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The anticarcinogenic potential of dietary natural compounds on colorectal carcinoma: Effects on signalling pathways related to proliferation and cell death
Cristina Pinto Ribeiro Xavier
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Tese de Doutoramento em Ciências
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The anticarcinogenic potential of dietary natural compounds on colorectal carcinoma: Effects on signaling pathways related to cell proliferation and cell death

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Abstract

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and the incidence is highly influenced by diet. Epidemiological studies have supported the idea that some dietary food components may influence the risk of CRC through modulation of several biological processes, including proliferation, survival and cell death. The PI3K/Akt and MAP kinases (ERK, JNK and p38) pathways are frequently altered in CRC and components of these pathways are important molecular targets for CRC treatment. Moreover, the apoptotic and non-apoptotic pathways of cell death have been shown good targets for anticancer drugs. The aim of the thesis was to identify potentially anticarcinogenic natural compounds and characterize their effects on signaling pathways related to proliferation and cell death, in CRC cell lines. The role of these natural compounds in combination with 5-fluorouracil (5-FU) was also evaluated.

Initially, the anticarcinogenic effect of some water extracts (prepared as a tea) in two human colon carcinoma-derived cell lines, HCT15 and CO115 was studied. In chapter II, we demonstrated the anticarcinogenic activity of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO), that seems to be due, at least partially, to the inhibition of the MAPK/ERK pathway, through effects upstream of BRAF. This effect was not due to rosmarinic acid (RA), the major phenolic compound present in these sage plants. In a subsequent study (chapter III), we showed the potential of *Hypericum androsaemum* (HA) in inhibiting cell proliferation and inducing apoptosis, at least in part, through inhibition of mutant BRAF and PI3K/Akt pathway, as well as, by the induction of p38 and JNK MAP kinases. As in the case of RA also here the main phenolic compound present in HA extract, chlorogenic acid, tested alone did not show any of those effects.

Taking into account the previous observations, we further evaluated the potential anticarcinogenic effects of quercetin (Q), luteolin (L) and ursolic acid (UA) in CRC cells. These phenolic compounds (Q and L) and triterpenoid (UA) are present (or appear as derivates) in the water extracts previously used, and have been demonstrated to modulate many steps of the carcinogenic process. In chapter IV, we found that Q and L have antiproliferative and proapoptotic effects that seem to be due, at least in part, to effects on

KRAS through regulation of both MAPK/ERK and PI3K pathways. UA demonstrated anticarcinogenic effects by acting on PI3K.

In chapter V, we performed a combined study of Q, L and UA with the most common chemotherapeutic agent used in CRC treatment, 5-FU. Resistance to 5-FU arises, especially in tumors with p53 mutations, make combinations with other drugs a necessary strategy to increase 5-FU's efficacy. In the first part of this study, we demonstrated that Q and L enhanced 5-FU-induced apoptosis and a synergistical effect was observed with Q in the p53 wild type CO115 cells. Q may increase 5-FU-induced apoptosis by modulating the mitochondrial pathway dependent on p53. In the second part, UA was observed to synergistically enhance 5-FU-induced apoptosis in the p53 mutant HCT15 cells. The increase on apoptosis was not dependent on caspases and it was almost completely abrogated by an inhibitor of JNK, suggesting that in these cells UA induces apoptosis through JNK activation. Moreover, the increase of total cell death and the accumulation of LC3, induced by UA, were also dependent on JNK activation.

In order to explore the remarkable induction of cell death by UA, which was not all explained by increased apoptosis, we verified the involvement of UA in the autophagic process (chapter VI). Interestingly, we found that UA inhibits autophagy in HCT15 cells at the maturation step, since no fusion events between lysosomes and autophagosomes were detected. Taking advantage of techniques established for MCF-7 breast cancer cells, including the tandem fluorescent construct tagged with LC3 and the measurement of LC3 turnover using a luciferase-based real time assay, the results above were confirmed. Furthermore, UA increased lysosomal membrane permeabilization and decreased the total lysosomal hydrolases activities, indicating a possible impact on lysosomal biogenesis and/or a direct destabilizing effect on lysosomal membranes.

In conclusion, this work adds SF, SO and HA to the list of potential plants to use in CRC dietary strategies, as well as, the natural compounds Q, L and UA present in human diet. These natural compounds control CRC progression by modulating important molecular targets, as well as, by enhancement of 5-FU efficiency. UA was the most promising compound, destabilizing the lysosomes and, consequently, inhibiting autophagy, which could sensitize cells to death and thus, may have a possible interest as adjuvant in cancer therapy.

Estudo do potencial anticarcinogénico de compostos naturais provenientes da dieta no cancro colorectal: Efeitos ao nível de vias de sinalização relacionadas com a proliferação e morte celular

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Resumo

O cancro colorectal (CRC) é o terceiro cancro mais comum a nível mundial estando a sua incidência fortemente influenciada pela dieta. Estudos epidemiológicos revelam que compostos presentes na dieta podem influenciar o risco de CRC, modulando vários processos biológicos, incluindo a proliferação, sobrevivência e morte celular. As vias de sinalização PI3K/Akt e MAP kinases (ERK, JNK e p38) estão frequentemente alteradas no CRC e por isso, componentes destas vias constituem alvos moleculares importantes para o seu tratamento. Além disso, as vias apoptóticas e não-apoptóticas de morte celular têm demonstrado ser bons alvos de drogas anticarcinogénicas. A presente tese teve como objectivo identificar compostos naturais com potencial anticarcinogénico e caracterizar os seus efeitos ao nível das vias relacionadas com proliferação e morte celular em linhas de CRC. O papel destes compostos em combinação com o 5-Fluorouracilo (5-FU) foi também abordado.

Inicialmente, os efeitos anticarcinogénico de alguns extractos aquosos (preparados como chás) foram estudados em duas linhas celulares humanas de carcinoma do cólon, HCT15 e CO115. No capítulo II, demonstrou-se a actividade anticarcinogénica das plantas *Salvia fruticosa* (SF) e *Salvia officinalis* (SO), em que o seu efeito pareceu dever-se, pelo menos em parte, à inibição da via MAPK/ERK, através de efeitos a montante do BRAF. O ácido rosmarínico (RA), composto fenólico maioritário presente nas *Salvias* não foi o responsável pelos efeitos observados. No capítulo III, demonstrou-se que o extracto aquoso de *Hypericum androsaemum* (HA) é capaz de inibir a proliferação celular e induzir a apoptose, pelo menos em parte, por inibir o BRAF mutado e a via PI3K/Akt, assim como, por induzir as MAP kinases p38 e JNK. Tal como no caso do RA, também o composto fenólico maioritário presente no extracto HA, o ácido clorogénico, testado sózinho, não mostrou nenhum destes efeitos.

Tendo em conta os resultados anteriores, foi-se de seguida avaliar o potencial anticarcinogénico da quercetina (Q), luteolina (L) e ácido ursólico (UA). Estes compostos fenólicos (Q e L) e triterpenoide (UA) encontram-se presentes (ou aparecem como derivados) nos extractos aquosos anteriormente estudados, e têm a capacidade em

modular várias etapas do processo carcinogénico. No capítulo IV, observou-se o efeito antiproliferativo e pró-apoptótico da Q e L, que foi relacionado, pelo menos em parte, com efeitos no KRAS regulando as vias PI3K e MAPK/ERK. O efeito anticarcinogénico do UA foi observado ao nível do PI3K.

No capítulo V, o efeito combinado da Q, L e UA com 5-FU, agente quimioterapêutico mais usado no tratamento do CRC, foi avaliado. O aumento das resistências ao 5-FU, especialmente em tumores com mutações no p53, leva a estratégias de combinações com outras drogas necessárias para aumentar a eficácia do 5-FU. Na primeira parte deste estudo, a Q e a L aumentaram a apoptose induzida pelo 5-FU, e um efeito sinergético foi obtido para a Q nas células CO115 com p53 normal. A Q provavelmente aumentou a apoptose induzida pelo 5-FU através da modulação da via mitocondrial dependente do p53. Na segunda parte, observou-se que UA sinergeticamente aumentou a apoptose induzida pelo 5-FU em células HCT15 com p53 mutado. O aumento da apoptose não foi dependente das caspases e foi quase completamente inibido por um inibidor da JNK, sugerindo que nestas células o UA induz apoptose via activação da JNK. Além disso, o aumento da morte celular total e a acumulação do LC3, induzidos pelo UA, foi também dependente da activação da JNK.

Por fim, para explorar o efeito marcante da indução da morte celular pelo UA, na qual não foi totalmente explicado pelo aumento da apoptose, foi estudado o involvimento do UA no processo autofágico (capítulo VI). Interessantemente, verificou-se que o UA inibiu a autofagia nas células HCT15 ao nível da maturação, uma vez que não foi detectado fusão entre lisossomas e autofagossomas. Estes resultados foram confirmados tirando vantagem de técnicas estabelecidas para a linha MCF-7 de cancro da mama, como a construção fluorescente tandem ligado ao LC3 e a medição do turnover do LC3 usando o ensaio de luciferase em tempo real. Adicionalmente, observou-se que UA aumentou a permeabilidade da membrana lisossomal e diminuiu a actividade total das hidrolases lisossomais, indicando um possível impacto na biogénese lisossomal e/ou uma destabilização directa na membrana dos lisossomas.

Em conclusão, este trabalho adiciona SF, SO e HA, à lista de potenciais plantas a usar em estratégias alimentares contra o CRC, assim como a Q, L e UA presentes na dieta. Estes compostos naturais controlam a progressão do CRC modulando importantes alvos moleculares e aumentando a eficiência do 5-FU. O UA foi o composto mais promissor, destabilizando os lisossomas e, consequentemente, inibindo a autofagia, e por isso, possuindo elevado interesse como adjuvante na terapia do cancro.

Contents

Abbreviations list

ACF	Aberrant crypt foci
AIF	Apoptosis-inducing factor
Akt	Protein kinase B (PKB)
AMPK	AMP-activated kinase
AOM	Azoxymethane
Apaf-1	Apoptotic protease activating factor-1
APC	Adenomatous polyposis coli
Atg	Autophagy-related
BAD	Bcl-$_{xL}$/Bcl-2-associated death promoter
BAK	Bcl-2-antagonist/ killer-1
BAX	Bcl-2-associated X protein
Bcl-$_{xL}$	Basal cell lymphoma-extra large
Bcl-2	B-cell lymphoma protein-2
Beclin 1	Bec-1, the human ortholog of murine Atg6
BID	Bcl-2 interacting domain
BIM	Bcl-2 interacting mediator of cell death
BRAF	v-raf murine sarcoma viral oncogene homolog B1
DIABLO	Direct IAP-binding protein with low pI
DNA	Deoxyribonucleic acid
DRAM	Damage-regulated autophagy modulator
CA	Chlorogenic acid
CDK	Cyclin-dependent Kinase
COX	Cyclooxygenase
CRC	Colorectal cancer
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EndoG	Endonuclease G
ERK	Extracellular-signal-regulated kinase
FASL	FAS ligand
FAP	Familial adenomatous polyposis
FdUTP	Fluorodeoxyuridine triphosphate

FOXO	Forkhead box
FUTP	Fluorodine triphosphate
GSK-3	Glycogen synthase kinase-3
GTP	Guanine triphosphate
HA	*Hypericum androsaemum*
hMLH1	Human mutL homolog 1
hMSH2	Human mutS homolog 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HP	*Hypericum perforatum*
IAPs	Inhibitor of apoptosis proteins
IGF-R	Insulin-like growth factor receptor
JNK	c-Jun N-terminal kinase
KRAS	v-ki-ras 2 Kirsten rat sarcoma viral oncogene homolog
L	Luteolin
LAMP-2	Lysosomal-associated membrane proteins 2
LC3	Light chain 3
MAPK	Mitogen-activated protein kinase
MAP1LC3	Microtubule-associated protein 1 light chain 3
MMP	Metalloproteinases
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	High frequency microsatellite instability
MSI-L	Low frequency microsatellite instability
mTORC1	Mammalian target of rapamycin complex 1
MSS	Microsatellite stability
NF-κB	Nuclear factor kappa B
NOXA	“damage”
PARP	Poly-(ADP-ribose) polymerase
PDG-R	Platelet-derivated growth factor receptor
PDK-1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology
PIP2	Phosphoinositol 4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PI3K	Phosphotidylinositol-3 kinase

PKA	c-AMP-dependent protein Kinase A
PKC	Protein Kinase C
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PUMA	p53-upregulated modulator of apoptosis
Q	Quercetin
RA	Rosmarinic acid
RALGDS	Ral guanine nucleotide-dissociation stimulator
Rheb	Ras homolog enriched in brain
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
SF	*Salvia fruticosa*
SMAC	Second mitochondrial-derived activator of caspase
SMAD	Mothers against decapentaplegic homolog (Drosophila)
SO	*Salvia officinalis*
TGF-β-RII	Transforming growth factor-β receptor II
TNF-α	Tumor necrosis factor-α
TORC2	Target of rapamycin complex 2
TP53	Tumor protein p53
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TRAIL-R	Tumor necrosis factor-related apoptosis inducing ligand receptor
TS	Thymidylate synthase
TSC	Tuberous sclerosis complex
UA	Ursolic acid
ULK1	Unc-51-like kinase 1
UVRAG	UV radiation resistance-associated gene
VEGF	Vascular endothelial growth factor
WCRF	World Cancer Research Fund
WE	Water extract
WNT	Wingless-int
4E-BP1	Eukaryotic initiation factor 4-E-binding protein 1
5-FU	5-Fluorouracil

Short Curriculum Vitae

Cristina Xavier was born in Braga, Portugal on the 27th of October 1983. She presently lives in Braga, where she works as a researcher in the Molecular and Environmental Biology Centre, under the supervision of Prof. Cristina Pereira-Wilson.

Her background includes a four-year degree in Applied Biology, by the School of Sciences, University of Minho, Portugal from 2001-2005. She took her final project in IPO (Institute Portuguese of Oncology), Porto, and, in the end, she graduated with 17 values (17/20). She has just submitted her PhD thesis entitled "The anticarcinogenic potential of dietary natural compounds on colorectal carcinoma: effects on signaling pathways related to proliferation and cell death" to the University of Minho. Her work has collaboration with the Cancer Genetics group, Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal; and the Apoptosis laboratory, Institute of Cancer Biology, Danish Cancer Society, Denmark.

In 2006, Cristina Xavier joined Cristina Pereira-Wilson's group as a researcher where she began her PhD thesis financed by a FCT (Portuguese Foundation for Science and Technology) grant. The aim of her thesis was to identify potential natural compounds with anticarcinogenic effects in colorectal cancer cell lines and their possible involvement in signaling pathways related to proliferation and cell death. During this phase, she worked at the Department of Biology, Braga, Portugal, and for minor periods in IPATIMUP, Portugal, and in Apoptosis laboratory, Institute of Cancer Biology, Danish Cancer Society, Denmark.

Cristina Xavier was involved in the organization of one international workshop with an experimental session, "Exploring molecular approach to cancer research", held from the 1st till 4th of May 2007 in Braga, Portugal. Additionally, she has been taking part of two FCT projects, as a member of the team. She has also become a member of different associations: SPB, EACR and AACR.

As a result of her research work, she attended several international and national meetings and congresses in the present field of research. She was also invited to an oral

communication in the 2º Congress of Iberoamerican of Phytotherapy, held from 8th till 10th of October 2009 in Lisbon, Portugal. Moreover, she was awarded two grants to participate in FEBS/EACR advanced lecture course and FEBS/ESF workshop. Presently, she is the author of two papers published in international peer-reviewed journals, one paper submitted and three papers under preparation for submission in international peer-reviewed journals.

The work performed during this PhD thesis resulted in the following publications:

PAPERS IN REFEREED JOURNALS

Xavier CP, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C (**2009**). *Salvia fruticosa*, *Salvia officinalis* and rosmarinic acid induce apoptosis and inhibit proliferation of Human Colorectal cell lines: the role in MAPK/ERK pathway. ***Nutrition and Cancer***, 61 (4): 564–571.

Xavier CP, Lima CF, Preto A, Seruca R, Fernandes-Ferreira M, Pereira-Wilson C (**2009**). Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells. ***Cancer Letters***, 281:162–170.

PAPERS SUBMITTED

Xavier CP, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C (**2010**). *Hypericum androsaemum* water extract inhibits mutant BRAF with inhibition of human colorectal cancer cells proliferation; submitted to *Journal of Biomedicine and Biotechnology*.

PAPERS IN PREPARATION

Xavier CP, Lima CF, Rohde M, Pereira-Wilson C (**2010**). Quercetin and luteolin enhance apoptosis induced by 5-Fluorouracil in MSI colorectal cancer cells.

Xavier CP, Lima CF, Pereira-Wilson C (**2010**). Ursolic acid synergistically enhances apoptosis induced by 5-FU through JNK pathway and induces LC3 accumulation in colorectal cancer cells.

Xavier CP, Corcelle E, Rohde M, Farkas T, Pereira-Wilson C, Jäättelä M (**2010**). The natural triterpenoid ursolic acid inhibits autophagy and induces lysosomal membrane permeabilization in breast and colon cancer cells.

ABSTRACTS IN INTERNATIONAL AND NATIONAL JOURNALS

Xavier CPR, Lima CF, Rohde M, Pereira-Wilson C (**2010**). Autophagy triggered by ursolic acid synergistically enhances 5-fluorouracil induced cell death in HCT15 (MSI p53 mutant) colorectal cancer cells. ***European Journal of Cancer (EJC) Supplements***, 8 (5): 66-67.

Xavier CPR, Pereira I, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C (**2009**). Involvement of PI3K/Akt and MAPK/ERK signalling pathways in anti-tumoral activity of Hypericum androsaemum extract in colorectal carcinoma cells. ***Revista de Fitoterapia***, 9 (S1): 87.

Xavier CPR, Lima CF, Pereira-Wilson C (**2009**). Quercetin, Luteolin and Ursolic acid are potent inducers of apoptosis in Colorectal Carcinoma Cells: Interaction with 5-Fluorouracil [Abstract nº

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Xavier CPR, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C (**2008**) Induction of apoptosis and inhibition of proliferation in colon cancer cells by Salvia fruticosa, Salvia officinalis and rosmarinic acid. ***Planta Medica***; 74 (9): 942-943.

