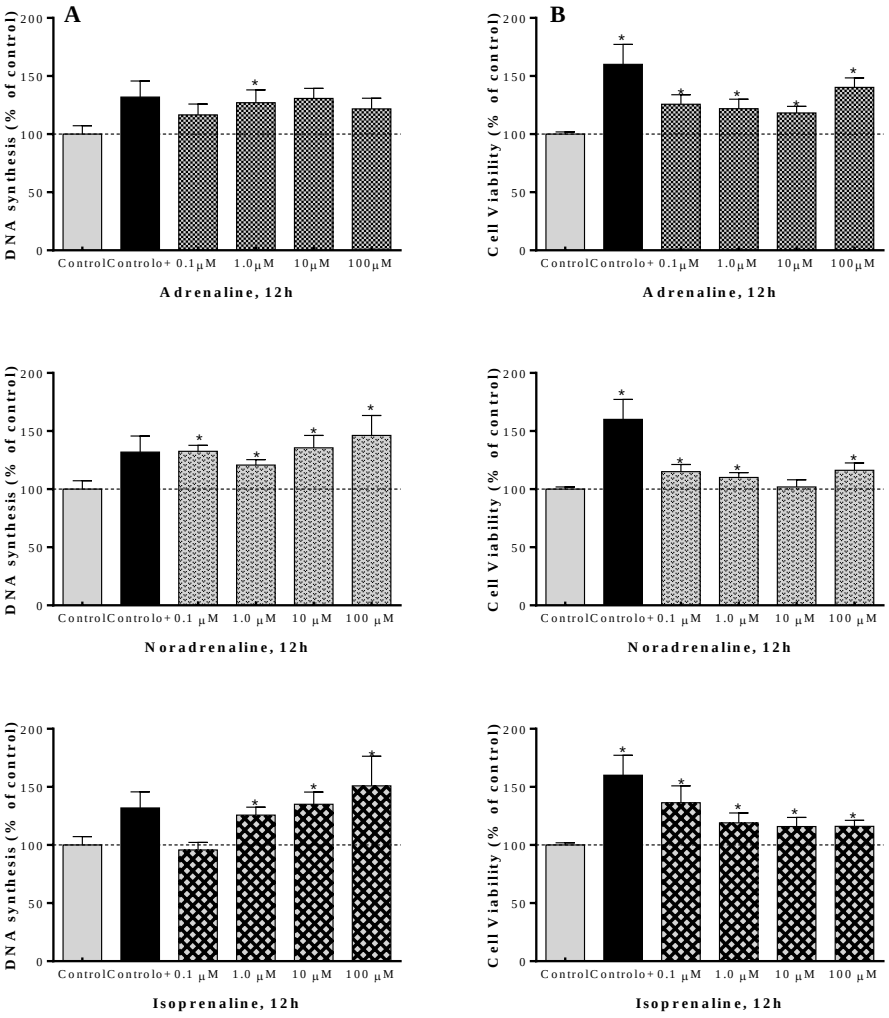

Figure 10. Effect of adrenaline (AD), noradrenaline (NA) and isoprenaline (ISO), at 0–100 μM, in human colon adenocarcinoma HT-29 cells proliferation (A) (n=6-12) and viability (B) (n=11-15), after incubation for 12 hours. Cell proliferation was assessed by BrdU incorporation and cell viability by a MTS assay, as described in the Materials and Methods section. Results are expressed as mean ± SEM. *$p<0.05$ comparing to control.

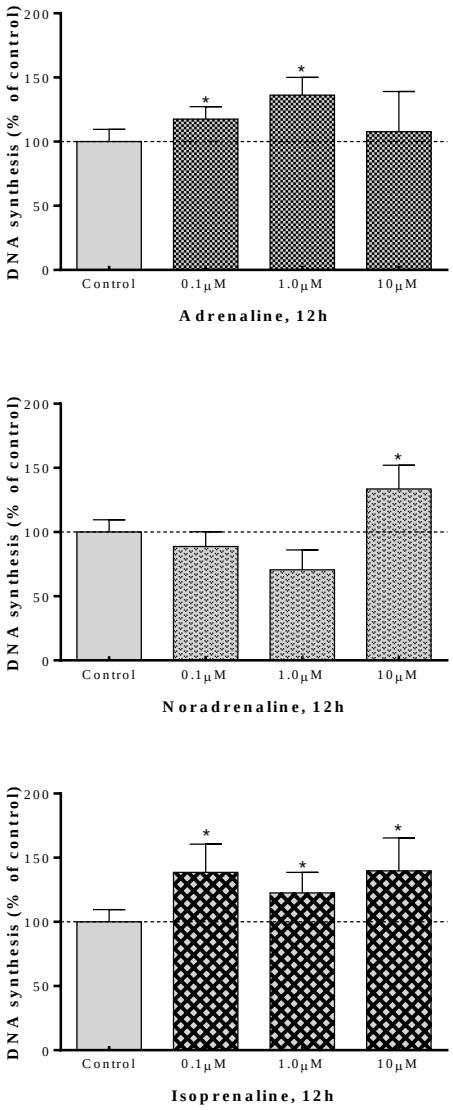

Figure 11. Effect of adrenaline (AD), noradrenaline (NA) and isoprenaline (ISO), at 0–10 μM, in human colon adenocarcinoma HT-29 cells proliferation after incubation for 12 hours. Cell proliferation was assessed by using the ^{3}H-Thymidine incorporation assay as described in the Materials and Methods section. Results are expressed as mean ± SEM (n=5-18). *$p<0.05$ comparing to control.

4.3. Effect of chronic treatment (24 hours) with adrenergic agonists on HT-29 cells proliferation and viability

We further tested the effect of AD and ISO on cell proliferation, and also the effect of the three adrenergic agonists under study in cell viability at 24 hours (Figure 12). Chronic treatment (24 hours) with the AR agonists induced a significant increase of HT-29 cell proliferation (Figure 12A). AD led to a significant increase of cell proliferation by 164.7% (n=18) and 145.5% (n=18), when used at 1μM and 10μM, respectively, whereas ISO enhanced HT-29 proliferation by 46.1% (n=10) and 48.6% (n=15), respectively at 1 and 10 μM, when compared to controls.

In relation to viability assessed by MTS assay, as we can see in Figure 12B, the results obtained with the treatment during 24 hours were less consistent in comparison with the treatment during 12 hours (Figure 10B), given that for all the adrenergic agonists only a few of the used concentrations were effective in increasing HT-29 viability.