Xavier CPR, Lima CF, Preto A, Seruca R, Fernandes-Ferreira M, Pereira-Wilson C. (**2008**) Quercetin, luteolin and ursolic acid are potent inhibitors of proliferation in colorectal carcinoma cells: new therapeutic tools? ***European Journal of Cancer (EJC) Supplements***; 6(9): 140.

COMMUNICATIONS IN NATIONAL AND INTERNATIONAL MEETINGS

Xavier CPR, Lima CF, Fernandes-Ferreira M, Pereira-Wilson C. Natural products as source of potential chemotherapeutic agents for the control of colon cancer progression – the case of *Salvia* extracts and some isolated compounds. Workshop in Medicinal and Aromatic Plants, University of Minho, Portugal, 19-23 July, **2010, oral**.

C.P.R. Xavier, C. Lima, M. Rohde, C. Pereira-Wilson. Autophagy triggered by ursolic acid synergistically enhances 5-fluorouracil induced cell death in HCT15 (MSI p53 mutant) colorectal cancer cells. 21st Meeting of the European Association for Cancer Research (EACR), Oslo, Norway, June 26-29, **2010, poster**.

C.P.R. Xavier, A.A. Ramos, D. Pedro, C.F. Lima, M. Fernandes-Ferreira, C. Pereira-Wilson. Chemoprevention and antitumor effects of *Salvia officinalis* extract on colon cancer: involvement of the MAPK/ERK pathway. Joint Doctoral Programme in Plant Biology – 1st Annual Workshop on Bioplant, Porto, Portugal, March 29-30, **2010, poster**.

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C.P.R. Xavier, C.F. Lima, C. Pereira-Wilson. Quercetin, luteolin and ursolic acid inhibit proliferation and induce apoptosis in KRAS, BRAF and p53 mutated human colorectal cancer cells: interaction with 5-fluorouracil. FEBS/EACR advanced lecture course - Molecular Mechanisms in Signal Transduction and Cancer, Island of Spetses, Greece, August 16-24, **2009, poster**.

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Ramos A, Pedro D, Lima CF, **Xavier CPR**, Fernandes-Ferreira M, Pereira-Wilson C. Nutrition and Chemopreventive of colon cancer. "DNA Repair, Damage Signaling and Carcinogenesis" – First German-French Meeting, Faculty of Pharmacy, Toulouse, France, September 15-19, **2007, poster**.

C.P.R. Xavier, C.F. Lima, R. Seruca, C. Pereira-Wilson. Involvement of the PI3K/Akt pathway on the antiproliferative effects of Salvia extract in HCT15 colon cells. Beatson International Cancer Conference, Molecular Cancer Therapies: New Challenge and Horizons, Cancer Research UK, Glasgow, Scotland, June 17-20, **2007, poster**.

Xavier CPR, Lima CF, Pereira-Wilson C. Effects of *Salvia* sp. and individual phenolic compounds (Luteolin, Ursolic acid and Quercetin) on HCT15 human colon cell line. An international Workshop with an experimental session about "Exploring molecular approaches to cancer research", Department of Biology, University of Minho, Portugal, May 1-4, **2007, oral**.

Preface

Several studies have been developed at the Department of Biology, in the University of Minho, with aromatic and medicinal plants that grow spontaneously or are cultivated in Portugal. Aromatic plants have a wide applicability, for example, in cosmetic, food, beverage and pharmaceutical industries. These plants are known to be rich in a number of bioactive compounds, which have demonstrated to possess different therapeutical properties. This research project arose from the interest to identify medicinal plants, such as *Salvia* and *Hypericum* species, with anticarcinogenic effects in colorectal cancer (CRC). The overall positive results obtained during the studies performed with these plants led us to elucidate which bioactive compounds, present in these water extracts, were behind these effects and their molecular targets. Thus, these natural compounds, which are also present in human diet, were studied.

The possible mechanism of action of the extracts and natural compounds were focused on some signalling pathways related to proliferation and cell death. Several molecular markers of these pathways have also been shown, by the research group of cancer genetics in IPATIMUP, to be important targets in CRC therapy, and thus being suggested for this work. In addition, effects on lysosomes and/or regulation of the autophagic process have also been shown to be relevant molecular targets. In the Apoptosis Laboratory, Institute of Cancer Biology at the Danish Cancer Society, it was possible to identify and characterize the effects of one compound on these targets. All these studies were performed *in vitro*, taking advantage of several cancer cell lines harbouring different mutations. However, we are aware that studies *in vivo* are needed and crucial to validate our findings.

In summary, this work was organized in seven chapters, where the first is a general introduction, with a brief review of the field from the last 10 years. The following five chapters are the results obtained during the four years, which were published or are soon to be published in peer-reviewed journals. Finally, in the last chapter, a global discussion of the work is presented to highlight the main findings and some implications of the results in cancer research. Additionally, future research goals are mentioned.

CHAPTER I

GENERAL INTRODUCTION

Overview of the past 10 years

1. CANCER

In the past decades cancer has emerged as a major health issue being responsible for more than 10% of deaths worldwide. Cancer arises as a result of a stepwise process called carcinogenesis, that consists in the accumulation of genetic mutations and epigenetic changes that compromise the control of cell proliferation, survival, differentiation, migration and interaction with neighbor cells [Pelengaris and Khan, 2006]. This multistep process can be divided in three phases: initiation, promotion and progression. A mutational event in a single cell results in irreversible changes that confer an intrinsic capacity to proliferate uncontrollably to form an adenoma (initiation). A continuous division of initiated cells facilitates the acquisition of further mutations and originates a mass of abnormal cells that gives rise to the carcinoma (promotion). Cells will then acquire the ability to invade and metastasize during the progression phase [Frank, 2007].

The carcinogenesis model proposed by Fearon and Vogelstein in 1990 has colorectal cancer (CRC) as a good model for the study of morphology and genetic stages in cancer progression [Frank, 2007]. Using this model, they identified a mutation in the tumor suppressor *adenomatous polyposis coli* (*APC*) gene as the first event involved in premalignant lesion to initiate the formation of a malignant tumor. Disruption of the APC pathway may be sufficient to start a small adenoma, affecting cell division through disturbing of the Wnt signaling. Then, mutations in the oncogene *v-ki-ras 2 Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) and in the tumor suppressor gene *tumor protein p53* (*TP53*) appear later in tumor progression, leading to the formation of a carcinoma. However, other genetic changes in other key important genes, including in DNA mismatch repair (MMR) genes, which result in multiple errors in repetitive DNA sequences during DNA replication, can also arise during the carcinogenesis process contributing to cancer development [Frank, 2007; Souglakos, 2007].

Genetic alterations can occur as germline mutations, resulting in inherited cancer predisposition that appear in hereditary tumors, or more commonly occur in somatic cells (somatic mutations), where their accumulation in association with several environmental factors give rise to sporadic tumors [Pelengaris and Khan, 2006; Souglakos, 2007]. The presence of inherited mutations can start cancer initiation, but

other modifications such as somatic mutations and epigenetic alterations are still needed for cancer to develop. Numerous studies now point to the crucial interplay between these last changes and environmental factors as key determinants of tumor progression [Pelengaris and Khan, 2006].

1.1. Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the third most common type of cancer worldwide being mainly a disease of industrialized countries, such as North American, parts of Europe, Australia, New Zealand and Japan, remaining relatively uncommon in low-income countries, such as in Africa and much of Asia [WCRF/AICR, 2007]. Although the overall 5-year survival rate of CRC has increased during the past 2 decades from 51% to 65%, its incidence and mortality are continually increasing [Gralow *et al.*, 2008]. Approximately 1 in 3 people who develop CRC die of this disease and 90% of CRC cases appear in patients after age 50 [Souglakos, 2007].

CRC is divided in sporadic, inherited and familial. The majority of the patients, around 70% of the cases, have sporadic CRC (nonhereditary) in which there is no evidence of CRC in family history. In this case, the patients are usually older than 50 years of age, environmental factors being most likely responsible for this high incidence. The other remaining types of CRC have an associated genetic component with patients having an inherited predisposition to develop CRC [Souglakos, 2007]. The two well-described CRC genetic syndromes are subdivided according to whether or not colonic polyps are manifested: the familial adenomatous polyposis (FAP) and the hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, which accounts about 1% and 3-4% of all CRC, respectively [Rustgi, 2007]. The familial CRC is the least well understood syndrome, where up to 25% of the affected patients have a family history, but present low risk of developing CRC compared to the inherited type [Souglakos, 2007].

Carcinogenesis in the colon epithelium results from the accumulation of multiple genetic mutations where the mutation in the *APC* gene has an important role in the early process of CRC, in both inherited and sporadic tumors (**Fig. 1**). Mutations in *ß-catenin* gene, which, as *APC*, are involved in Wnt signaling, have also been demonstrated to play a key role in the first events of CRC, appearing in most sporadic CRC with normal

or wild-type (wt) *APC*. Subsequently, mutations in *TP53* and *KRAS* genes appear later during adenoma progression. Other genetic alterations in *Bcl-2-associated X protein (BAX)*, *SMAD4*, *transforming growth factor β receptor II (TGFβ-RII)*, *cyclooxygenase 2 (COX2)* and *phosphotase and tensin homologue (PTEN)* genes had also found to be frequently involved in CRC development [Soreide *et al.*, 2006; Souglakos, 2007]. Additionally, alterations in genes encoding enzymes involved in the DNA MMR system, which lead to microsatellite instability (MSI), have been demonstrated to contribute to the CRC progression, in both hereditary and sporadic CRC. In HNPCC cases, 90% of the tumors are MSI due to germline mutations in *hMSH2* and *hMLH1* mismatch repair genes, while in sporadic CRC approximately 15% of tumors have MSI due to alterations in *hMLH1* gene [Soreide *et al.*, 2006].

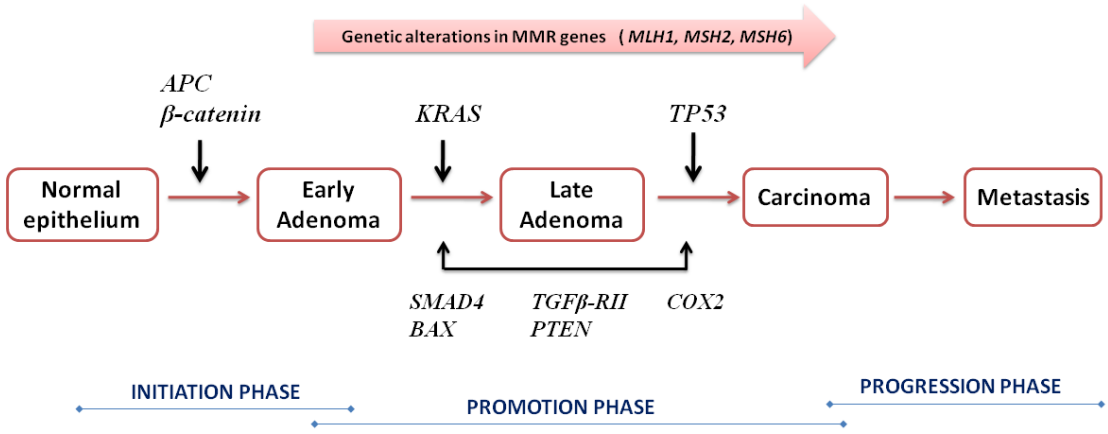

Fig. 1. Schematic representation of some key genetic alterations during colorectal cancer (CRC) carcinogenesis. Mutations in the *adenomatous polyposis coli* (*APC*) and *ß-catenin* genes occur in the initiation process of CRC carcinogenesis, while mutations in the *KRAS* and *TP53* genes appear during the adenoma progression. Mutations in *Bcl-2-associated X protein (BAX)*, *SMAD4*, *transforming growth factor β receptor II (TGFβ-RII)*, *cyclooxygenase 2 (COX2)* and *phosphotase and tensin homologue (PTEN)* genes have also found to be involved in CRC development. Genetic alterations in DNA mismatch repair genes (MMR), such as *MLH1*, *MSH2* and *MSH6*, also aride during the carcinogenesis process. Adapted from [Soreide *et al.*, 2006; Souglakos, 2007].

1.2. CRC and Nutrition

1.2.1. Importance of Diet

Genetic and environmental risk factors modulate CRC, diet being considered the most important environmental factor contributing to the high CRC incidence [Davis and Hord, 2005; Souglakos, 2007]. Many studies have reported a relationship between

nutritional factors and CRC, supporting the idea that dietary constituents may affect CRC by reducing or enhancing its risk [Ryan-Harshman and Aldoori, 2007]. Recently, an international review from the World Cancer Research Fund (WCRF) heightened the implication of food and nutrition in CRC, although there are some inconsistencies between different studies [WCRF/AICR, 2007].

The WCRF adverts that there are convincing evidences that red and processed meat are risk factors of CRC, while diets containing dietary fibre, garlic, milk and calcium most likely protect against this disease. Additionally, the report of the WCRF indicates that, although with limited evidence, fish and foods containing folate, vitamin D and selenium may also protect against CRC. The benefits of fruit and vegetables consumption have appeared controversial among different studies, but there seems to be a significant association between their consumption and a reduced risk of CRC incidence [WCRF/AICR, 2007].

More recently, different reports discussed the effects of diet in CRC. One study showed that high consumption of fruit and vegetables is associated with a reduced risk of CRC, although their results suggest that probably only specific types of fruit and vegetables, or their related nutrients, may confer CRC protection [van Duijnhoven *et al.*, 2009]. Other recent report demonstrated that an intake of fish was statistically associated with a decrease risk of having CRC [Hall *et al.*, 2008]. A diet poor on calcium and vitamin D consumption was also showed to induce CRC tumors on long-term feeding in mice, implying that their intake may protect against CRC development [Newmark *et al.*, 2009]. In aggrement with that, calcium intake was able to reduce CRC promotion induced by red meat in rats [Pierre *et al.*, 2008]. Besides the previous report of WCRF 2007, these examples shows that consumption of fruits, vegetables, fish, calcium and vitamin D could be included in the list of foods that convincingly reduce the risk of CRC.

Overall, an appropriate diet may minimize CRC risk, suggesting the possible benefit of preventive strategies based on dietary supplements and a potential therapeutic role for the compounds responsible for these effects.

1.2.2. Variability in Nutritional Responses

The variability of results concerning the effects of dietary constituents on CRC, and the variation in CRC incidence among and within populations with similar dietary patterns, suggests the involvement of individual responses to a dietary constituents [Davis and Milner, 2004; Davis and Hord, 2005]. In fact, it has been found that several food components are able to modify the expression of a number of genes, which suggest an interaction between nutrition/diet and the individual's genome (nutrigenomic), as well as, to influence protein expression (proteomic). However, for a bioactive food component to influence a key molecular event in a cell or organism, at a given moment (metabolomic), it is necessary to reach the target at an effective concentration and with an appropriate timing of exposure [Davis and Milner, 2004; Davis and Hord, 2005].

Several types of diets and food constituents have been found to alter the expression of certain genes and/or proteins, allowing the identification of genetic susceptibilities towards these particular dietary components. For example, Diergaarde *et al*, showed that red meat consumption could enhance the development of MSI-L and MSS carcinomas in particular, whereas fruit consumption can decrease this risk of MSI-H carcinomas that exhibit alterations in *hMLH1* [Diergaarde *et al.*, 2003]. In addition, the authors also demonstrated that consumption of fruits and dietary fiber might also decrease the risk of CRC in individuals with HNPCC [Diergaarde *et al.*, 2007]. A study performed with different types of vegetables pinpointed their capacity to modulate specific genes involved in the prevention of CRC, such as *CASP3* [van Breda *et al.*, 2005]. Another study demonstrated an increase of PKC isozyme expression in rats fed with red meat, suggesting a possible mechanism of red meat on CRC carcinogenesis [Pajari *et al.*, 2000]. Interestingly, the consumption of beef, pork and other meat products were associated with CRC tumors with wild-type *KRAS* gene, suggesting that they may exert their action through a pathway independent of a *KRAS* mutation [Brink *et al.*, 2005].

Thus, all these studies suggest that certain genes can be modulated and modified by specific dietary components, being essential to consider the genomic profile of each individual to determine who may or may not respond to CRC dietary interventions.

2. CANCER CELL SIGNALLING RELATED TO PROLIFERATION AND CELL DEATH

Carcinogenesis involves an accumulation of genetic alterations that in turn lead to alterations in important biological pathways of cell proliferation and cell death, such as phosphotidylinositol-3 kinase (PI3K)/protein kinase B (PKB/Akt), mitogen-activated protein kinases (MAPKs) [extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38] and apoptotic and non-apoptotic pathways.

2.1. RAS Signaling

The small GTP-binding protein Ras is a common upstream molecule of several signaling pathways, including PI3K/Akt, MAPK/ERK and RAlGDS (Ral guanine nucleotide-dissociation stimulator). Three Ras proteins, H-Ras, K-Ras and N-Ras, have been identified differing in the ability to activate different pathways. *KRAS* is the most frequently mutated *RAS* isoform in human cancers. Ras is activated by changes from the inactive Ras-GDP conformation to active Ras-GTP form through farnesylated or geranylgeranylated in cysteine residue. Ras is able to bind to the p110 catalytic subunit of class I PI3K, resulting in its translocation to the plasma membrane and consequent PI3K activation. Ras is also able to recruit the protein Raf to the plasma membrane and thus activate the MAPK/ERK pathway (**Fig. 2**) [McCubrey *et al.*, 2006; Schubbert *et al.*, 2007].

The upregulation of PI3K/Akt and Raf/MAP/ERK pathways by the oncogene *RAS* cooperates to regulate various cellular responses and generate resistance to therapy [Barault *et al.*, 2008]. Moreover, it was found that Ras could downregulate PTEN expression through the Raf/MAPK/ERK pathway promoting activation of the PI3K/Akt pathway, which strengthens the connection between these two pathways [Vasudevan *et al.*, 2007].

2.1.1. Mutations on *KRAS* in CRC

It is known that about 30% of sporadic CRC tumors have mutations in *KRAS* [Oliveira *et al.*, 2007; Barault *et al.*, 2008]. In addition, CRC patients that display *KRAS* mutation alone or in combination with *BRAF* mutation (~0.4%) have bad prognosis and

poor survival [Oliveira *et al.*, 2007]. It has been demonstrated that *KRAS* mutations have effects at tumor initiation, on cell growth and progression, as well as, in promoting invasion, angiogenesis and resistance to CRC therapy [Jiang *et al.*, 2009].