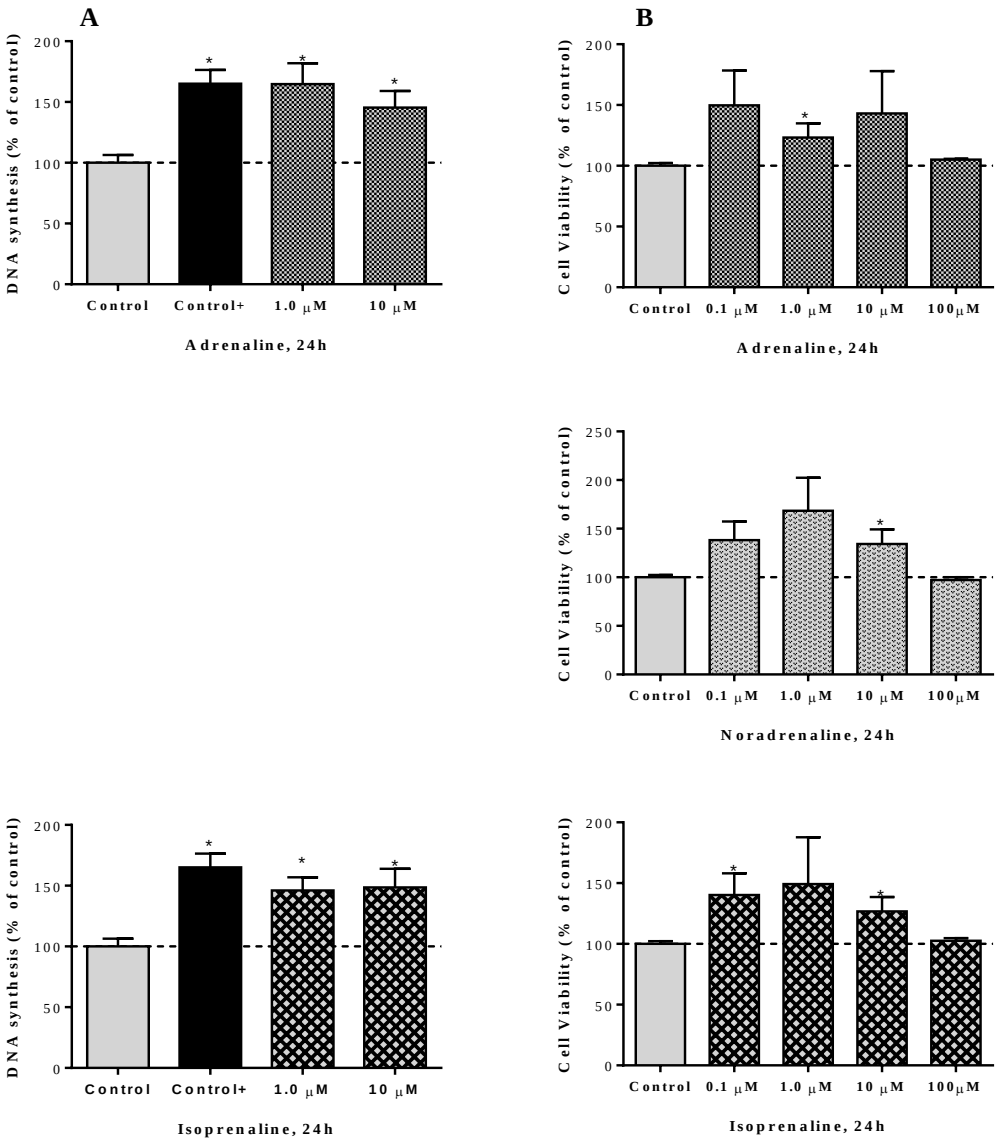

Figure 12. Effect of adrenaline (AD), noradrenaline (NA) and isoprenaline (ISO), in human colon adenocarcinoma HT-29 cells at 0–10 μM (n=6-18) on proliferation assessed by BrdU incorporation assay (A) and at 0–100 μM (n=3-14) on viability assessed by MTS assay (B) as described in the Materials and Methods section, after incubation for 24 hours. Results are expressed as mean ± SEM, *p<0.05 comparing to control.

4.4. Determination of IC50 values for β-blockers in HT-29 cell line

In order to calculate the IC50 values (the drug concentration for 50% block) for the effect of β-blockers on cellular survival, cells were treated under control conditions (without drugs) and with increasing concentrations of either PRO, CAR, ATE or ICI (0.1, 1, 5, 10, 20, 50 and 100 µM) for 24 hours. The concentration-response curves of cell proliferation are shown in Figure 13 and IC50 values in Table 3.

As shown in Figure13, PRO potently inhibited the proliferation of HT-29 cells at concentrations over 50 µM, being its IC50 for PRO 65.4 µM. HT-29 proliferation was inhibited in a concentration-dependent manner by CAR after exposure during 24 hours. Among the β-blockers tested, CAR was proved to be the most potent with an IC50 of 8.0 µM. ATE when used at the highest concentration (100 µM) significantly decreased HT-29 survival being its IC50 value 52.9 µM. CAR and ICI showed similar IC50 values, respectively 8.0 µM and 8.9 µM.

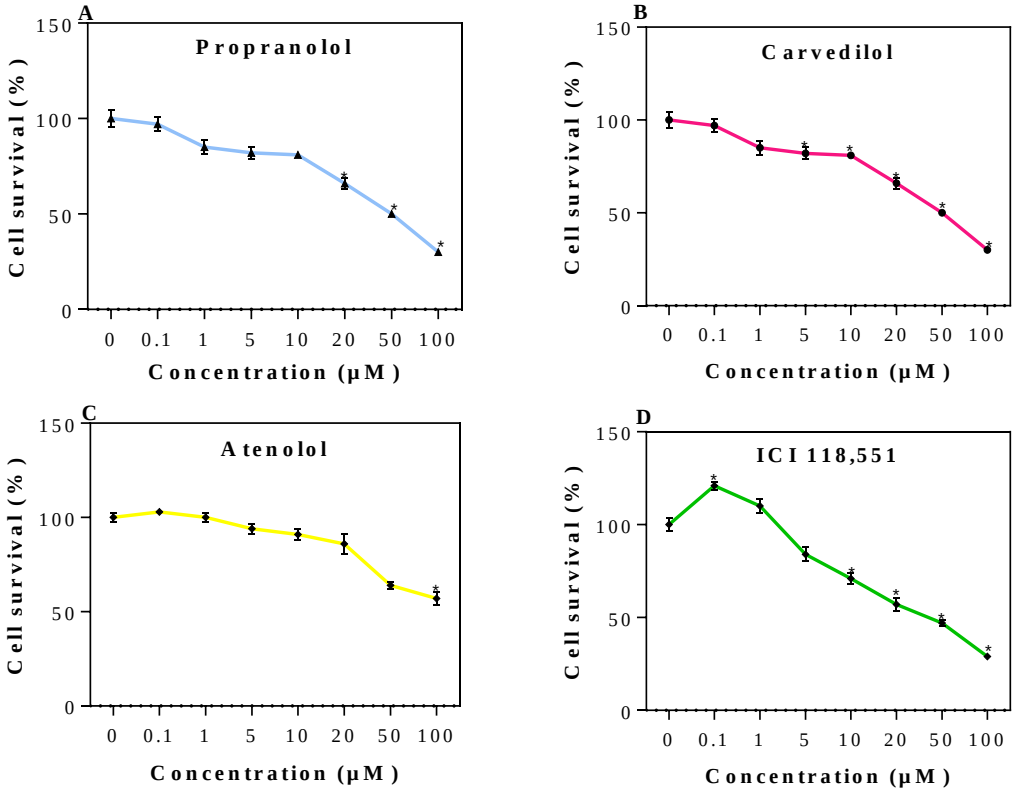

Figure 13. Concentration-response curves for HT-29 cell survival. Cells were treated with increasing concentrations (0, 0.1, 1, 5, 10, 20, 50 and 100 μM) of each β-blocker, propranolol (PRO) (A), carvedilol (CAR) (B), atenolol (ATE) (C) and ICI 118,551 (ICI) (D) during 24 hours and viability was assessed by a MTT assay. Results are expressed as mean ± SEM and normalized to 100% of control groups (without drugs). *p<0.05 comparing to control.

Table 4. Effect of β-blockers on HT-29 proliferation

Cell type	β-Blockers	IC_{50} μM	95%	n
HT-29	**Propranolol**	65.4	33.7 – 126.9	10-12
	Carvedilol	8.0	6.0 – 10.6	12
	Atenolol	52.9	21.7 – 128.7	6-12
	ICI 118,551	8.9	6.5 – 12.0	12

Shown are IC50 values with the corresponding 95% confidence intervals.

4.5. Effect of β-blockers on HT-29 cells proliferation

To elucidate the role of β-AR upon cell proliferation induced by AR activation, the agonists AD and ISO (a nonselective versus a β-selective agonist) were employed simultaneously with the following β-blockers PRO (50 µM), CAR (5 µM), ATE (50 µM) or ICI (5 µM).The concentrations used for β-blockers were selected based on the IC50 values, previously calculated.

4.5.1. Propranolol

The role of β-ARs in proliferation is supported by the results presented on Figure 14 where can be seen that PRO, a non-selective β-AR antagonist, abolished the proliferation increase induced by both AD and ISO. AD-induced cell proliferation was markedly reduced by PRO to 11.8 ± 3.4% (n=6) and 32.0± 9.7% (n=5), when AD was respectively used at 1 and 10 µM. Cells treated with PRO, at 50 µM, greatly decreased cell proliferation evoked by ISO to 20.1± 2.1% (n=5) and 23.3± 5.2% (n=5), when ISO was respectively used at 1 and 10 µM. Furthermore, as we can see in the same graph, PRO per se induced a significant proliferation decrease to 44.2± 9.6% (n=6), when compared with control group.

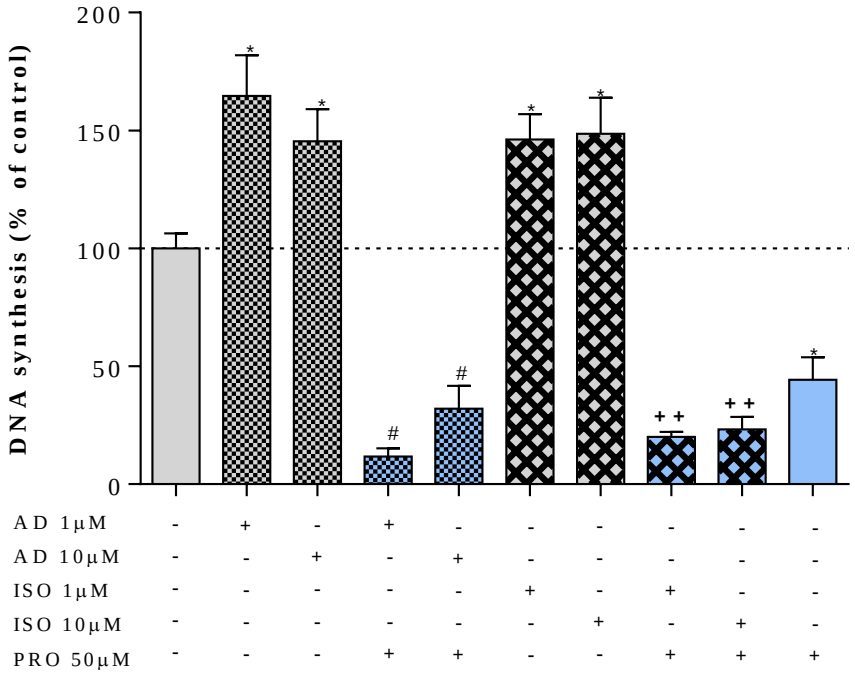

Figure 14. Effect of the non-selective β-adrenoceptor antagonist propranolol (PRO) at 50 μM on adrenaline (AD), 1 and 10 μM, and isoprenaline (ISO), 1 and 10 μM, induced HT-29 cell proliferation. Cells were pretreated with PRO for 45 minutes before incubation, and simultaneously with AD and ISO for 24 hours. Cell proliferation was measured by BrdU incorporation assay, as described in the Materials and Methods section. *p<0.05, significantly different from the untreated control group. #p <0.01, significantly different from the respective concentration AD-treated group and ++p <0.01 significantly different from the respective concentration ISO-treated group.