2.2. PI3K/Akt Pathway

2.2.1. PI3K

The phosphotidylinositol-3 kinases (PI3Ks) are a family of lipid kinases that are classified according to sequence homology and subtract specificity into class I, class II and class III. Class I PI3K is the major class and is involved in cell growth, proliferation and apoptosis [Zhao and Vogt, 2008]. Class I PI3K, a heterodimeric protein, is composed by a p110 catalytic subunit and a p85 regulatory subunit, both of which expressed in various isoforms. This class of PI3K can be activated by receptor tyrosine kinases (RTK), such as PDG-R, EGF-R or IGF-R through interaction with phosphotyrosine residues, and by G protein-coupled receptors, such as RAS. The activation of PI3K leads to the conversion of the lipid substrate phosphoinositol 4,5-bisphosphate (PIP2) to the second messenger, the phosphatidylinositol-3,4,5-triphosphate (PIP3) at the inner side of the plasma membrane (**Fig. 2**). PIP3 binds to target proteins that contain pleckstrin homology (PH) domains, such as PKA, PKC and Akt/PKB, the latter being the primary downstream mediator of PI3K effects [Vara *et al.*, 2004; Michl and Downward, 2005; Zhao and Vogt, 2008].

2.2.2. Akt

Akt is the human homologue of the viral oncogene v-Akt. Three members of the Akt family (Akt1, Akt2 and Akt3) have been identified and are ubiquitously expressed with their levels variable depending upon the tissue type [Vara *et al.*, 2004; Blanco-Aparicio *et al.*, 2007]. The PH domain of Akt binds to PIP3 inducing conformational changes in the molecule, which make a threonine residue (T308) accessible for phosphorylation by the activated protein serine/treonine kinase 3´-phosphoinositide-dependent kinase-1 (PDK-1). Full activation of Akt is also associated with phosphorylation of a serine 473 (S473), located in the C-terminal tail of Akt, by the target of rapamycin complex 2 (TORC2). Phosphorylations of T308 and S473 residues are essential for a complete activation of Akt function [Vara *et al.*, 2004; Michl and

Downward, 2005; Blanco-Aparicio *et al.*, 2007; Manning and Cantley 2007]. Subsequently, Akt phosphorylates a number of subtracts that play a central role in cell proliferation and cancer progression (**Fig. 2**).

Akt can promote cell growth and affect the cell metabolism by indirectly activating the mammalian target of rapamycin complex 1 (mTORC1) and inhibiting the glycogen synthase kinase-3 (GSK-3). In addition, Akt is also able to phosphorylate the cyclin-dependent kinase inhibitors p27 and p21 affecting cell cycle progression. Akt can also block the function of proapoptotic proteins by directly phosphorylating and inhibiting BAD, procaspase-9 and the transcription factor Forkhead box (FOXO), as well as, by phosphorylating MDM2 and promoting its translocation to the nucleus where it negatively regulates p53 expression [Vara *et al.*, 2004; Manning and Cantley, 2007].

2.2.3. PTEN

The activation of Akt has also been partially related to the loss of *PTEN* (phosphatase and tensin homologue deleted on chromosome 10), a tumor suppressor gene that functions as an intracellular inhibitor of the PI3K pathway. PTEN dephosphorylates PIP3 to generate PIP2, acting as a negative regulator for PI3K-induced signaling (**Fig. 2)** [Vara *et al.*, 2004; Blanco-Aparicio *et al.*, 2007]. In addition, it was also reported that PTEN mediates effects, independently of Akt, on cell cycle and apoptosis [Blanco-Aparicio *et al.*, 2007].

2.2.4. PI3K/Akt Deregulation in CRC

Nearly 40% of CRC tumors have been demonstrated to have alterations in one of the genes related with PI3K signaling [Parsons *et al.*, 2005]. Approximately 30% of CRC tumors have showed somatic mutations in *PIK3CA* gene that encodes the p110α subunit of class I PI3K [Samuels and Velculescu, 2004; Michl and Downward, 2005; Barault *et al.*, 2008]. This mutation has been demonstrated to occur as a primary genetic event in CRC, having a relevant importance in the carcinogenic process [Velho *et al.*, 2008].

CRC cell lines with *PIK3CA* mutation have shown the capacity to proliferate and survive under environmental stresses [Wang *et al.*, 2007], as well as, to be more resistant to apoptosis and to have high metastatic potential [Guo *et al.*, 2007]. Moreover,

mutation in *PIK3CA* gene have been shown to occur more frequently in combination with other mutations in other components of the PI3K pathway, such as *KRAS* or *BRAF*, than in isolation, suggesting a possible synergistic effect in the signaling pathways controlled by them for an efficient malignant transformation [Velho *et al.*, 2005; Oda *et al.*, 2008]. More recently, a mutation in *PIK3R1* gene, which encodes the p85α regulatory subunit, was found, which turns it to a novel oncogene with important effect on CRC development [Li *et al.*, 2008].

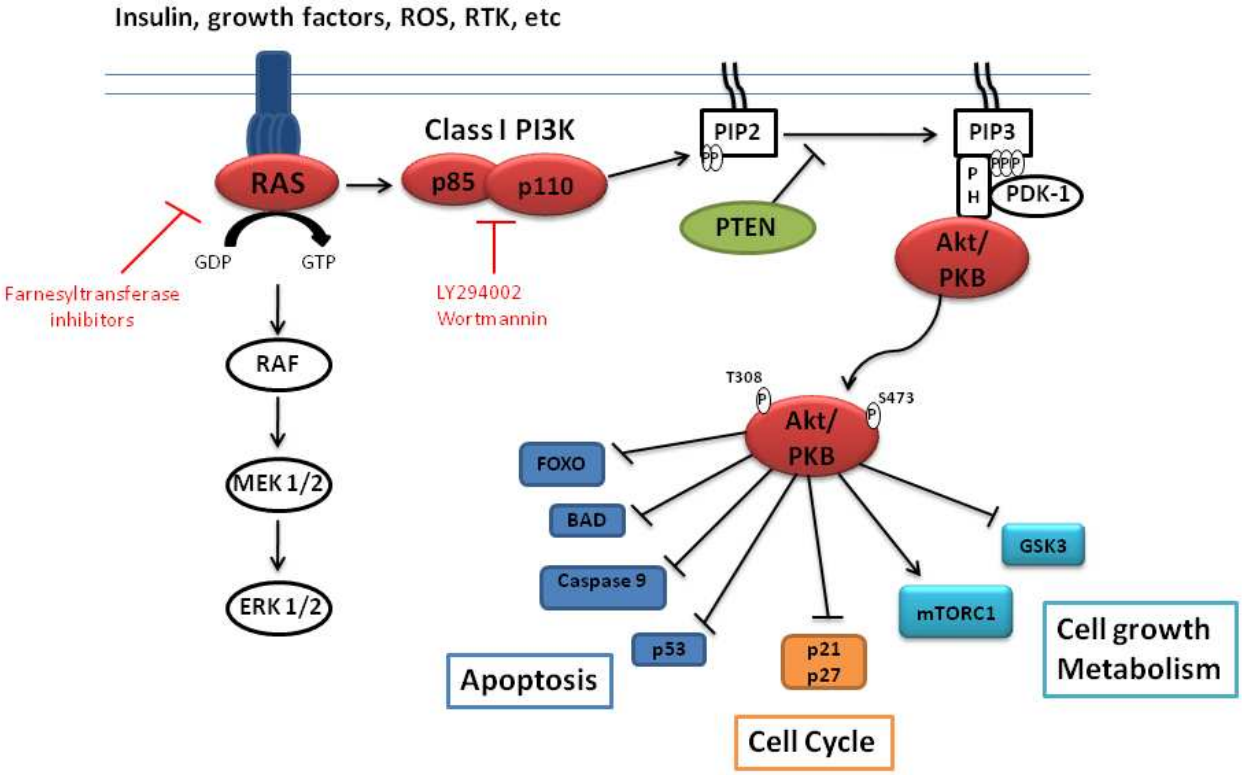

Fig. 2. Schematic representation of PI3K/Akt and MAPK/ERK pathways. The G protein-coupled receptor RAS activated by, for example, growth factors and receptor tyrosine kinases (RTK), changes from the inactive RAS-GDP to active GTP, being able to activate the class I phosphotidylinositol-3 kinases (PI3K) and the protein RAF. RAF phosphorylates MEK1/2 and consequently activates ERK1/2 pathway. The Class I PI3K, composed by the p110 catalytic and p85 regulatory subunits, leads to the conversion of the phosphoinositol 4,5-biphosphate (PIP2) to the phosphatidylinositol-3,4,5-triphosphate (PIP3), which could be reverted by the negative regulator *PTEN* (phosphatase and tensin homologue deleted on chromosome 10). The pleckstrin homology (PH) domain of Akt binds to PIP3 being activated by phosphorylations on T308 and S473 residued, and its phosphorylation by the protein serine/treonine kinase 3´-phosphoinositide-dependent kinase-1 (PDK-1) lead to the Akt activation. Then, Akt phosphorylates a number os substratcts, such as Bad, Forkhead box (FOXO), caspase-9, p53, p21, p27, mammalian target of rapamycin complex 1 (mTORC1) and glycogen synthase kinase-3 (GSK-3), which are involved on various cellular functions, apoptosis, cell cycle regulation, cell growth and metabolism. The protein RAS could be inhibited by farnesyltransferase inhibitors and the PI3K pathway could be surpressed by several drugs such as, LY294002 and wortmannin. Adapted from [Manning and Cantley, 2007; Zhao and Vogt, 2008].

Mutations in the *AKT* gene are not common in humans, however, some studies have reported an up-regulation of Akt in about 50% of CRC carcinomas, demonstrating its essential role in CRC progression [Itoh *et al.*, 2002; Roy *et al.*, 2002; Khaleghpour *et al.*, 2004]. More recently, Carpten *et al.* found an *AKT 1* mutation in CRC in the PH domain of Akt [Carpten *et al.*, 2007]. This mutation results in an Akt conformational change and its increased membrane association, which lead to a constitutive activation of Akt. Alterations in PTEN tumor suppressor function by loss or reduction of protein expression has also been demonstrated to be frequent in sporadic CRC tumors [Goel *et al.*, 2004; Nassif *et al.*, 2004]. A deficient PTEN expression was associated with a predictor of local recurrence in CRC patients [Colakoglu *et al.*, 2008].

Overall, alterations in components of the PI3K pathway have been demonstrated to occur with high frequency in CRC tumors contributing for its progression and consequently with impact on therapy resistance. Thus, this pathway became an attractive target for the development of novel anticancer agents to treat CRC.

2.3. MAP Kinase Pathways

The mitogen-activated protein kinases (MAPKs) are a large family of serine-threonine kinases that are often altered in human cancer [McCubrey *et al.*, 2006]. There are three major subfamilies of MAPK related to cancer: the extracellular-signal-regulated kinase (ERK)1/2, the c-Jun N-terminal kinase (JNK)1/2/3 and the p38 MAP kinase (p38) α/β/γ/δ.

The MAPK activity is regulated through a cascade of activations, in which MAPKs are phosphorylated by the MAPK kinase (MAPKK, MKK or MEK), which themselves are phosphorylated by MAPKK kinase (MAPKKK or MEKK). These MAPKKK are each capable to regulate multiple MAPKKs that lead to the activation of different families of MAPK, resulting in the activation of ERK1/2, p38 or JNK [Cuevas *et al.*, 2007]. These pathways are regulated by diverse extracellular and intracellular stimuli, including growth factors, cytokines, hormones and various cellular stresses. In turn, MAPK proteins phosphorylate a number of substrates involved in several cellular events, such as cell proliferation, survival, apoptosis, cell migration, differentiation and inflammatory response [McCubrey *et al.*, 2006; Cuevas *et al.*, 2007].

2.3.1. RAF/ERK Pathway

MAPK/RAF/ERK is an important pathway involved in cell proliferation, apoptosis and cell cycle progression being activated primarily by mitogenic stimuli, such as growth factors [McCubrey *et al.*, 2006].

The mammalian Raf protein is a serine/threonine kinase present in three forms, A-Raf, B-Raf and C-Raf. These proteins recruited to the membrane bind to Ras and are subjected to phosphorylations/dephosphorylations on different domains and to dissociation with the Raf kinase inhibitory protein (RKIP) promoting Raf activation [McCubrey *et al.*, 2006]. When activated, the protein Raf, a MAPKKK, can phosphorylate two MAPKK proteins, MEK1 and MEK2, which consequently activates the MAP kinases ERK1 and ERK2 (**Fig. 3**). [McCubrey *et al.*, 2006]. Subsequently, ERK phosphorylates important cytoplasmic and nuclear targets [Cuevas *et al.*, 2007].

In the cytosol, ERK can activate NF-κB by phosphorylating and activating the inhibitor κB kinase, as well as, phosphorylate caspase 9 on residue Thr125 leading to its inactivation. In the nucleus, ERK1/2 promotes the phosphorylation of many transcription factors, including c-Jun, c-Myc and c-Ets-1 that are required for activator protein 1 (AP-1) expression [McCubrey *et al.*, 2006].

2.3.2. JNK and p38 Pathways

The JNK and p38 MAP Kinases, also called stress-activated protein kinases (SAPK), are activated by environmental and genotoxic stresses and they regulate apoptosis, survival and inflammation. The effects of these kinases, that have been shown to be cell type-specific and dependent on intensity and duration of the stimuli, function either as tumor suppressors or oncoproteins [Wagner and Nebreda, 2009].

The JNK protein kinase is encoded by three genes, *MAPK8*, *MAPK9* and *MAPK10*, which encodes JNK1, JNK2 and JNK3, respectively, and they are alternatively spliced to create at least 10 isoforms. The proteins JNK1 and JNK2 are believed to be expressed in almost every cell and tissue type, whereas the JNK3 protein is mainly found in neuronal tissues and in the heart. JNK are mainly activated by the upstream MAPKK proteins, MEK4 and/or MEK7, that in turn activates a number of proteins (**Fig. 3**) [Weston and Davis, 2007; Wagner and Nebreda, 2009].

The three JNKs have been shown to be involved in stimulating apoptotic signaling through different mechanisms. JNK can directly modulate the activities of

mitochondrial pro- and antiapoptotic proteins through phosphorylation of several members of the Bcl-2 family, such as Bcl-2, BAD, BIM, as well as, stimulating the release of cytocrome c [Dhanasekaran and Reddy, 2008]. Activated JNK cal also translocate to the nucleus where it activates a number of transcription factors, such as c-Jun, AP-1 and p53, increasing the expression of pro-apoptotic genes [Dhanasekaran and Reddy, 2008]. JNK have also been shown to be involved in inflammation process since it can be activated by TNF-α, a proinflammatory cytokine [Wagner and Nebreda, 2009].

The p38 MAP Kinases are encoded by four genes, *MAPK14*, *MAPK11*, *MAPK12* and *MAPK13*, which encode p38α, p38β, p38γ, p38δ, respectively, and are mainly activated by MEK3, MEK6 and in some cases by MEK4 (**Fig. 3**). The p38α is highly abundant in most of cell types being the best known p38 MAPK, whereas p38β seems to be expressed at very low levels and p38γ and p38δ expressions have more restricted pattern of expression [Wagner and Nebreda, 2009].

It has been shown that p38 MAPK activates a wide range of subtracts, such as transcription factors, protein kinases, cytosolic and nuclear proteins [Coulthard *et al.*, 2009]. The p38 plays an important role in inflammation by modulating the transcription factor NF-κB and regulating the induction of the pro-inflammatory mediator cyclooxygenase 2 (COX-2) and the production of many cytokines, such as TNFα and interleukins [Wagner and Nebreda, 2009]. In addition, p38 is also envolved in the negative regulation of cell cycle progression, as well as, in the induction of apoptosis and modulation of cell migration and differentiation [Coulthard *et al.*, 2009; Wagner and Nebreda, 2009].

Since JNK and p38 MAPK pathways can be activated by the same stimulus and share several upstream regulators, they may cooperate to synergistically activate the same subtracts. Nevertheless, there are evidences indicating that the two stress-activated pathways could also have opposite effects or that p38 negatively regulate JNK activity in several contexts [Wagner and Nebreda, 2009].

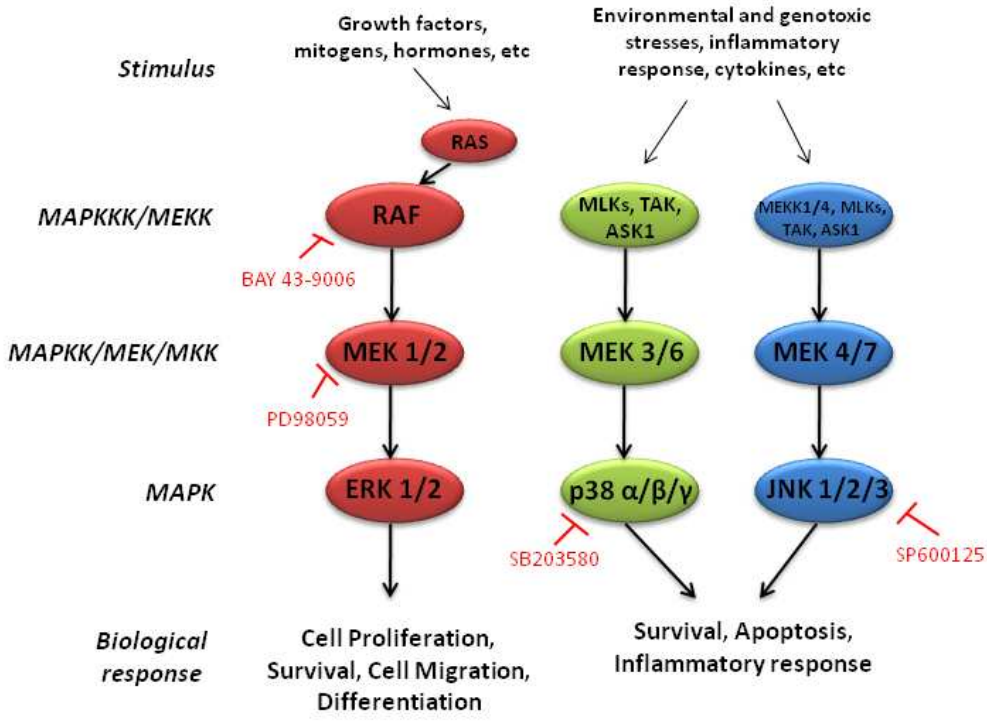

Fig. 3. Schematic representation of MAPK signaling pathways (ERK, p38 and JNK). RAS activates RAF that in turns phosphorylates MEK1/2 and consequently activates ERK1/2 pathway. ERK pathway is involved in various biological responses, such as cell proliferation, survival, cell migration and differentiations and it could be inhibited by the grugs BAY 43-9006 and PD98059. The JNK and p38 pathways are stimulated by various stresses that lead to effects on survival, apoptosis and inflammatory response. The p38 isoforms are mainly activated by MEK3 and MEK6 and could be inhibeted by SB203580, while the JNK 1/2/3 are mainly activated by MEK4 and MEK7 and could be inhibited by SP600125. Adapted from [Malemud, 2007].