4.5.2. Carvedilol

The response profile of CAR, a potent non-selective β and α_1-AR antagonist, in reverting the proliferative effects of both AD and ISO, was quite similar to the results obtained with PRO. Indeed, as shown in Figure 15, CAR was able to strongly inhibit the proliferative effect evoked by both agonists.

CAR decreased the proliferation induced by AD to 28% (n=6) and 56% (n=6), when AD was respectively applied at 1 and 10 µM, and to 27% (n=6) and 36% (n=6) when ISO at 1 and 10 µM was used. Contrary to PRO, CAR per se did not significantly affect the proliferation of HT-29 cells.

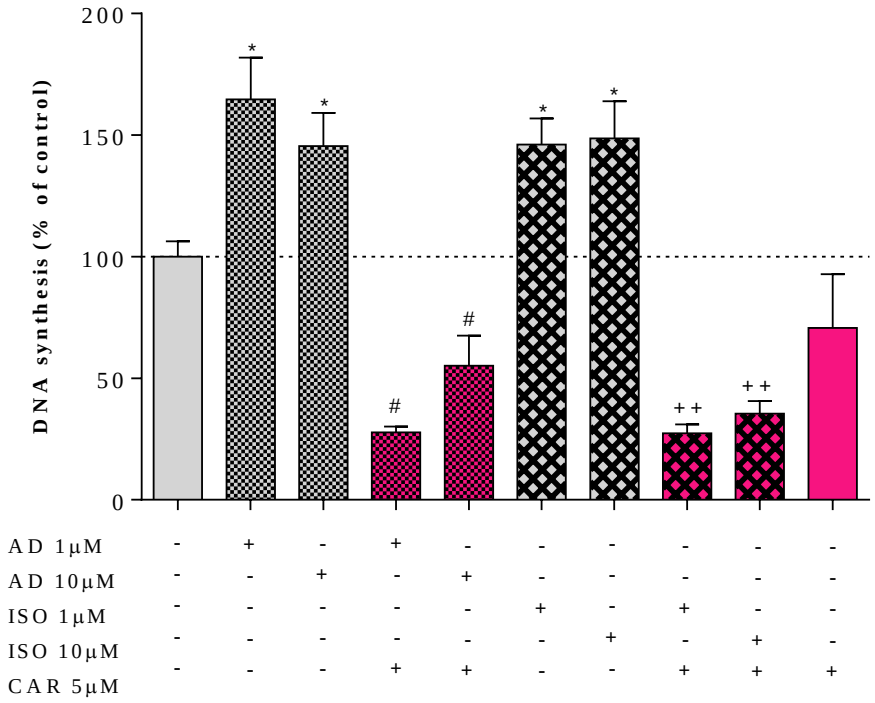

Figure 15. Effect of the non-selective β and α_1-adrenoceptor antagonist carvedilol (CAR; 5 µM) on adrenaline (AD) at 1 and 10 µM, and isoprenaline (ISO), at 1 and 10 µM on HT-29 cell proliferation. Cells were pretreated with CAR for 45 minutes before incubation, and simultaneously with, AD and ISO for 24 hours. Cell proliferation was measured by BrdU incorporation assay, as described in the Materials and Methods section. *p<0.05, significantly different from the untreated control group. #p <0.01, significantly different from the respective concentration AD-treated group and ++p <0.01 significantly different from the respective concentration ISO-treated group.

4.5.3. Atenolol

To elucidate the role of β1-AR in HT-29 proliferation, we have used ATE, a β_1 – selective antagonist, as previously mentioned for the other related experiments, before and simultaneously with the treatment with both AR agonists. Figure 16 shows that ATE significantly blocked AD and ISO-induced cell proliferation, confirming the involvement of β_1 subtype in promoting tumor cell proliferation. ATE significantly decreased cell proliferation induced by AD at 1 and 10 µM to 55.7± 5.6% (n=6) and 53.4± 3.6% (n=6), respectively and to 45.8± 9.2% (n=6) and 32.1± 4.6% (n=6) for ISO respectively at 1 and 10 µM. Furthermore, when applied alone, ATE decreased proliferation to 55.4± 13.9% (n=6), comparing to control.

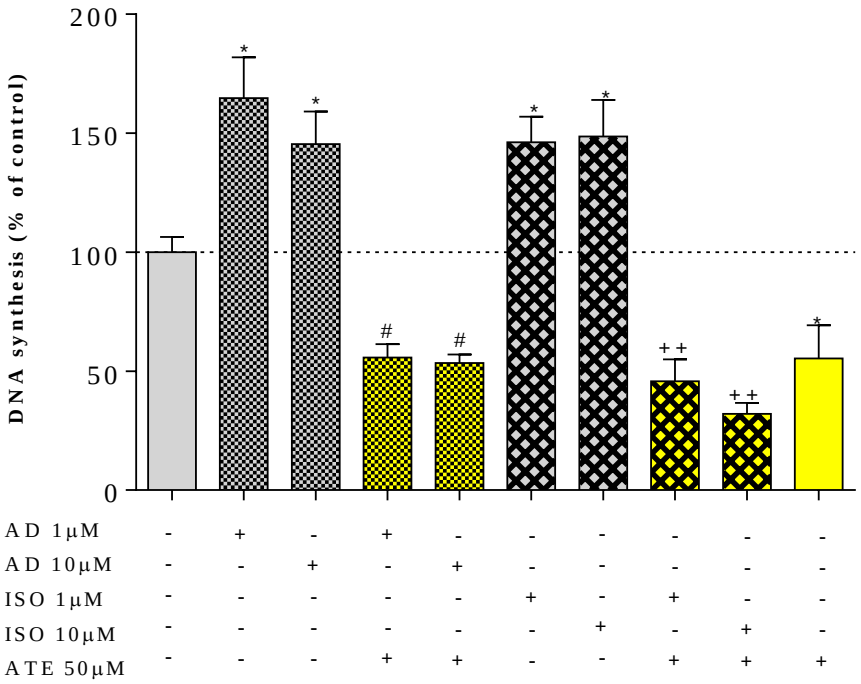

Figure 16. Effect of the selective β_1-adrenoceptor atenolol (ATE; 50 µM) on adrenaline (AD), at 1 and 10 µM, and isoprenaline (ISO), at 1 and 10 µM, on HT-29 cell proliferation. Cells were pretreated with ATE for 45 minutes before incubation, and simultaneously with AD and ISO for 24 hours. Cell proliferation was

measured by BrdU incorporation assay, as described in the Materials and Methods section. *p<0.05, significantly different from the untreated control group. #p <0.01, significantly different from the respective concentration AD-treated group and ++p <0.01 significantly different from the respective concentration ISO-treated group.

4.5.4. ICI 118,551

To confirm the involvement of β_2 –AR in HT-29 proliferation, cells were incubated with ICI, a β_2–selective antagonist, either alone or with the AR agonists. As we can see in Figure 16, ICI did not significantly affect the proliferation induced by AD, whereas diminished to 64.1 ± 11.1% (n=7) the effect evoked by ISO 10 μM, reinforcing the involvement of β_2 -AR subtype in this process. ICI per se had no effect on HT-29 cell proliferation.