2.3.3. Activation of MAP Kinases in CRC

Activation of MAPK/ERK pathway has been demonstrated to be involved in CRC progression. Mutations upstream of this pathway are frequent in CRC, including mutations in *KRAS* and *BRAF* genes, participating in the upregulation of ERK signaling [Fang and Richardson, 2005]. About 5-10% of sporadic CRC tumors have mutations in *BRAF* [Oliveira *et al.*, 2007; Barault *et al.*, 2008] and these mutation have been correlated with cell growth and inhibition of apoptosis [Ikehara *et al.*, 2005]. Moreover, MEK has also shown to be frequently phosphorylated in CRC tumors, which constitutivly activates the MAPK/ERK pathway [Lee *et al.*, 2004].

Mutations in JNK and p38 are not frequent in CRC, instead, alterations in the expression of these proteins and/or mutations in components of JNK and p38 pathways have been demonstrated. Loss-of-function mutation in the *MKK4* gene, which activates both JNKs and p38α, were found in ~5% of CRC tumors [Wagner and Nebreda, 2009].

A recent study, however, found a significant downregulation of MAPK activity in some CRC samples, suggesting that CRC tumors must be carefully evaluated for MAP kinases inhibitor therapy [Gulmann *et al.*, 2009].

The importance of SAPK in CRC has been demonstrated in several studies. It was shown that mutated KRAS activates p38γ, by inducing its expression, this being essential to maintain the Ras mutated phenotype and promoting CRC carcinogenesis [Tang *et al.*, 2005]. Moreover, a cross talk between JNK and Wnt signaling was also found where an activation of JNK increases the expression of Wnt target genes, contributing to accelerate CRC tumorogenesis [Sancho *et al.*, 2009].

2.4. Apoptosis

Apoptosis is a mechanisms of cell death in which several molecules are activated or inactivated working as a team for a controlled cellular self-destruction called programmed cell death. Apoptosis is essential to maintain homeostasis by removing unwanted, injured and infected cells and its dysfunction or deregulation is implicated in cancer progression [Degterev and Yuan, 2008]. During apoptosis, typical cellular morphologic changes occur, including nuclear chromatin condensation and fragmentation, membrane blebbing, cell shrinkage, loss of contact to its neighboring cells and the formation of apoptotic bodies [de Bruin and Medema, 2008; Degterev and Yuan, 2008].

On the other hand, necrosis, another form of cell death, differently from apoptosis, is an uncontrolled form of death due to a violent environmental perturbation. In this case, an early disruption of the cell membrane occurs accompanied by organelle swelling, mitochondrial dysfunction and a collapse of cell structure, which result in the release of the cellular contents to the milieu causing damage to surrounding cells and a strong inflammatory response [de Bruin and Medema, 2008; Degterev and Yuan, 2008].

2.4.1. Classical Apoptotic Pathways

The classical apoptotic pathways can be initiated via different stimuli, at the plasma membrane by death receptors (extrinsic pathway) or at the mitochondria (intrinsic pathway), and involve the activation of proteolytic enzymes called cysteine aspartic acid specific proteases (caspases) [Degterev and Yuan, 2008].

Caspases are synthesized as inactive proenzymes, being activated by autoproteolytic cleavage or being cleaved by other caspases at specific aspartic acid residues. They are subdivided, depending on their activity and subcellular localization, into proinflammatory and proapoptotic caspases. The latter are grouped as initiator (caspases 8, 9 and 10) or activator (caspases 3, 6 and 7) caspases. At the end, caspases modulate the activity of a variety of substrates, including the poly-(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in initiating DNA repair, that losses its repair activity when cleaved by caspases [Degterev and Yuan, 2008].

2.4.1.1. The Mitochondrial (intrinsic) Pathway

The intrinsic pathway of apoptosis is activated by a variety of stress stimuli, including DNA damage, heat and ultraviolet radiation through p53 tumor suppressor protein. This activation leads to the insertion of the pro-apoptotic B-cell lymphoma protein-2 (Bcl-2) family proteins Bax and Bak into the outer mitochondrial membrane (OMM) causing mitochondria damage and the release of cytochrome c to the cytosol. The activity of Bax and Bak is modulated by the anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-Xl and Mcl-1 that prevent cytochrome c release, whereas the pro-apoptotic BH3 members of the Bcl-2 family, such as Bad, Bik, Bim, Bid, Puma and Noxa restored Bax and Bak activation. It is the ratio between anti-apoptotic and pro-apoptotic members of Bcl-2 family that tightly regulate the permeability of the OMM and the release of cytochrome c. Subsequently, cytochrome c interacts with the apoptotic protease activating factor-1 (Apaf-1) and recruits the initiator pro-caspase-9, in the presence of ATP, to promote the formation of the apoptosome. This interaction leads to the cleavage and activation of caspase 9 that mediates the activation of the effector caspases 3, 6 and 7 to execute apoptosis (**Fig. 4**) [Ghavami *et al.*, 2009; Pradelli *et al.*, 2010].

In addition to cytochrome c, mitochondria can also release a large number of other pro-apoptotic molecules, including second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI (Smac/Diablo), Omi/high temperature requirement protein A (HtrA2), endonuclease G (EndoG) and apoptosis-inducing factor (AIF) (**Fig. 4**). Smac/Diablo and Omi/HtrA2 promote caspase activation through inhibition of the inhibitor of apoptosis proteins (IAPs), while AIF and EndoG are able to cause DNA damage without caspase involvement [Ghavami *et al.*, 2009; Pradelli *et al.*, 2010].

The nuclear factor-κB (NF-κB) is a transcription factor that has been implicated in apoptosis by promoting and regulating the transcription of important anti-apoptotic genes such as the Bcl-2 family member Bcl-Xl [Naugler and Karin, 2008].

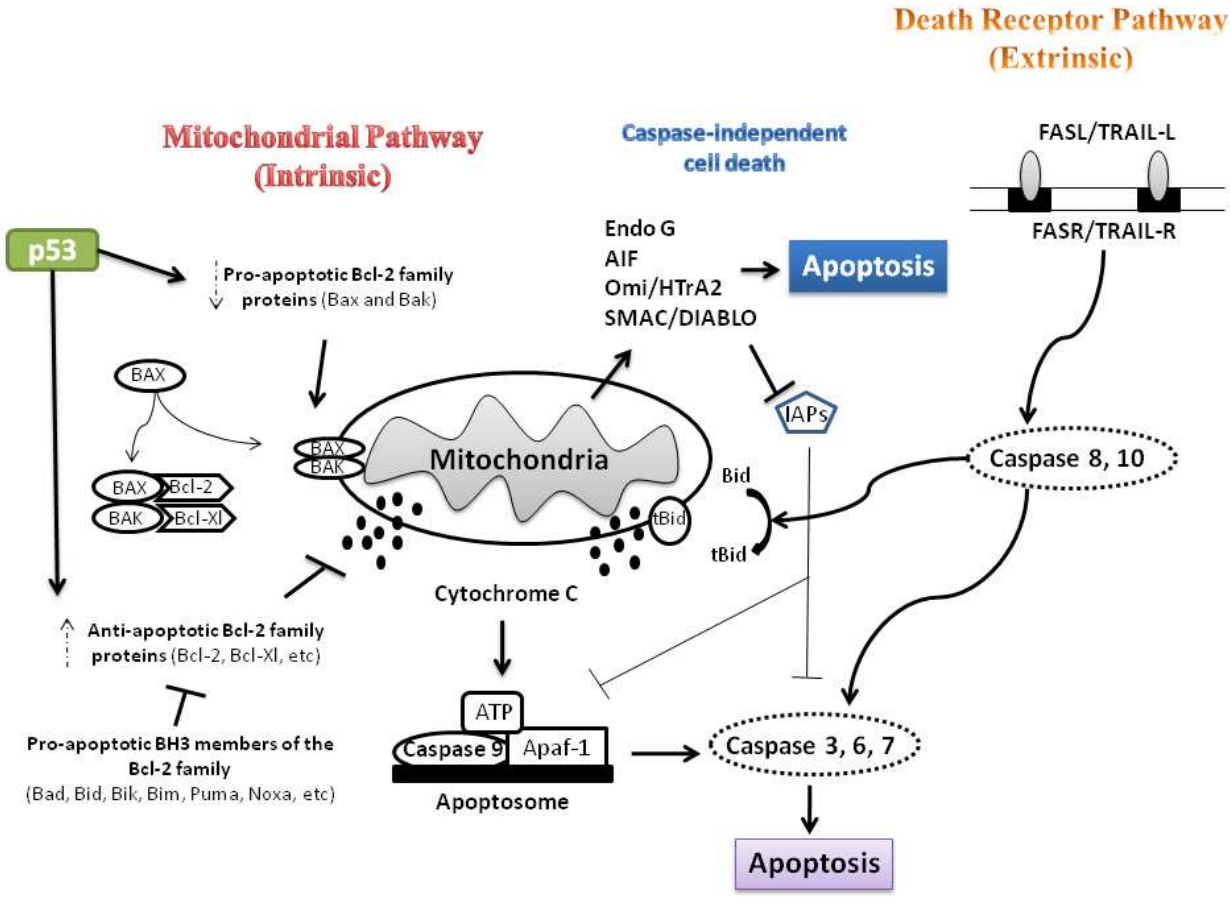

Fig. 4. The molecular mechanisms of apoptosis. Apoptosis pathways can be initiated at the plasma membrane by death receptors (extrinsic pathway) or at the mitochondria (intrinsic pathway). Stimulation of death receptors, such as FASR or TRAIL-R, results in the formation of death inducing signaling complex and the recruitment of the initiators caspases 8 or 10. Caspase 8 cleaves and activates the effector caspases 3, 6 and 7 to induce apoptosis, as well as, cleaves the pro-apoptotic protein Bid, generating the truncated form (tBid) that cause mitochondria damage and release of cytochrome c. The mitochondrial membrane permeabilisation is regulated by the pro-apoptotic B-cell lymphoma protein-2 (Bcl-2) family proteins Bax and Bak and the pro-apoptotic BH3 members of the Bcl-2 family, such as Bad, Bik, Bim, Bid, Puma and Noxa, which cause the release of cytochrome c, and the anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-Xl and Mcl-1 that prevent this occurrence. The protein p53 can interact with members of the Bcl-2 family to induce the release of cytochrome c. Following, in the cytosol, cytochrome c interacts with the apoptotic protease activating factor-1 (Apaf-1) and recruits the initiator caspase-9, in the presence of ATP, to promote the formation of the apoptosome, that in turns activate the effectors caspases 3, 6 and 7 to execute apoptosis. The transmembrane channels across the mitochondrial outer membrane can also release other proteins, such as second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI (Smac/DIABLO), Omi/high temperature requirement protein A (HtrA2), endonuclease G (EndoG) and apoptosis-inducing factor (AIF) that translocate to the nucleus to induce apoptosis without caspase involvement, or they could also activate caspase pathway by inhibiting the inhibitor of apoptosis proteins (IAPs). Adapted from [Ghavami *et al.*, 2009; Qiao and Wong, 2009].

2.4.1.2. The Death Receptor (extrinsic) Pathway

In the extrinsic pathway, apoptosis is mediated by the activation of the transmembrane death receptors located in the cell membrane and they are activated by extracellular ligands. The binding of TNF family members, such as Fas-L or TRAIL-L, to their receptors causes the formation of death inducing signaling complexes and the recruitment, cleavage and activation of the initiator caspases 8 or 10. These caspases, in turn activate a cascade of other caspases that leads to the activation of the effector caspases 3, 6 and 7 to execute cell death. Thus, both classical apoptotic pathways (intrinsic and extrinsic pathway) converge in the activation of the same activator caspases. In addition, these apoptotic pathways could also crosstalk at the level of the mitochondria, since caspase 8 can also cleave and activate the pro-apoptotic protein Bid, generating the active or truncated form (tBid) that in turn cause mitochondria damage and release of cytochrome c (**Fig. 4**) [Ghavami *et al.*, 2009; Pradelli *et al.*, 2010].

2.4.2. Others Relevant Pathways to Apoptosis

2.4.2.1. Lysosomal Cell Death Pathway

Lysosomes are the main compartment for intracellular degradation and subsequent recycling of cellular constituents. They have a number of lysosomal hydrolases, such as cathepsin proteases, capable of digesting all major cellular macromolecules, being implicated in the control of cell death at several levels [Boya and Kroemer, 2008; Kirkegaard and Jaattela, 2009]. Lysosomal cell death pathway can activate apoptotic effectors, such as mitochondria and/or caspases, triggering cells to death by classical apoptosis. An induction of lysosomal membrane permeabilization (LMP) causes the release of cathepsins to the cytosol, which can activate pro-apoptotic Bcl-2 proteins, such as Bid, Bax and Bak, leading to mitochondrial membrane permeabilization and the release of cytochrome c, and subsequently, to the induction of apoptosis [Boya and Kroemer, 2008; Kirkegaard and Jaattela, 2009]. However, a massive induction of LMP results in an uncontrolled death by necrosis [Boya and Kroemer, 2008]. In addition, lysosomes are also able to break down the extracellular matrix, stimulating angiogenesis and migration, downregulate signaling from receptor tyrosine kinases, as well as, participate in the final step of the autophagic process [Kirkegaard and Jaattela, 2009].

A wide range of apoptotic stimuli can induce the LMP [Boya and Kroemer, 2008; Kirkegaard and Jaattela, 2009]. The TNF death receptor family in the presence of the death domain-containing receptor interacting protein-1 (RIP-1) can trigger LMP and cell death. There are also evidences that p53 can induce LMP upstream of mitochondrial pathway. Oxidative stress has also been shown to induce LMP through generation of reactive oxygen species (ROS), which cause oxidation of lysosomal membrane lipids, resulting in the destabilization of the lysosomal membrane and leading to the release of lysosomal contents. Moreover, PI3K pathway has also been reported to function as an LMP inhibitor by regulating the lysossomal compartment through control of maturation, size, activity and stabilization of lysosomes [Boya and Kroemer, 2008; Kirkegaard and Jaattela, 2009].

The induction of LMP has recently emerged as an effective way to kill cells, especially cancer cells resistant to apoptosis, and a number of compounds that induce lysosomal cell death are being discovered [Groth-Pedersen and Jaattela, 2010].

2.4.2.2. PI3K and MAPK Pathways

PI3K and MAPK pathways may also be implicated in the induction of apoptosis, since they are able to regulate the Bcl-2 family proteins and the protein p53. The protein Akt is able to phosphorylate BAX by inhibiting its conformational changes, which avoid its translocation to the mitochondria and cytochrome c release. Akt also phosphorylates Mdm2 by enhancing its nuclear localization that, in consequence, negatively regulates p53 expression [Manning and Cantley, 2007; Stiles, 2009].

It has also been demonstrated that ERK1/2 is able to phosphorylate pro-apoptotic Bcl-2 proteins, such as Bad and Bim, promoting their degradation and, thus, preventing cytochrome release [Balmanno and Cook, 2009]. In addition, JNK and p38 kinases were also shown to regulate the expression of Bcl-2 proteins, such as Bax and BAD, modulating the membrane mitochondrial permeabilization [Wagner and Nebreda, 2009].

2.4.2.3. Role of p53 in Apoptosis

The tumor suppressor p53 mediates critical functions within cells, including inhibition of proliferation by induction of cell cycle arrest and induction of apoptotic cell death [Chari *et al.*, 2009]. The p53 protein has a very short life and it is usually present at low levels within normal cells, although it can be transiently stabilized and

activated in response to several stresses, including hypoxia, heat shock and DNA damage agents. The stability of p53 is regulated by the E3 ubiquitin ligase and by the protein MDM2 that mediate the ubiquitination of p53 and allow its degradation by the proteosome, maintaining low the levels of p53. Moreover, MDM2, which can ubiquitinate itself and regulate its own stability, is a transcription target of p53 in a mechanism where p53 controls the expression of its own regulator [Chari *et al.*, 2009].

Upon p53 activation, this protein translocates to the nucleus where it binds to specific DNA sequence elements within the regulatory regions of target gene promoters regulating the transcription of several genes, such as *Apaf-1*, *BAX*, *PUMA*, *NOXA*, *cathepsin D*, *TNF* family members and some *caspases* [Chari *et al.*, 2009]. In addition to its transcriptional regulator activity, p53 can also directly activate components of the apoptotic machinery through interaction with the members of the anti-apoptotic and pro-apoptotic Bcl-2 family proteins to induce the mitochondrial pathway [Vaseva and Moll, 2009] .

2.4.3. Deregulation of Apoptosis in CRC

Deregulation of apoptosis has been linked to cancer development and resistance to CRC treatment [Qiao and Wong, 2009]. In CRC, alterations in caspase activation, the central modulators of apoptosis, have been identified, including mutations in *caspase 3*, *caspase 7* and *caspase 8* [Hector and Prehn, 2009], as well as, mutations in *caspase 4* and *caspase 5* [Ghavami *et al.*, 2009]. Alterations in the Bcl-2 family members, such as high expressions of Bcl-2 and Bcl-Xl anti-apoptotic proteins and decreased expressions of the pro-apoptotic proteins Bax and Bak have also been demonstrated in CRC [Hector and Prehn, 2009]. In fact, overexpression of Bcl-2 protein was related with negative prognostic factor in CRC [Qiao and Wong, 2009].