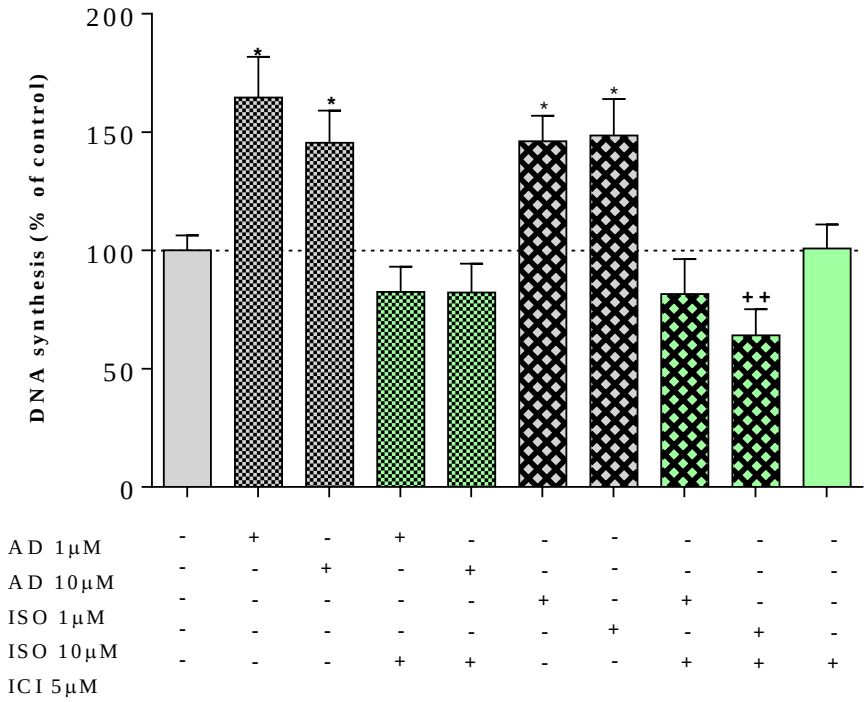

Figure 17. Effect of the selective β_2-adrenoceptor ICI-118,551 (ICI, 5μM) on adrenaline (AD), 1 and 10 μM, and isoprenaline (ISO), at 1 and 10 μM, induced HT-29 cell proliferation. Cells were pretreated with ICI for 45 minutes before incubation, and simultaneously with AD and ISO for 24 hours. Cell proliferation was measured by BrdU incorporation assay, as described in the Materials and Methods section. *p<0.05, significantly different from the untreated control group. #p <0.01, significantly different from the respective concentration AD-treated group and ++p <0.01 significantly different from the respective concentration ISO-treated group.

All together, these results suggest that PRO is the most effective β-blocker in reverting the proliferative effect of the adrenergic agonists under study, probably due to its ability to bind both to β_1 and β_2.

V. CHAPTER

DISCUSSION

5. DISCUSSION

Stress has been proposed to play a central role in the incidence and development of cancer (36). However, the molecular and cellular mechanisms by which stress increases the risk of some cancers and their prognosis remain understudied. Interestingly enough, it has been postulated that endogenous CA mediate the association between stress response and poor cancer outcomes (39,47). It hence appears that the most common human cancers are not only stimulated by stress neurotransmitters in the systemic circulation, but also produce their own CA. Moreover, growing evidences suggest that exposure to chronic stress impairs the immune system, alter cancer cell functions and tumor development (36,101). Epidemiologic studies also have associated the use of β-blockers in clinical settings to reduced rates of progression for several solid tumors (76). β-blockers are currently one of the most used drugs to treat angina and cardiac arrhythmias, as well as hypertension, cardiac failure, glaucoma, migraine and anxiety, but new therapeutic options have been advanced (77). Indeed, several findings suggest that β-AR blockers may be inexpensive and safe therapeutic agents for cancer, but their impact on cancer survival is not known. In the majority of the mentioned studies, no distinction was made between the use of β-blockers with selective β_1 or β_2 receptor activity, and the signaling pathways implicated in these responses are poorly understood.

In this study, we addressed the effect of the stress hormones, AD and NA, and ISO, a synthetic β non-selective agonist, and several β-blockers, on colon cancer cells proliferation, a critical component of the carcinogenesis cascade. As previously mentioned, CA exert their biological effects through binding to AR. In tumoral cells, β-AR are the key receptors in mediating the effects of CA. Indeed, the expression of β-AR has been identified in normal colon tissue and in colon cancer cells, including HT-29 cells, the experimental model used in this study, being β_2-AR the predominant receptor subtype in these cells (14). In fact, several studies have recognized β-AR activation as a central mediator of stress effects on cancer growth. The activation of these receptors in a variety of cancer cell types in different tumorigenic processes, including tumor cell proliferation (12),

migration (15) apoptosis (110), angiogenesis (61) and differentiation (18). Therefore, β-AR blockade with pharmacological agents could potentially be used to alleviate the effects of stress upon cancer growth and progression. In line with this, some studies have found that β-AR blockade may suppress cancer cell invasion and inhibit adrenergic driven metastasis (88).

In our study, the results obtained with the adrenergic agonists, not only confirmed that stress hormones affect colon cancer cells proliferation, but also suggest a prominent role for β-AR in this process. AD and ISO, as previously shown by other authors (12,14,81) significantly augmented HT-29 proliferation, most probably through β-AR. Indeed, both agonists have a high affinity for β-AR, strongly expressed in these cells, and in our study evoked a similar proliferative response. Based on the above observations, we explored the AR subtypes involved in AD and ISO effects, by using β-blockers with distinct profiles for AR.

Our results show that the β-blockers under study significantly reduced the proliferation induced by both AD and ISO, reinforcing that these agonists mainly acted through β-AR. Altogether, our results clearly indicate the involvement of both β-AR subtype's β_1 and β_2 in promoting colon cancer cell proliferation. PRO, the nonselective β-AR antagonist, was the most potent β-blocker in reverting AD effects upon cell proliferation, probably by its ability to bind to both β_1 and β_2, as already highlighted by other reports (81). Indeed, the proliferation increase evoked by AD was strongly abolished by PRO, less by CAR, even less by ATE and was not affected by ICI. According to β_2 involvement in this effect, the β_1-blocker (ATE) was a week antagonist for AD actions; however, the selective β_2-blocker (ICI) had no effect. On the other hand, with the exception of ICI, which had a moderate effect, all the other β-blockers markedly inhibited the proliferation induced by ISO. Thus, contrary to other reports (12,14,81), ATE, the selective β_1 antagonist, was more effective than the selective β_2-antagonist, ICI, in reverting both AD and ISO induced cell proliferation.

As previously referred, β-blockers are not solely antagonists for the G-protein pathways, but they may indeed independently modulate more than one pathway, and behave as partial agonist, inverse agonists or pure antagonists in each pathway, increasing the complexity of their actions (82). Thus, biased agonism might have outstanding implications for β-AR blockers therapeutic use in cancer, since distinct signaling through these pathways is thought to have specific functional consequences (82). All the β-AR blockers tested were already recognized as being inverse agonists (84). The finding that both PRO and ATE used alone were able to decrease HT-29 cell proliferation, suggests that they acted as inverse agonists, a characteristic already described by some authors in other experimental models for both drugs acting via β_1 and β_2-AR (84). Activation of β-ARs result in an increase of cAMP intracellular concentrations and cell proliferation, two processes reversed by treatment with either β_1-or β_2-AR antagonists (93). In fact, PRO and ATE acting as inverse agonists through binding to both β-AR lead to a decrease of cAMP accumulation (84), an outcome that could explain the proliferation decrease induced by these drugs in our study. On the other hand, neither the β_2 selective agonist (ICI) nor CAR had an antiproliferative effect when used alone, despite both have been described as being able to diminish cAMP levels (102). Thus, as suggested by others (77), β-blockers seem to have complex profiles for cAMP modulation and Erk1/2 activation at both β_1 and β_2-AR. Moreover, CAR is able to activate different signaling pathways depending on the cell type (102), and till now its effect upon HT-29 proliferation was unknown. In fact, CAR behaved similarly to PRO when used simultaneously with both agonists, but, contrary to data obtained with other cell types for both β_1 and β_2, probably had no effect as inverse agonist.

Several researchers have been studying the downstream signaling pathways involved in β-AR mediated tumor growth. Among the mammalian MAPK pathways, ERK is the best studied, and the deregulation of this pathway occurs in approximately one-third of all human cancers (103). Some studies have concluded that β-AR mediated ERK1/2 activation could be one of the mechanisms underlying stress-induced cancer cell growth in vivo, suggesting for instance that β-AR blockade may be an effective approach for patients with stress-related colon cancer (81). On the other hand, in 2006, Shenoy *et al* (104)

showed in tumoral models that ISO through binding to β_2-AR leads to the activation of a G protein- independent ERK pathway, but dependent on β-arrestin. Indeed, the pathway whereby growth factors and mitogens activate ERK signalling is of particular relevance to cancer (103). All the β-blockers used in our study, with the exception of ICI, were already described as being capable to activate ERK pathway. However, as referred before, when used alone, ATE and PRO decreased cell proliferation and CAR had no effect.

Three of these drugs (CAR, PRO and ATE) are widely used clinically. In fact, similar studies have shown that these and other β-blockers reveal new clinical applications in medicine, which is very attractive for commercial purposes. On the other hand, deepening the knowledge of β signaling pathways involved in cancer may allow finding and selecting appropriate inhibitors both to prevent and treat cancer. In addition, corroborating the putative use of β-blockers as therapeutic agents in cancer, at least three phase II clinical studies assessing the safety and efficacy of β-blockers in breast, colorectal and ovarian cancers are currently running (105).

VI. CHAPTER

GENERAL CONCLUSIONS AND FUTURE WORK

6. GENERAL CONCLUSIONS AND FUTURE WORK

In conclusion, our work showed that all the adrenergic agonists tested increased the proliferation of HT-29 cells. All the β-blockers reverted, with less or more potency, the proliferative effect of the agonists, confirming the role of β-AR in cancer biology. This study reinforces several lines of evidence suggesting that stress hormones have an important role in carcinogenesis, namely in colon cancer proliferation, and that β-blockers can be used to revert these effects.

Cancer cell proliferation is a complex process that involves several pathways and different molecules; therefore further approaches are essential to evaluate other β-blockers, and also the intracellular mechanisms underlying, not only the effect of endogenously CA, greatly released under stress conditions, but also of adrenergic agonists in clinical use. A better knowledge about these mechanisms might contribute to the discovery of new promising targets for cancer prevention and treatment.

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