The death receptors Fas and TRAIL have been shown to be constitutively expressed in CRC [Qiao and Wong, 2009]. Moreover, mutations in the tumor suppressor gene *TP53* have been reported in around 45% of CRC cases and clinical evidences suggest that p53 mutations associate with lower survival for patients treated by chemotherapy [Iacopetta, 2003].

2.5. Autophagy

Autophagy, which means self-eating in Greek, is responsible to the elimination of damaged structures and cell organelles, as well as, pathogens and long-lived proteins, contributing to the homeostatic function of the cell under normal conditions. Under stress, such as starvation, growth factors deprivation and/or hypoxia, autophagy is induced to provide essential nutrients and remove damaged constituents in order to promote cell survival. Autophagy is therefore a mechanism of survival that maintains the metabolic activity of the cell through lysosomal recycling of intracellular nutrients [Mathew *et al.*, 2007; Brech *et al.*, 2009; Morselli *et al.*, 2009].

In cancer cells, autophagy has been suggested to have a dual role. In one way, autophagy can stimulate oncogenesis by promoting cancer cells survival allowing their adaptation to adverse metabolic conditions. On the other hand, autophagy can also function as a tumor suppressor by inducing cellular self-degradation that leads to autophagic cell death [Brech *et al.*, 2009; Morselli *et al.*, 2009].

2.5.1. The Autophagic Process

Autophagy is characterized by the capacity of cells to sequester and engulf cellular proteins, organelles and cytoplasm in an isolate membrane or phagophore that closure to form a double membrane structure named autophagosome. These autophagosomes undergo a maturation process fusing with lysosomes to form the autolysosomes, where the sequestered contents are then degraded by lysosomal hydrolyses and the amino acids and sugars recycled into the cytosol. The formation of the autophagosome is controlled by autophagy-related (atg) proteins that participate in the sequestration of intracellular constituents and in the elongation of the phagophore membrane to form the autophagosome. The fusion between autophagosomes and lysosomes is required for the completion of the autophagic process [Mathew *et al.*, 2007; Mizushima, 2007; Brech *et al.*, 2009].

The mechanism that leads to the formation of autophagosomes was first identified in yeast, where it was discovered over more than 30 Atg genes. Many Atg homologs have subsequently been identified and characterized in higher eukaryotes, suggesting that autophagy is a highly conserved pathway through evolution [He and Klionsky, 2009]. The first identified regulator of autophagy was Beclin 1 that functions

as a haploinsufficient tumors suppressor. Beclin 1 is the human homologue of Atg6/Vps30, which is a key regulator of autophagy in yeast. Beclin 1 was also found to be crucial for autophagy induction in mammalian cells, since it is involved in autophagosome formation by recruiting proteins from the cytosol, which will be used for autophagic degradation, or in supplying the autophagic pathway with membrane components. The expression level of Beclin 1 is therefore usually increased during autophagy [Brech *et al.*, 2009]. Beclin-1 is part of the class III PI3K complex, where it allows the recruitment of others essential Atg proteins to the membrane and produces the lipid PI3 required for the formation of the phagophore. Moreover, Beclin 1 also contains a BH3 domain that mediates its interaction with Bcl-2 and other anti-apoptotic proteins inhibiting its function, whereas its dissociation is required for autophagic induction [Mathew *et al.*, 2007; Brech *et al.*, 2009; He and Klionsky, 2009].

The microtubule-associated protein 1 light chain 3 (MAP1LC3) is an ubiquitin-like protein Atg8 that is cleaved during the autophagic process producing an active cytosolic precursor form, LC3-I, which subsequently conjugates with a phospholipid via a ubiquitylation-like system to form the active LC3-II. LC3-II then becomes a component of the inner membrane of the autophagosome and, following fusion with lysosomes, LC3-II is rapidly degraded by lysosomal enzymes [Mathew *et al.*, 2007; He and Klionsky, 2009; Mizushima *et al.*, 2010]. The relative amounts of LC3-II reflect the abundance of autophagosomes, which can occur in both induction and inhibition of the autophagic process. The level of mammalian polyubiquitin-binding protein p62/sequestosome 1 (SQSTM1), also called p62, which is associated with ubiquitinated proteins and interact with LC3-II incorporating into autophagolysosomes, has been demonstrated to be a reflex of autophagic fluxes [Pankiv *et al.*, 2007; Mizushima *et al.*, 2010].

2.5.2. Some Modulators of Autophagy

Autophagy is a multistep process that could be altered in cancer cells at the levels of induction, regulation and lysosomal degradation [Hoyer-Hansen and Jaattela, 2008]. Several cancer-associated alterations in genes that regulate various signaling cascades such as PI3K, MAPK and p53 pathways, have been implicated in up- or down-regulation of autophagy [Brech *et al.*, 2009; Corcelle *et al.*, 2009; Maiuri *et al.*, 2009; Morselli *et al.*, 2009].

2.5.2.1. PI3K Pathway

The best known regulator of autophagy in human cells is the mammalian target of rapamycin (mTOR) kinase, a downstream component of the class I PI3K pathway, which downregulates autophagy when activated in response to growth factor signaling, nutritional status and energy level [Brech *et al.*, 2009; Morselli *et al.*, 2009]. Activated Akt promotes phosphorylation of the protein encoded by the tuberous sclerosis complex (TSC) 2 tumor suppressor gene, blocking the interaction between TSC2 and TSC1 and preventing the formation of the TSC1/TSC2 complex. The inhibition of this complex prevents the stimulation of the GTPase activity of Rheb, causing the presence of the active GTP-bound form that directly binds and activates the mammalian target of rapamycin complex 1 (mTORC1). Consequently, mTORC1 takes part in multiple signaling cascades that control cell growth, protein synthesis and promote mRNA translation through phosphorylation of its downstream targets, the kinase p70S6K1 and the elongation factor 4E-BP1. In addition, mTORC1 is also involved in the autophagic process by directly interacting with atg proteins (**Fig. 5**). The mTORC1 interacts with the autophagic regulator kinase ULK1 and the protein atg13 leading to the inactivation of the complex Atg13-ULK1 causing suppression of autophagy [Brech *et al.*, 2009; Corcelle *et al.*, 2009; He and Klionsky, 2009].

Nevertheless, studies have demonstrated that distinct classes of PI3K control autophagy in opposite directions. While products of class I PI3K have showed to inhibit autophagy, class III PI3K has been demonstrated to promote autophagy. The class III PI3K is part of a complex of proteins where Beclin-1, UVRAG and Bif-1 belong, which are essential to the formation of autophagosome [Brech *et al.*, 2009; Corcelle *et al.*, 2009; Mizushima *et al.*, 2010].

2.5.2.2. MAPK Pathways

Similar to Akt, ERK1/2 has also been reported to regulate the formation of autolysosomes by inhibiting the complex TSC1/TSC2 through TSC2 phosphorylation [Ma *et al.*, 2005] and consequently inducing the suppression of autophagy. Moreover, it was also demonstrated that ERK1/2 regulates the maturation of autophagosomes by affecting the lysosomal membrane protein LAMP-2, promoting the formation of large defective autolysosomes and thus blocking autophagy (**Fig. 5**) [Corcelle *et al.*, 2007; Corcelle *et al.*, 2009].

It has also been demonstrated that p38 pathway playa a role in autophagy by inhibiting the formation of autolysosomes, functioning as a negative regulator of autophagy [Corcelle *et al.*, 2007; Webber and Tooze, 2010]. Thus, ERK and p38 kinases can control autophagy, however, possesing opposing effects (**Fig. 5**) [Corcelle *et al.*, 2007; Corcelle *et al.*, 2009].

Recent studies have also demonstrated the role of JNK pathway in autophagy induction via different forms. Activation of JNK can upregulate Beclin-1, since JNK phosphorylates Bcl-2 in three residues resulting in the dissociation of the complex Bcl-2/Beclin 1 and the release of Beclin 1 (**Fig. 5**) [He and Klionsky, 2009]. In addition, the JNK target, c-Jun transcription factor, has also been shown to upregulate the transcription of autophagic genes, such as Beclin-1, and thereby increasing autophagy [He and Klionsky, 2009; Li *et al.*, 2009]. Recently, it was also found that JNK can induce the lysosomal protein damage-regulated autophagy modulator (DRAM) that play a key role in controlling autophagic cell death [Lorin *et al.*, 2010].

2.5.2.3. p53 Protein

Recent evidences indicate that p53 regulates autophagy exerting, however, contradictory effects dependent on its localization in the cell [Tasdemir *et al.*, 2008]. In the cytoplasm, p53 despite inducing mitochondrial membrane permeabilization and apoptosis, it can inhibit autophagy. In the nucleus, p53 acts as an inducer of apoptosis and an autophagy-promoting transcription factor [Tasdemir *et al.*, 2008]. Moreover, it was also demonstrated that some variants of p53 mutations that localize the protein to the cytoplasm repress autophagy, while p53 mutants with nuclear distribution failed to inhibit autophagy [Morselli *et al.*, 2008].

In the cytoplasm, basal levels of p53 mediate an inhibition of autophagy directly at the endoplasmatic reticulum. However, an activation of p53 by cellular stresses and oncogenic signals have been reported to induce autophagy [Vousden and Ryan, 2009]. It has been demonstrated that p53 activates the AMP-activated kinase (AMPK), which is known to phosphorylate TSC2 or directly inhibit mTORC1, inducing autophagy (**Fig. 5**) [Feng *et al.*, 2005b; Corcelle *et al.*, 2009]. Other p53 target genes such as Bax and PUMA, besides contributing to the induction of apotosis, have also recently been shown to be positive regulators of autophagy [Yee *et al.*, 2009].

In the nucleus, p53 can promote autophagy via transcriptional upregulation of the gene coding for the lysosomal protein DRAM (damage-regulated autophagy

modulator), a p53 target gene, which also contributes to damage-induced cell death [Crighton *et al.*, 2006].

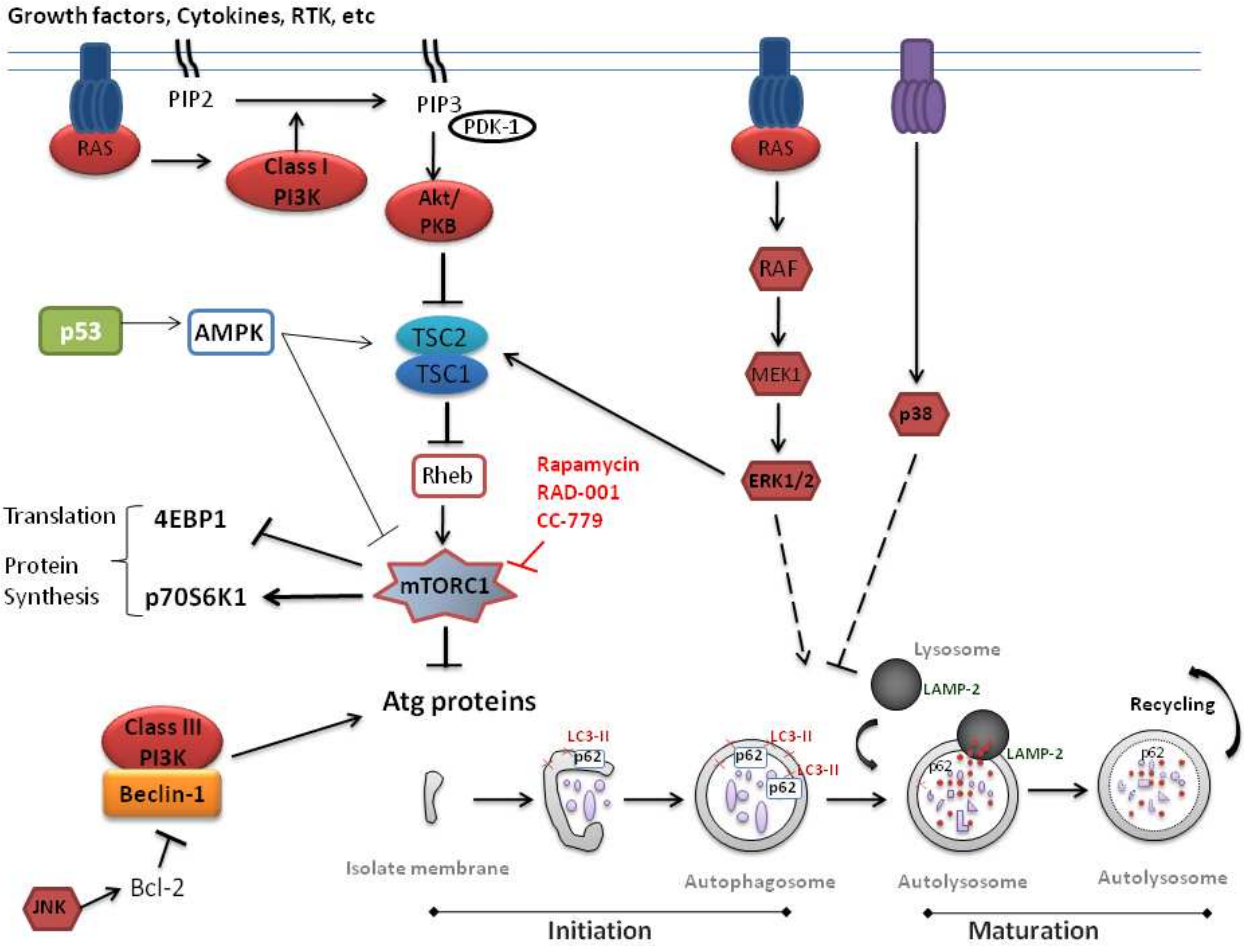

Fig. 5. Schematic model of the autophagic process and their regulation by the major signaling pathways in mammals. Autophagy begins with an isolate membrane that sequesters and engulfed cellular proteins, organelles and cytoplasm to form a double membrane structure named autophagosomes, during the initiation phase. These autophagosomes fuse with lysosomes to undergo a maturation process forming the autolysosomes. Then sequestered contents are degraded by lysosomal hydrolyses and the amino acids and sugars recycled into the cytosol. The autophagic process is regulated by several important signaling pathways. The activation of the class I PI3K inhibits the formation of the complex tuberous sclerosis complex 1/2 (TSC1/TSC2) that in turn activates Rheb. This protein induces the mammalian target of rapamycin complex 1 (mTORC1) that is involved in protein synthesis and mRNA translation through phosphorylation of its downstream targets, the kinase p70S6K1 and the elongation factor 4E-BP1. In addition, mTORC1 also inhibits the autophagy-related proteins (atg) causing the suppression of autophagy. The mTORC1 can be inhibited by various drugs, such as rapamycin and its analogues, CCI-779 and RAD-001. Contrarily, an activation of the class III PI3K promotes autophagy by inducing the atg proteins. This is negatively regulated through Beclin-1 interaction with Bcl-2, which could be disrupted by the JNK pathway to induce autophagy. The activation of the AMP-activated kinase (AMPK) by p53 can also induce the formation of the complex TSC1/TSC2 promoting autophagy. Additionally, ERK and p38 pathways have been showed opposite effects in the control of autophagy during the maturation step. Adapted from [Brech *et al.*, 2009; Corcelle *et al.*, 2009; He and Klionsky, 2009].

2.5.3. Impact of autophagy in CRC

The autophagic process is regulated by important signaling pathways that are frequently altered in CRC. Activating mutations in class I PI3K, Akt and Ras, or inactivating mutation in PTEN, have been shown to decrease autophagy through activation of mTORC1 [Maiuri *et al.*, 2009; Morselli *et al.*, 2009].

In advanced CRC cases, a high activity of mTOR has been observed, which is associated with increased *in vitro* and *in vivo* cell growth, demonstrating the importance of mTOR pathway in CRC progression [Zhang *et al.*, 2009a] and the possible role of autophagy in CRC. In addition, an inhibition of p38α in CRC cell lines showed to cause autophagic cell death, suggesting an involvement of the p38 pathway in autophagy in CRC [Comes *et al.*, 2007].

3. THERAPY IN CRC

3.1. Some Compounds Modulators of Cancer Cell Signaling

3.1.1. Inhibitors of KRAS

KRAS is an important therapeutic target due to the fact that activates important signaling pathways. Farnesyltransferase inhibitors have been developed to inhibit RAS (**Fig. 2**), however, pre-clinical results were disappointing because Ras could bypass farnesyltransferase blockage through the other related enzyme, the geranylgeranyltransferase [Schubbert *et al.*, 2007; Sousa *et al.*, 2008].

3.1.2. Inhibitors of PI3K Pathway

The strategy to target PI3K pathway consists in the use of inhibitors for individual components of this pathway, such as PI3K, Akt, PDK-1 and mTOR, [Cheng *et al.*, 2005; Granville *et al.*, 2006]. A number of drugs have been developed and some of them are in preclinical evaluation or in clinical trial showing synergistic effect with different types of therapies and the ability to overcome therapy resistances [Hennessy *et al.*, 2005; LoPiccolo *et al.*, 2008]. However, it should be kept in mind that targeting PI3K pathway is likely to be most effective in cases where tumors bear activation of this pathway, suggesting that a proper selection of patients is important [LoPiccolo *et al.*, 2008]. Interestingly, it was found recently that PDK-1 is not only important in

regulating components downstream of PI3K signaling but also in phosphorylating components downstream of MAPK pathway, showing the importance of PDK-1 as a target in CRC therapy regulating two different pathways [Lu *et al.*, 2010].

The best known pharmacological inhibitors of PI3K are wortmannin and LY294002 (**Fig. 2**), which target the p110 catalytic subunit of class I PI3K with antitumor activity *in vitro* and *in vivo* [Hennessy *et al.*, 2005; LoPiccolo *et al.*, 2008]. Wortmannin, a natural fungal metabolite, although it was shown its ability to inhibit PI3K at low concentrations, was found to be soluble in organic solvents and insoluble in water, which may limit its use in clinical trials [Cheng *et al.*, 2005]. LY294002 is a derivate of the flavonoid quercetin, which is effective in inhibiting PI3K, requiring, however, a higher concentration when compared with wortmannin. LY294002 has low solubility in water and, it not only inhibits the ATP binding site of PI3K, but also other downstream components of the PI3K pathway [Cheng *et al.*, 2005; Granville *et al.*, 2006].

Despite the poor solubility and high toxicity of wortmannin and LY294002, these commercially available PI3K inhibitors in combination with chemotherapeutic agents have been shown effective results, demonstrating the advantage of their use in combination therapies. These compounds are not clinically useful but could provide powerful tools to study the cellular effects of PI3K inhibition and to develop other PI3K inhibitors [LoPiccolo *et al.*, 2008]. Derivates of LY294002 and wortmannin are being developed to bypass their undesirable properties [Hennessy *et al.*, 2005; Granville *et al.*, 2006].

3.1.3. Inhibitors of BRAF

A number of RAF inhibitors have been developed and some of them have entered clinical trials [Halilovic and Solit, 2008]. One of the first RAF inhibitors in clinical trial was sorafenib (BAY 43-9006), however this drug showed to inhibit both wt and mutant BRAF (V600E), with also effects on another subset of RTK (**Fig. 3**). A second generation of RAF inhibitors have been developed that have greater selectivity for mutant BRAF [Halilovic and Solit, 2008]. In fact, more recently, it was found that ATP-competitive RAF inhibitors block MAPK signaling pathway, decreasing tumor growth in mutant BRAF (V600E) tumors, but surprisingly, they activate RAF-MEK-ERK signaling in mutant KRAS and wt BRAF tumors, enhancing tumor growth

[Hatzivassiliou *et al.*, 2010; Poulikakos *et al.*, 2010]. These findings suggest RAF inhibitors are only effective in tumors in which BRAF is mutated.

3.1.4. Inhibitors of MAPK Pathways

Several compounds have been found to inhibit the enzyme activity of MAP kinases [Malemud, 2007]. One of them, PD98059 (**Fig. 3**), showed to inhibit MEK1 and MEK2 and partially MEK5 with an impact on ERK signaling [McCubrey *et al.*, 2006]. Second-generation MEK inhibitors have also been developed with better pharmacological properties, higher effect against MEK activity and longer duration of suppression, demonstrating good results in clinical trials [Fang and Richardson, 2005]. Alterations in PI3K pathway, such as mutations in *PIK3CA* gene, have been recently found to be a major mechanism of acquired resistance to MEK inhibitors, suggesting a combination therapy of PI3K and MEK inhibitors to increase sensitivity to MEK inhibition [Wee *et al.*, 2009].

Inhibitors of p38α and JNK have been developed to trigger death in cancer cells when combined with other chemotherapeutic drugs [Coulthard *et al.*, 2009; Wagner and Nebreda, 2009]. SP600125 is a commonly used JNK inhibitor demonstrating, however, little specificity and selectivity for the different JNK isoforms [Wagner and Nebreda, 2009]. SB203580 is an inhibitor of p38α/ß that acts through blockage of the ATP binding site [Coulthard *et al.*, 2009] (**Fig. 3**). Although there are a number of p38 and JNK inhibitors in ongoing clinical trials, it is essential to take in consideration the type of tumor for JNK and p38 targeted therapy [Wagner and Nebreda, 2009].

3.1.5. Potential Molecular Targets to Cell Death

Most of the chemotherapeutic drugs induce cell death by apoptosis through receptor or mitochondrial pathways. Molecules involved in these pathways have been demonstrated to be good targets in the discovery of new anticancer drugs [Qiao and Wong, 2009; Tan *et al.*, 2009]. The alteration of the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins, the cell surface death receptors, such as Fas, TRAIL and TNF and inhibition of components of the PI3K and MAPK signaling have been shown to be good strategies to trigger apoptosis [Qiao and Wong, 2009; Tan *et al.*, 2009]. Currently, strategies to restore p53 function and/or to specifically target

regulators of p53, such as Mdm2, have appeared [Vaseva and Moll, 2009], with a number of novel compounds demonstrated to modulate p53 [Wang and Sun, 2010].

Furthermore, lysosomes have also been increasingly recognized as a promise target for cancer treatment, by allowing cell death to occur even in cells with defects in the classical apoptotic pathways [Kirkegaard and Jaattela, 2009]. Various drugs have been identified that affect the integrity of the lysosome membranes by inducing LMP, which results in cathepsin-mediated cell death [Kroemer and Jaattela, 2005].

3.1.6. Inducers/Inhibitors of Autophagy

The impact of autophagy on cancer cells seems to depend on tumor type, in its intrinsic properties and on the nature of the cytotoxic therapy that is combined with it [Brech *et al.*, 2009]. Many compounds present in clinical have shown the ability to inhibit or induce autophagy with anticancer effects. Inhibitors of autophagy have been shown more likely to succeed when they are combined with other types of cytotoxic drugs that activate a protective autophagy. On the other hand, inducers of autophagy that trigger autophagic cell death in various cancer cells, may succeed when tumors have defects on the apoptotic machinery [Hoyer-Hansen and Jaattela, 2008].

Rapamycin, produced naturally by the bacterium *Streptomyces hygroscopicus*, was the first identified compound to target mTOR, which possesses poor aqueous solubility and strong immunosuppressive properties. Rapamycin was found to inhibit mTOR with anti-proliferative effects against several cancer cell lines and to induce autophagy *in vitro* and *in vivo*. Various analogues of rapamycin, such as CCI-779 and RAD-001, have been developed and entered in clinical trials with promising results when combined with other chemotherapeutic drugs (**Fig. 5**) [LoPiccolo *et al.*, 2008]. On the other hand, a number of different autophagic inhibitors were also developed. The drug 3-methyladenine is one example that showed to specifically inhibit autophagy through effects on class III PI3K [Tan *et al.*, 2009].

3.2. Clinical Chemotherapeutic Drugs

In CRC, chemotherapeutic agents have been developed over time and they have showed different mechanisms of action. The genetic variability is an important factor that regulates the response and toxicity of a drug and the introduction of genetic tests to

individualize treatment will allow better response to these therapeutic agents [Bhushan *et al.*, 2009]. Currently, drugs such as 5-Fluorouracil (5-FU), oxaliplatin, irinotecan, capecitabine, bevacizumab cetuximab and panitumumab, are used as clinical options and in most of the cases in combinations to increase CRC treatment efficacy [Bhushan *et al.*, 2009; De Dosso *et al.*, 2009; Segal and Saltz, 2009].

3.2.1. 5-Fluorouracil (5-FU)

The best known and most used chemotherapeutic agent in CRC treatment is 5-FU that has been used in clinical since more than 40 years [Bhushan *et al.*, 2009]. 5-FU is a nucleoside analog that incorporates its active metabolites, the fluorodeoxyuridine triphosphate (FdUTP) and fluorodine triphosphate (FUTP), into RNA and DNA leading to the disruption of their synthesis. In addition, 5-FU inhibits the enzyme thymidylate synthase (TS) that is essential in DNA synthesis and repair [Warusavitarne and Schnitzler, 2007; Bhushan *et al.*, 2009]. Resistance to 5-FU appears in tumors with alterations in enzymes associated with 5-FU mechanisms of action, including high TS expression [Warusavitarne and Schnitzler, 2007; Bhushan *et al.*, 2009]. Moreover, 5-FU is also able to induce apoptosis through intrinsic and extrinsic pathways and thus defects in these apoptotic pathways contribute to 5-FU resistance [Hector and Prehn, 2009].

Currently, the response rate to 5-FU has increased when used in combination with other chemotherapeutic drugs, such as irinotecan and oxiplatin [De Dosso *et al.*, 2009; Segal and Saltz, 2009].

3.2.2. Other Chemotherapeutic Drugs

A number of drugs had appeared to improve survival rate being used alone or in combination with 5-FU. Capecitabine is a prodrug that is converted to 5-FU at the site of the tumor and administered orally, demonstrating a good response rate [De Dosso *et al.*, 2009; Segal and Saltz, 2009]. Irinotecan, a derivate from the natural alkaloid camptothecin, was a second-line agent following failure of 5-FU. This chemotherapeutic drug has effect by interacting with the enzyme topoisomerase I, which causes transient single-strand DNA breaks that are stabilized by irinotecan, leading to DNA fragmentation and cell death.[Bhushan *et al.*, 2009; Segal and Saltz, 2009]. Oxaliplatin is a platinum compound that forms a cross-link between complementary DNA strands, thereby blocking DNA replication and transcription

leading to apoptosis [Bhushan *et al.*, 2009; De Dosso *et al.*, 2009; Segal and Saltz, 2009].

More recently, inhibitors of growth factor receptors have appeared. Bevacizumab, a monoclonal antibody directly targets the vascular endothelium growth factor (VEGF), was shown to inhibit angiogenesis or new blood vessel formation. Cetuximab and panitumumab, two monoclonal antibodies developed to target the human epidermal growth factor receptor (EGFR), showed to block the binding of EGF to its receptor, which is overexpressed in up to 80% of CRC tumors and responsible for a poor prognosis [Bhushan *et al.*, 2009; De Dosso *et al.*, 2009; Segal and Saltz, 2009].

3.2.3. Resistance and Limitations

Even though a wide range of chemotherapeutic drugs are available for the treatment of CRC, resistances and limitations to the above drugs have been shown. Different types of tumors have different behaviors and consequently have different responses to CRC therapy. It is also essential to keep in mind that the use of a specific drug that inhibits only one target may not be enough against CRC progression. Thus, it is important to know the biology of the tumor and its mutations in signaling mediators to choose the best treatment.

Mutations in the MMR genes and in the *TP53* have been shown to influence the cellular response to some chemotherapeutic agents [Warusavitarne and Schnitzler, 2007; Bhushan *et al.*, 2009]. CRC cells with loss of MMR system, especially when combined with loss of p53, have showed to generate high cisplatin resistance during sequential drug exposure [Lin and Howell, 2006]. In addition, despite some controversy, evidences have also demonstrated a reduced response of CRC MSI tumors to 5-FU treatment [Jover *et al.*, 2006; Warusavitarne and Schnitzler, 2007]. CRC tumors with mutations in *TP53*, *TS* enzyme and/or alterations on Bcl-2 family proteins have also shown to be resistant to 5-FU-induced apoptosis [Violette *et al.*, 2002; Adamsen *et al.*, 2007; Warusavitarne and Schnitzler, 2007]. Moreover, poor prognosis and reduced sensitivity to 5-FU were also shown to CRC tumors with MSI status and p53 overexpression or high TS activity, compared with tumors without these alterations [Mori *et al.*, 2004; Warusavitarne and Schnitzler, 2007]. The use of TS inhibitors, such as CDK inhibitors, in combination with 5-FU has been shown to reduce TS expression, improving 5-FU responses [Takagi *et al.*, 2008].

The use of drugs inhibiting only one signaling pathway has failed in some subsets of tumors. For example, deregulation of the Ras/Raf/MAPK/ERK pathway in cancer cells has been associated with resistance to PI3K inhibitors, suggesting combined targeting of PI3K and MEK to an effective anticancer strategy [Yu *et al.*, 2008]. Another example is the resistance of CRC tumors to cetuximab and panitumumab, which happen when tumors harbor *PIK3CA* mutation/*PTEN* loss and/or *RAS*/*BRAF* mutations. These mutations are present downstream of EGFR and, therefore, related signaling pathways are activated even if EGF receptor is inhibited [Jhawer *et al.*, 2008; Laurent-Puig *et al.*, 2009; Siddiqui and Piperdi, 2010]. In fact, panitumumab was the first drug to be approved for use only in cases without mutation of RAS, being required the applicability of genetic tests before EGFR therapy [Baselga and Rosen, 2008]. MEK inhibitors, such as BRAF inhibitors, have also shown to be good candidates in cases where MAPK/ERK1/2 is activated, however, they demonstrate less favorable response in the presence of RAS or tyrosine kinases mutations, such as PI3K, where these alternative downstream pathways could substitute ERK in maintaining cell survival [Balmanno and Cook, 2009].

4. POTENTIAL OF NATURAL COMPOUNDS TO MODULATE MOLECULAR SIGNALING PATHWAYS IN CRC

Cancer cells are known to have alterations in multiple signaling pathways, which could lead to a disappointing inefficiency of specific synthetic inhibitors used as anticarcinogenic agents in cancer treatment, which only target one pathway. On the other hand, many natural compounds have been shown to modulate multiple cellular signaling pathways being usually classified as multi-target agents [Surh, 2003; Aggarwal and Shishodia, 2006; Sarkar *et al.*, 2009], being therefore interesting compounds for anticancer interventions. In fact, an enormous progress in the characterization of natural products-related anticancer effects has been made. Not surprisingly, 60% of all cancer drugs that are used clinically are either natural products or owe their origin to a natural source [Heinrich *et al.*, 2003]. Thus, natural compounds are continuing to be widely studied as possible anticancer agents and/or as enhancers of therapeutic responses of chemotherapeutic drugs when combined with them.

In CRC, several bioactive food components have been shown to modulate cell signaling pathways related to proliferation and cell death, being the PI3K/Akt and MAPKs (ERK, JNK and p38) pathways, as well as, molecules involved in apoptosis the most promising targets of these compounds [Aggarwal and Shishodia, 2006; Khan *et al.*, 2008; Rajamanickam and Agarwal, 2008; Ramos, 2008; Sarkar *et al.*, 2009]. In addition, effects on the autophagic process have also been demonstrated for some natural compounds, which may be used in order to sensitize cells to death [Singletary and Milner, 2008].

4.1. Medicinal Plants

The National Cancer Institute (NCI) identified a large number of plant-based foods with anticancer properties through beneficial effects in a single or multiple molecular targets [Surh, 2003]. With respect to tea (from the plant *Camellia sinensis*), although some studies did not found positive effects, many epidemiological and laboratory studies have been found a positive correlation between tea consumption and reduction of human cancer risk, including CRC [Yang and Landau, 2000; Yang *et al.*, 2007].

Many other plant extracts have been shown to provide a rich source of bioactive food compounds especially of phenolic compounds, which are considered to play an important role as anticarcinogenic agents due to their antioxidant properties [Yang *et al.*, 2001; Yang *et al.*, 2008]. Reactive oxygen species (ROS) and free radicals, produced by cancer cells, activate a number of genes and signal transduction pathways that mediate cancer cell proliferation and survival. Therefore, radical scavenging activity by the phenolic compounds present in plant foods by reducing ROS levels contribute to a decrease in the activity of redox-sensitive pathways, thus decreasing cancer progression [Loo, 2003; Fruehauf and Meyskens, 2007]. In addition, direct interactions between plant food constituents and multiple key elements in signaling transduction pathways have been reported [Surh, 2003; Ramos, 2008].

4.1.1. Genus *Salvia*

The word *Salvia* comes from the Latin *salvare* that means “to save”. Plants of the genus *Salvia* (family Lamiaceae) are aromatic plants that have been used by folk

medicine since Roman times due to its antiseptic, anti-inflammatory and hypoglycemic properties, among others. About 100 *Salvia* species have been characterized. They are plants native to the Mediterranean region and are cultivated nowadays all over the world [Topcu, 2006]. Studies have shown biological antioxidant effects of *Salvia* (sage), which have become an important source of bioactive compounds with health benefits for cosmetic and pharmaceutical industries [Lima, 2006; Lima *et al.*, 2007].

Due to the increasing scientific evidences of natural antioxidants in inhibiting cancer cell proliferation [Loo, 2003], sage plants have been suggested to be a natural source of potential antitumor agents. The anticarcinogenic effect of sage was demonstrated in several species, such as *Salvia miltiorrhiza* and *Salvia menthaefolia*, where their antiproliferative activity on several types of tumor cells was osberved [Liu *et al.*, 2000; Fiore *et al.*, 2006].

4.1.1.1. *Salvia fruticosa* and *Salvia officinalis*

The anticancer activity of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO), to our knowledge, were never reported until now. The compositions of the water extracts (tea) produced from these plants and used in this work are presented in **Table 1**. Rosmarinic acid (RA) is the major phenolic compound present in both sages, constituting about 58% of all phenolic compounds present in SF water extract and 70% in SO water extract [Lima, 2006; Lima *et al.*, 2007]. In addition, derivatives of luteolin, 6-hidroxyluteolin-7-glucoside (in SF) and luteolin-7-glucoside (in SO) are present as the major flavonoids in the sage extracts. Both sages also contain an important bioactive triterpenoid, ursolic acid, in their composition, although present at very low concentration in the water extracts (Braga *et al.*, unpublished results).

Studies have demonstrated that RA, in the water extracts, is a major contributor to the antioxidant activity at cellular level of SO [Lima *et al.*, 2007]. In fact, this phenolic compound has shown to possess high antioxidant and anti-inflammatory activities [Lima *et al.*, 2006]. Effects of RA on several signaling pathways in cancer cells were also reported. RA showed to inhibit ERK phosphorylation in colon and breast cancer cells [Scheckel *et al.*, 2008] and activate PKA without effects on Akt and p38 in melanoma cells [Lee *et al.*, 2007]. Studies also reported effects of RA on induction of apoptosis via mitochondrial pathway in human hepatoma cells [Lin *et al.*, 2007] and human Jurkat lymphoma cells [Kolettas *et al.*, 2006]. Therefore, RA could be one of the bioactive compounds present in sages.

Table 1 - Composition (µg/mg extract) in phenolic compounds and ursolic acid of *Salvia fruticosa* and *Salvia officinalis* water extracts after lyophilization ([Lima, 2006; Azevedo, 2008] and Braga P. *et al*., unpublished results).

Component	*S. fruticosa* (µg/mg extract)	*S. officinalis* (µg/mg extract)
Phenolic compounds		
Phenolic acids		
rosmarinic acid	71.49	52.00
caffeic acid	0.13	0.82
ferulic acid	0.03	0.52
3-caffeoylquinic acid	tr[a]	tr[a]
5-caffeoylquinic acid	tr[a]	tr[a]
Flavonoids		
6-hydroxyluteolin-7-glucoside	22.66	-
not identified flavone*	28.64	-
apigenin-7-glucoside	0.59	0.43
luteolin-7-glucoside	tr[a]	19.74
4',5,7,8-tetrahidoxiflavone	-	0.90
Triterpenoides		
ursolic acid	0.05	0.03

*Quantified as apigenin-7-glucoside
[a] Trace amounts: compounds present in concentration below 0.01µg/mg extract

4.1.2. Genus *Hypericum*

Plants of the genus *Hypericum* (family Hypericaceae), which contain a variety of phenolic compounds, are been attributed some medicinal properties. There are about twenty seven species of this genus, where *H. androsaemum* and *H. perforatum* are widely used as herbal drugs in Portugal [Valentao *et al.*, 2003].

4.1.2.1. ***Hypericum androsaemum***

Hypericum androsaemum (HA) is a medicinal plant, native to Europe and Asia and traditionally used in Portugal as diuretic, hepatoprotector, cholagogue, and also used in kidney failure and in the relief of digestive tract disorders [Guedes *et al.*, 2004]. Studies *in vitro* have confirmed its reputed antioxidant and hepatoprotective activities [Valentao *et al.*, 2002; Valentao *et al.*, 2004b], although these results were not observed in *in vivo* studies [Valentao *et al.*, 2004a]. The effects of HA have been attributed, at least in part, to the presence of several flavonoids, such as quercetin and its glycosides,

and phenolic acids, such as chlorogenic acid [Valentao *et al.*, 2004b]. However, the anticancer activity of HA was never been studied, as far as we know.

The composition of HA water extract (tea) produced from this plant and used in this work is presented in **Table 2**. Chlorogenic acid (CA; 5-caffeoylquinic acid) and its isomer (3-caffeoylquinic acid) are the main phenolic compounds present, and quercetin and derivatives of quercetin represent the major flavonoids. Studies have demonstrated that CA has high antioxidant properties and antitumor effects in several cancer cells [Jin *et al.*, 2005; Belkaid *et al.*, 2006], where effects on MAPK and NF-κB signaling were reported [Feng *et al.*, 2005a]. Anticarcinogenic effects of CA in CRC, however, have not been observed [Xie *et al.*, 2009; Park *et al.*, 2010].

4.1.2.2. ***Hypericum perforatum***

Hypericum perforatum (HP), also known as St. John´s wort, is the *Hypericum* specie more studied and used for its medicinal properties. This plant is found throughout the world and it is known for its high pharmacological activities, including antidepressant, antiviral and antibacterial properties [Barnes *et al.*, 2001]. The anticarcinogenic activity of HP has been reported in several cancer cell types [Martarelli *et al.*, 2004; Roscetti *et al.*, 2004; Skalkos *et al.*, 2005; Stavropoulos *et al.*, 2006].

The composition of HP water extract (tea) produced from this plant and used in this work is presented in **Table 2**. This plant has the glycoside of quercetin, quercetin 3-rutinoside (also known as rutin), as the major constituent. Hypericin, which is also present in this plant, has been found in some *Hypericum* species (its main source), being extensively studied for its enormous applications in the photodynamic therapy, including in cancer therapy. This compound, when irradiated with visible light, is capable to generate ROS that are toxic when produced at high levels leading cells to death [Barnes *et al.*, 2001; Kitanov, 2001]. Although the anticancer effects of HP could be related with the presence of hypericin even if in small amounts, other studies have demonstrated that different types of compounds could be responsible for the plant's anticancer effects [Roscetti *et al.*, 2004; Skalkos *et al.*, 2005]. Effects on MAP Kinases have also been reported for this plant [Karioti and Bilia, 2010].

4.1.2.3. ***Hypericum undulatum***

Hypericum undulatum (HU), a plant native to Europe and north of Africa, is one of the less studied herbal plants, being used for the same ailments as HP. A study *in*

vitro found high antioxidant activity of HU, as well as, an inhibitory effect on acetylcholinesterase with beneficial effects on memory [Ferreira *et al.*, 2006]. The composition of HU water extract (tea) produced from this plant and used in this work is presented in **Table 2**. This plant has glycosides of quercetin, quercetin 3-galactoside (also known as hyperoside) and quercetin 3-glucoside (also known as isoquercitrin), as the major constituents. Hypericin is also present in HU's composition.

Table 2 – Composition (µg/mg extract) in phenolic compounds of *Hypericum androsaemum, Hypericum perforatum and Hypericum undulatum* water extracts after lyophilization (Lima E. *et al*., unpublished results).

Component	*H. androsaemum* (µg/mg extract)	*H. perforatum* (µg/mg extract)	*H. undulatum* (µg/mg extract)
Phenolic compounds			
Phenolic acids			
3-caffeoylquinic acid (chlorogenic acid isomer)	19.64	0.81	8.40
5-caffeoylquinic acid (chlorogenic acid)	34.18	4.94	6.45
Flavonoids			
Quercetin 3-rutinoside (rutin)	2.73	38.07	-
Quercetin 3-galactoside (hyperoside)	16.35	16.00	34.12
Quercetin 3-glucoside (isoquercitrin)	5.41	6.47	21.70
Quercetin 3-rhamnoside (quercitrin)	-	0.24	1.97
Quercetin	1.32	3.39	4.97
Amenthoflavone	tr [a]	0.33	0.30
Hyperforin	-	-	-
Hypericin	-	0.03	0.03

[a] Trace amounts: compounds present in concentration below 0.01µg/mg extract

4.2. Flavonoids

Phenolic compounds are highly abundant in fruits, vegetables, cereals and medicinal plants with more than 8000 different phenolic compounds described. They are characterized by the presence of one or more phenolic rings being divided in different classes based on their chemical structure. The most abundant dietary phenolic compounds are the flavonoids (such as quercetin, resveratrol, kaempferol, genistein, myricetin and luteolin) and phenolic acids (such as rosmarinic acid and clorogenic

acid), that accounts of 60% and 30%, respectively, of all polyphenols [Nichenametla *et al.*, 2006; Ramos, 2008].

Flavonoids are the largest class of phenolic compounds with more than 5000 varieties described and ubiquitously distributed in most of plants [Yang *et al.*, 2001]. Studies *in vitro* and *in vivo* have shown the potential of some flavonoids to modulate many steps of the carcinogenic process, although being dependent on tissue, cell type and doses [Nichenametla *et al.*, 2006; Ramos, 2008].

4.2.1. Quercetin

Quercetin (3,3´,4´,5,7-pentahydroxyflavone; **Fig. 6**) is one of the main flavonoids present in the human diet and it can be found in many fruits, vegetables and beverages, particularly in onions, apples, cherries, broccoli, tomatoes, barriers, tea, red wine and leek [Ramos, 2008].

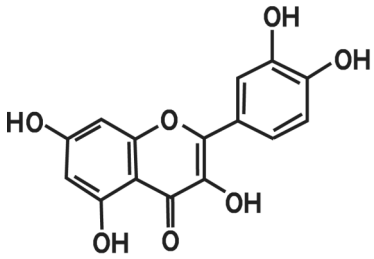

Fig. 6. Chemical structure of quercetin.

Quercetin (Q) usually occurs in the diet as glycosides in which at least one hydroxyl group is substituted by a sugar. Onion is the major vegetable source of quercetin glycosides. The sugar group is frequently bound at the 3-position forming different conjugates, such as quercitrin, isoquercitrin, hyperoside and rutin. These conjugated metabolites are found in circulating blood, being stable precursors that could be converted to the active aglycone (*i.e.* quercetin without a sugar group) and exert its function at the target site [Murakami *et al.*, 2008]. Q is known for its prominent dietary antioxidant activity and its strong anti-inflammatory capacities [Boots *et al.*, 2008].

Studies with Q have demonstrated its anticarcinogenic potential in many types of cancer cells. The ability of Q to inhibit several tyrosine kinases especially PI3K, has been shown and compounds such as LY294002, a PI3K inhibitor, have been designed based on quercetin's structure [Walker *et al.*, 2000]. Effects of Q in suppressing survival and inducing apoptosis through PI3K/Akt and/or MAPK/ERK pathways have been found for several types of cancer cells, such as hepatoma [Granado-Serrano *et al.*, 2008], neural [Spencer *et al.*, 2003], skin epidermal [Lee *et al.*, 2008] and breast [Gulati *et al.*, 2006], while in lung cancer cells ERK activation was required for quercetin-induced apoptosis [Nguyen *et al.*, 2004].

In CRC, effects of Q on PI3K/Akt and MAPK/ERK signaling pathways were also reported. Studies on gene expression have demonstrated effects of Q in genes involved in the MAPK signal pathways [van Erk *et al.*, 2005], as well as, in cell cycle and apoptosis regulation genes [van Erk *et al.*, 2005; Murtaza *et al.*, 2006]. Proteomic studies also showed effects of Q via inhibition of Akt [Kim *et al.*, 2005b] and MAP kinase p38 [Wenzel *et al.*, 2004], as well as, induction of the mitochondrial pathway of apoptosis [Kim *et al.*, 2005b; Volate *et al.*, 2005]. One of the most relevant reported effects of Q is its ability to effectively inhibit the expression of the 3 types of RAS proteins (H-Ras, K-Ras and N-Ras) in different CRC cell lines [Ranelletti *et al.*, 2000]. A specific inhibition on mutated RAS was then described where Q only decreased RAS protein level in cells expressing oncogenic RAS [Psahoulia *et al.*, 2007b]. In addition, it was found that Q sensitizes CRC cells to apoptotic death in tumors cells resistant to death receptor TRAIL directed therapies [Psahoulia *et al.*, 2007a].

Studies *in vivo* were also performed to validate the anticarcinogenic activity of Q in CRC. This flavonoid showed to decrease 75% of aberrant crypt foci (ACF) incidence in azoxymethane (AOM)-induced CRC mice [Volate *et al.*, 2005]. A more recent study [Warren *et al.*, 2009] also showed a reduction on ACF development in AOM model, however, without effects on PI3K/Akt signaling. In contrast with data from cell culture experiments, in this *in vivo* study a decrease in COX-1 and COX-2 expression was found, suggesting that effects on proliferation and apoptosis may result from the ability of Q to suppress the expression of proinflammatory mediators. This was confirmed by another study where authors showed that AMPK-COX-2 signaling is important in quercetin-mediated cancer control [Lee *et al.*, 2009].

4.2.2. Luteolin

Luteolin (3´,4´,5,7-tetrahydroxyflavone; **Fig. 7**) is an important member of the flavonoid family being present in various fruits and vegetables, such as parsley, thyme, celery, oregano, green chili and peppers [Ramos, 2008]. Luteolin (L) usually occurs in the diet as luteolin-7-O-glucoside, being biotransformed into the aglycone, luteolin, in the intestine by microorganisms and hydrolases. This compound is structurally related with Q but has no

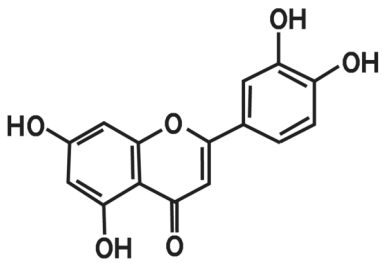

Fig. 7. Chemical structure of luteolin.

hydroxyl group at position 3, which renders it more lipophilic. This higher lipophilicity may explain the potent intracellular antioxidant activity because it may confer better access to intracellular targets within the cell [Lima *et al.*, 2006]. Besides its antioxidant activity, some studies have demonstrated the anticarcinogenic activity of L in several cancer cell lines, such as leukemia [Ko *et al.*, 2002], pancreatic [Lee *et al.*, 2002] and human hepatoma [Chang *et al.*, 2005; Lee *et al.*, 2005; Lee *et al.*, 2006] cells. Effects on PI3K/Akt and MAPK/ERK pathways [Lee *et al.*, 2006], JNK signaling [Lee *et al.*, 2005] and the involvement of the mitochondrial pathway [Chang *et al.*, 2005; Lee *et al.*, 2005] were also reported to contribute to cell growth inhibition and induction of apoptosis in human hepatoma cells.

In CRC, some reports have also shown that L has anticarcinogenic effects. In particular, studies demonstrated apoptosis induction by L mediated through downregulation of Bcl-2 and Mdm-2 proteins, by increased caspase activities [Lim do *et al.*, 2007] and by inhibition of TNF [Shi *et al.*, 2004]. Effects on cell cycle arrest were also found in different CRC cell lines [Wang *et al.*, 2004; Lim do *et al.*, 2007]. It was also found that L is able to inhibit NF-κB signaling and suppress MAP kinases [Kim *et al.*, 2005a]. Activation of JNK was also reported [Shi *et al.*, 2004]. In addition, L was able to sensitize colon cancer cells to cisplatin-induced apoptosis, one of the most commonly cancer therapeutic agent, via increase of p53 protein level and activation of JNK pathway [Shi *et al.*, 2007].

Studies *in vivo* were also performed where L was able to reduce the incidence of ACF and enhance the activity of antioxidants enzymes in AOM-induced colon carcinogenesis mice [Ashokkumar and Sudhandiran, 2008]. In addition, L decreased the multiplicity of foci and their incidence in 1,2-dimethyl hydrazine (DMH)-induced tumorigenesis in mice [Manju and Nalini, 2007], confirming the potential of this natural compound as possible anticancer agent in CRC.

4.3. Triterpenoids

Triterpenoids are compounds structurally related to steroids synthesized by great number of plants. They are especially abundant in ginseng, legumes and oats, among others. Currently, more than 80 different triterpenoid structures have been identified,

which are spread in the plant kingdom in the form of free acid or aglycones and their importance in medicine have been growing [Ikeda *et al.*, 2008].

4.3.1. Ursolic acid

Ursolic acid (**Fig. 8**), a natural pentacyclic triterpenoid carboxylic acid, is the major component of some traditional medicinal herbs, such as rosemary, sage, olive, oregano, and it is also found in some fruits, such as apple, blueberry, cranberry and guava.

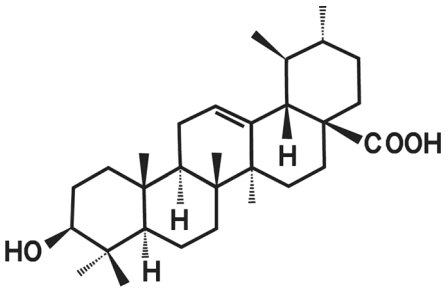

Fig. 8. Chemical structure of ursolic acid.

A wide range of biological functions has been known for ursolic acid (UA), such as anti-inflammatory, hepatoprotective and anticancer combined with low toxicity [Ikeda *et al.*, 2008]. The potential of UA to modulate several important signaling pathways related to proliferation and cell death was also demonstrated in several types of cancers with few studies reported in CRC.

In CRC, UA showed the ability to suppress MAP Kinases [Shan *et al.*, 2009], activate caspases [Andersson *et al.*, 2003; Shan *et al.*, 2009] and to induce cell cycle arrest [Li *et al.*, 2002]. In other cell types, such as prostate cancer cell lines [Kassi *et al.*, 2007; Zhang *et al.*, 2009b; Zhang *et al.*, 2010a; Zhang *et al.*, 2010b] and leukemia cells [Liu and Jiang, 2007], UA induced apoptosis through phosphorylation of Bcl-2, activation of caspases and induction of JNK pathway, while in breast cancer cells [Yeh *et al.*, 2010] UA suppressed the JNK pathway. Effects on PI3K/Akt and/or MAPK/ERK pathways are also reported in prostate [Zhang *et al.*, 2010a], breast [Yeh *et al.*, 2010], endometrial [Achiwa *et al.*, 2007] and ovarian [Wang *et al.*, 2009] cancer cells, as well as, effects on cell cycle progression in lung cancer cells [Hsu *et al.*, 2004]. In addition, effects on angiogenesis through inhibition of VEGF levels and effects on the expression of matrix metalloproteinases MMP-2 and MMP-9 proteins were reported in B16F-10 melanoma cells [Kanjoormana and Kuttan, 2010].

UA has also been linked to anti-inflammatory activity in several studies in melanoma [Manu and Kuttan, 2008], leukemia [Shishodia *et al.*, 2003] and breast cancer [Yeh *et al.*, 2010] cells, where an inhibition of the nuclear transcription factor NF-κB activity by UA was reported. More recently, UA, at low doses, was also found to

be able to sensitized cancer cell lines to chemotherapeutic agents, such as taxol and cisplatin, through suppression of NF-κB, helping in the induction of apoptosis and the reduction of the necessary drug doses for cancer treatment [Li *et al.*, 2010]. An effect on mitochondrial membrane permeability through decrease of sodium pump NA^+-K^+ ATPase by UA was recently reported in several liver cancer cell lines [Yan *et al.*, 2010].

4.4. Natural Compounds in Clinical Trials

For a compound to enter clinical trial many steps are required. The compound must be promising in *vitro* and *in vivo* to proceed for in-depth efficacy evaluation concerning toxicity and pharmacokinetics. The compounds that prove to have low toxicity and high efficiency in animal models are allowed to enter for clinical evaluation in studies involving humans. Only compounds successfully arriving the phase III are considered as possible future chemotherapeutic drugs [Kakizoe, 2003]. It is not high the number of natural compounds that have passed through this process.

4.4.1. Curcumin

Curcumin, a polyphenol, is an active principle of the herb *Curcuma longa*, commonly known as turmeric and it is one of the most well studied natural compounds. Curcumin possesses anticancer effects on different stages of carcinogenesis in several cancer cell lines and exhibits antitumor activity in animal models, including in CRC. Curcumin is able to downregulate the activity of multiple kinases and to interact with important targets, affecting different signaling pathways. Current clininal trials of curcumin are ongoing for different diseases, including pancreatic and colorectal cancers, oral premalignat lesions and in conditions linked to inflammation such as psoriasis and alzheimer´s disease. Although with poor bioavailability, curcumin was remarkably well tolerated and safe in phase I clinical trial, thus a promising outcome is awaited [Goel *et al.*, 2008; Hatcher *et al.*, 2008]. Currently, some analogues of curcumin, such as JC-9, have progressed to phase II clinical trials against prostate cancer, demonstrating candidates to anticancer drugs [Lee, 2010].

4.4.2. Resveratrol

Resveratrol is a natural polyphenol found in grapes, red wine, berries, peanuts and other plants, which was first described as a phenolic component of the medicinal herb hellebore (*Veratrum grandiflorum*). The ability of resveratrol to bind several biomolecules involved in different signaling pathways has been shown to result in diverse biological effects with interest for anticancer treatment. Resveratrol has anticancer effects *in vitro* and *in vivo*, with effects on cell proliferation and death, inflammation, angiogenesis and metastasis processes. Although data in humans have revealed that resveratrol is pharmacologically quite safe, few clinical studies in humans have been performed. Clinical trials for patients with cancer and type 2 diabetes are underway. Because of its low bioavailability, structural analogues of resveratrol are also being improved as potential therapeutic agents for cancer treatment [Aggarwal *et al.*, 2004; Marques *et al.*, 2009].

Natural compounds are possible pharmacological agents' themselfes or can be used as leads to design new compounds based on their structures creating synthetic analogues with better pharmacological activities.

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CHAPTER II

ANTICARCINOGENIC EFFECTS OF *SALVIA FRUTICOSA, SALVIA OFFICINALIS* AND ROSMARINIC ACID

Salvia fruticosa, Salvia officinalis and Rosmarinic acid induce apoptosis and inhibit proliferation of human colorectal cell lines: The Role in MAPK/ERK pathway

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Epidemiological studies have shown that nutrition is a key factor in modulating sporadic colorectal carcinoma (CRC) risk. Aromatic plants of the genus *Salvia* (sage) have been attributed many medicinal properties, which include anticancer activity. In the present study, the antiproliferative and proapoptotic effects of water extracts of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO) and of their main phenolic compound rosmarinic acid (RA) were evaluated in two human colon carcinoma-derived cell lines, HCT15 and CO115, which have different mutations in the MAPK/ERK and PI3K/Akt signalling pathways. These pathways are commonly altered in CRC, leading to increased proliferation and inhibition of apoptosis. Our results show that SF, SO, and RA induce apoptosis in both cell lines, whereas cell proliferation was inhibited by the two sage extracts only in HCT15. SO, SF, and RA inhibited ERK phosphorylation in HCT15 and had no effects on Akt phosphorylation in CO115 cells. The activity of sage extracts seems to be due, at least in part, to the inhibition of MAPK/ERK pathway.

INTRODUCTION

Cancer is an important health problem and one of the most common forms is colorectal carcinoma (CRC). Phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathways play critical roles in cell proliferation and survival and are frequently activated in CRC (1–3). Deregulation of these pathways is also thought to determine response to treatment (4). Mutations of KRAS and BRAF in sporadic CRC (70–80% of total cases) (5) are alternative in which the former constitutively activates both MAPK/ERK and PI3K/Akt pathways, and the latter activates the MAPK/ERK pathway (3,4,6–8). As presented by Schubbert et al. (9), mutations in CRC of either KRAS or BRAF genes occur in 32% and 14% of cases, respectively. Studies have also shown that CRC is frequently associated with mutations in genes that encode for PI3K, p110 catalytic subunit PI3KCA, and PTEN (an endogenous inhibitor of PI3K activity), resulting in an overexpression of Akt (10–13). Considering the high incidence of CRC, inhibitors of these pathways are actively being searched for use in the control of cancer progression (14–16).

Epidemiologic studies have shown that Western type diets, poor in vegetables and fruits, are risk factors known to be associated with CRC, suggesting that nutritional factors may also be preventive and also helpful in the control of cancer (17–19). In fact green and black tea consumption has been shown to be effective in the initiation, promotion, and progression stages of carcinogenesis, although effects on colon cancer are inconclusive (20). Plants of the genus *Salvia* (sage) such as *Salvia miltiorrhiza* and *Salvia menthaefolia* have also been suggested to have anticancer properties based on antiproliferative activity on tumor cells (21,22). In addition, reactive oxygen species (ROS) have been reported to play a role in signalling transduction enhancing proliferation and survival of cancer cells. Antioxidant phytochemicals, through their ROS scavenging activity, may suppress altered redox-sensitive signalling events in cancer (23,24).

Salvia fruticosa (SF) and *Salvia officinalis* (SO), poorly studied with regard to their anticancer activity, are Mediterranean medicinal and aromatic plants that contain rosmarinic acid (RA; Fig. 1) as major phenolic compound in their water extracts. RA constitutes about 58% of all phenolic compounds present in SF water extract and 70% in SO water extract (25,26). This phenolic

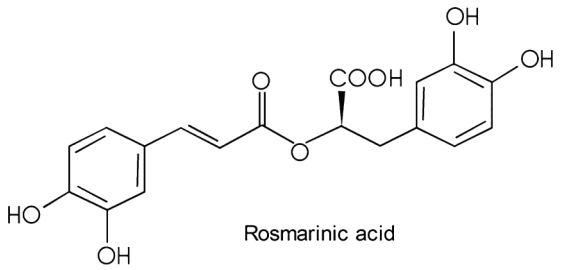

FIG. 1. Chemical structure of rosmarinic acid.

compound has high antioxidant and anti-inflammatory activities (22,27), but little is known about its effects on cancer cells and especially on CRC.

In the present article, we report on the antiproliferative and proapoptotic effects of 2 *Salvia* water extracts, SF and SO, and their major phenolic compound, RA, in 2 human colon cancer-derived cell lines, HCT15 and CO115, through effects on the MAPK/ERK and PI3K/Akt pathways and caspase-mediated apoptosis. These 2 cell lines possess different activating mutations in these 2 pathways: HCT15 has a KRAS (G13D) mutation (28), whereas CO115 has a BRAF (V599E) mutation (29).

In view of these genetic differences, we further speculate on the mechanisms behind the antiproliferative and proapoptotic effects of sage extracts and RA and the involvement of PI3K/Akt and MAPK/ERK signalling pathways in these effects.

MATERIAL AND METHODS

Reagents and Plant Extracts

All reagents and chemicals used were of analytical grade. Wortmannin (W), RA, and staurosporine were purchased from Sigma-Aldrich (St. Louis, MO) and PD-98059 (PD) was from Calbiochem (San Diego, CA). The primary antibodies anti-phospho-Akt (Ser473), anti-Akt total, anti-phospho-PTEN (Ser380/Thr382/383), anti-PTEN total, anti-p44/42 MAPK total, and anti-cleaved caspase-9 (Asp315) were purchased from Cell Signalling (Danvers, MA); the anti-phospho-ERK and caspase-3 (H-277) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and the anti-β -actin was from Sigma-Aldrich. The secondary antibodies HRP donkey antirabbit and sheep antimouse were from GE Healthcare (Bucks, United Kingdom).

The water extracts of Salvia fruticosa and Salvia officinalis were prepared as previously described by Lima et al. (30) by pouring boiling water onto the dried plant material (at a ratio of 150 ml of water to each 2 g of plant) and allowing it to steep for 5 min. After filtering, the water extract was lyophilized to dryness. The extracts of both sages were made using batches of the plants whose composition, in terms of phenolics compounds, have already been published (25,26). In brief, SF water extract contains as major phenolic compound RA (71.5 μg/ml), 6-hydroxyluteolin-7-glucoside (22.7 μg/ml), a not identified flavone heteroside (28.6 μg/ml), and the remaining phenolic compounds represent 0.8 μg/ml. SO water extract contain as major phenolic compounds RA (52.0 μg/ml), luteolin-7-glucoside (19.7 μg/ml) and the remaining phenolic compounds represent 2.7 μg/ml.

Stocks solutions of PD and W were made in dimethyl sulfoxide (DMSO), and aliquots were kept at –20°C. Therefore, DMSO (0.5%) was included in cell culture for the other conditions (controls and extracts/RA) to exclude any possible DMSO effect.

Cell Culture

HCT15 and CO115 human CRC-derived cell lines were a gift from Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained in culture at 37°C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 0.1 mM pyruvate, 1% antibiotic-antimycotic solution (Sigma-Aldrich), and 10% fetal bovine serum (FBS; EU standard; Cambrex, Verviers, Belgium). Cells were seeded onto 6-well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/well. Incubations with different concentrations of sage extracts and RA were performed in serum free medium for 48 h to quantify BrdU incorporation and TUNEL positive cells and for 24 h for Western blot analysis.

Assessment of Proliferation by BrdU Incorporation

Preliminary experiments using the MTT assay were performed in order to choose concentrations of SF and SO extracts that inhibited around 50% cell proliferation without cytotoxic effects. RA was tested in similar concentrations to the ones found in the extracts at the concentrations used and also did not induce cytotoxic effect. After 45 h of treatment with sage extracts or RA at different concentrations, bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the culture medium to give a final concentration of 10 μM and then incubated for another 3 h. Both adherent and nonadherent cells were collected from each sample, fixed with 4% paraformaldehyde for 15 min at room temperature, and then attached into a polylysine-treated slide using a Shandon Cytospin (Thermo Fisher Scientific Inc, Waltham, MA). Cells were incubated with HCl 2 M for 20 min, washed in PBS containing 0.5% Tween-20 and 0.05% BSA (TPBS-B) and then incubated with monoclonal mouse anti-BrdU antibody (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature. After washing in TPBS-B, cells were incubated with antimouse IgG FITC-conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature, washed again, and then incubated with Hoechst for nuclei staining. The percentage of proliferating cells was calculated as the ratio between BrdU positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least 3 independent experiments.

Assessment of Apoptosis by TUNEL Assay

Cells treated as above for 48 h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for

15 min at room temperature and then attached into a polylysine-treated slide using a Shandon Cytospin. Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany) following the manufacture's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst) from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least 3 independent experiments.

Protein Extraction and Western Blotting

After 24 h of treatment with sage extracts or RA at the highest concentration used in the BrdU and TUNEL assay, cells were washed with PBS and lysed for 15 min at 4°C with ice cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA), supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with BSA as a protein standard. Twenty micrograms of protein for each sample were separated by SDS gel electrophoresis and then electroblotted to a Hybond- P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (wt/vol) non-fat dry milk or BSA, incubated with the primary antibody, and followed by the secondary antibody conjugated with IgG horseradish peroxidase. Membranes were washed 3 times with TPBS between the different incubations. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β -actin was used as a loading control. Results are presented as mean ± SEM of at least 3 independent experiments.

Statistical Analysis

One-way ANOVA followed by the Student–Newman–Keuls test was used to perform statistical analysis for BrdU, TUNEL, and Western blot data. GraphPad Prism 4.0 software (San Diego, CA) was used, and P values ≤ 0.05 were considered statistically significant.

RESULTS

Effects on Cell Proliferation

To test the effects of SF, SO, and RA on cell proliferation of human colon cancer cells, 2 different colon carcinoma-derived cell lines, HCT15 and CO115, were used.

Based on preliminary experiments using the MTT assay (data not shown) in which cells were incubated with several concentrations of sage extracts for 48 h, concentrations of each extract that were not cytotoxic and inhibited cell proliferation around 50% were chosen for the subsequent studies. Since RA is the main phenolic compound of these extracts, we also tested RA in similar concentrations to the ones found in the extracts under our experimental conditions.

The effects of sage extracts and RA on cell proliferation of both cell lines were tested using the BrdU incorporation assay. As shown in Fig. 2A, a significant inhibition of HCT15 cell proliferation by both SF and SO was observed at all concentrations tested. Levels of BrdU incorporation significantly decreased from 26.2% in the control to 4.7% in HCT15 cells treated with 50 μg/ml of SF and SO extracts. In CO115 cells, SF and SO did not significantly inhibit cell proliferation (Fig. 2B). No significant inhibition of cell proliferation was observed in neither of the cell lines when treated with RA (Fig. 2). Comparing the effects of sage extracts in the 2 cell lines, we observed that SF extract was somewhat more active than SO, and HCT15 cells were more sensitive to the sage extracts.

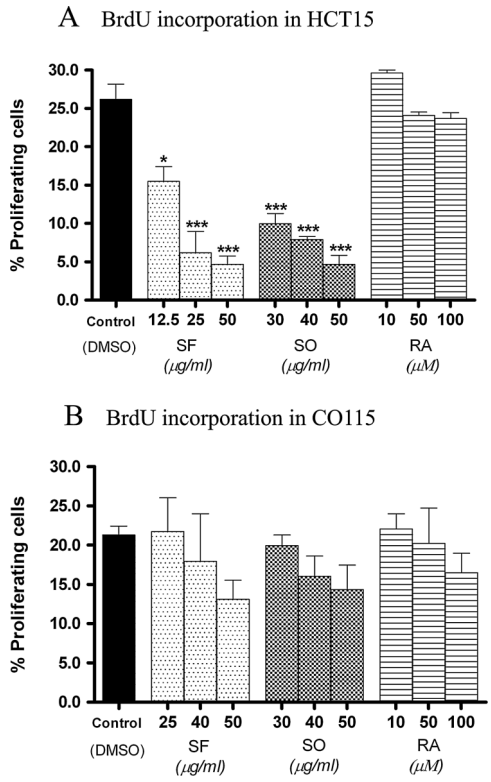

FIG. 2. Effect of different concentrations of *Salvia fruticosa* (SF), *Salvia officinalis* (SO), and rosmarinic acid (RA) for 48 h on bromodeoxyuridine (BrdU) incorporation in A: HCT15 and B: CO115 cells. Values are mean ± standard error of the mean of at least 3 independent experiments. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ when compared to control. DMSO, dimethyl sulfoxide.

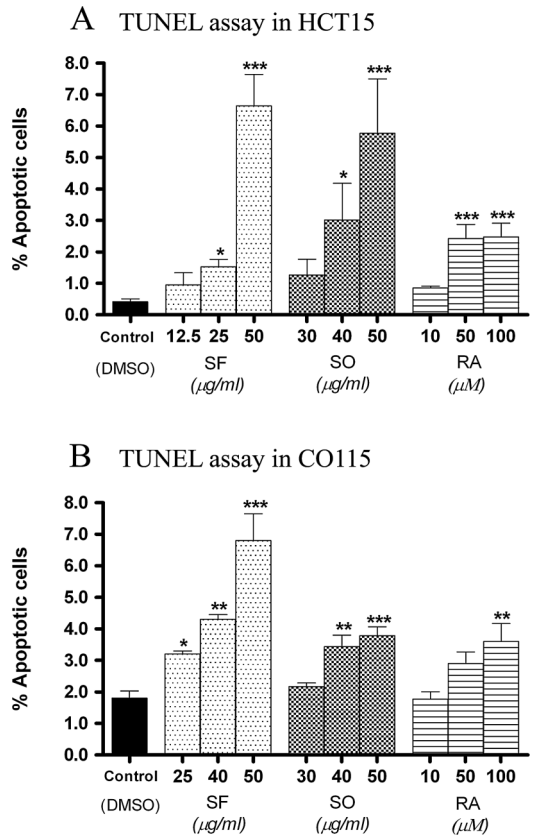

FIG. 3. Effect of different concentrations *Salvia fruticosa* (SF), *Salvia officinalis* (SO), and rosmarinic acid (RA) on apoptosis for 48 h as assessed by the TUNEL assay of A: HCT15 and B: CO115 cell lines. Values are mean ± standard error of the mean of at least 3 independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ when compared to control. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DMSO, dimethyl sulfoxide.

Effects on Apoptosis

The ability of SF, SO, and RA to induce apoptosis in human CRC-derived cells were studied using the TUNEL assay. As shown in Fig. 3, both *Salvia* extracts and RA significantly induced apoptosis in a concentration-dependent manner in both HCT15 and CO115 cells. Apoptotic cells in HCT15 increased from 0.4% in the control to 6.6%, 5.8%, and 2.5% in SF, SO, and RA treatments, respectively, at the higher concentrations tested (Fig. 3A). In CO115 cells, apoptotic cells increased from 1.8% in the control to 6.8%, 3.8%, and 3.6% in the conditions treated with the higher concentrations tested of SF, SO, and RA, respectively (Fig. 3B). Since the basal levels of apoptosis were higher in the CO115 cell line, overall it seems that the HCT15 cells were more sensitive to the extracts and RA. Again, SF extract showed to be more active than SO extract and RA alone.

The involvement of caspases 3 and 9 in the apoptosis induction by sage extracts and RA was also studied by Western blot. After 24 h of treatment with the highest concentrations used of SF, SO, and RA, we did not observe cleaved caspase-9 and caspase-3 in either cell line in contrast with the reference compound, staurosporine (data not shown).

Effects on MAPK/ERK Pathway

The effects of sage extracts and RA for 24 h were studied on the MAPK/ERK pathway by Western blot. *Salvia* extracts and RA significantly decreased phospho-ERK protein levels in HCT15 cells (Fig. 4A), whereas no effects were observed in CO115 cells (Fig. 4B). The reference inhibitor of phospho-ERK, PD-98059 (PD), was effective in both cell lines (Fig. 4) in a similar way to SF, SO, and RA in HCT15 cells.

Effects on PI3K/Akt Pathway

The effects of sage extracts and RA on the expression of phospho-Akt and phospho-PTEN (a negative regulator of PI3K/Akt pathway) were also tested. Phospho-Akt was observed in CO115; however, it was not detected in HCT15 in medium with and without serum (data not shown). Neither of the *Salvia* extracts nor RA inhibited significantly the expression of phospho-Akt in CO115 cells (Fig. 5A). A significant inhibition of Akt phosphorylation was observed for the reference PI3K inhibitor, wortmannin (W). HCT15 cells expressed phospho-PTEN, and this expression was not significantly changed by *Salvia* extracts, RA or W (Fig. 5B). CO115 cells did not express phospho-PTEN or total PTEN in medium with and without serum (data not shown).

DISCUSSION

To assess the potential of sage in the control of CRC progression, the antiproliferative and proapoptotic effects of *Salvia fruticosa* (SF) and *Salvia officinalis* (SO) water extracts and their main phenolic compound, rosmarinic acid (RA), were studied in two human CRC-derived cell lines, HCT15 and CO115. Both sage water extracts (SF and SO) were effective in inhibiting proliferation in a concentration-dependent manner in HCT15 but not in CO115 cells. SF, SO, and RA induced apoptosis. SF was more effective than SO with regard to both antiproliferative and proapoptotic effects. To identify the bioactive compound behind these effects, sage's major phenolic compound (RA) was tested individually at concentrations similar to those present in the extracts. However, RA was found not to have antiproliferative activity but to be proapoptotic in both cell lines, although to less extent than sage extracts. In view of these results, it seems that other active compounds present in the extracts may be responsible for the antiproliferative and proapoptotic effects of SF and SO.

The 2 cell lines used harbor different activating mutations: HCT15 has a KRAS (G13D) activating mutation (28) with potential to constitutively activate both PI3K/Akt and MAPK/ERK pathways, whereas CO115 harbors a BRAF (V599E) mutation (29) that affects the MAPK/ERK pathway. The highest sensitivity of HCT15 could be a result of these genetic differences. HCT15 cells, even though presenting an activating mutation of

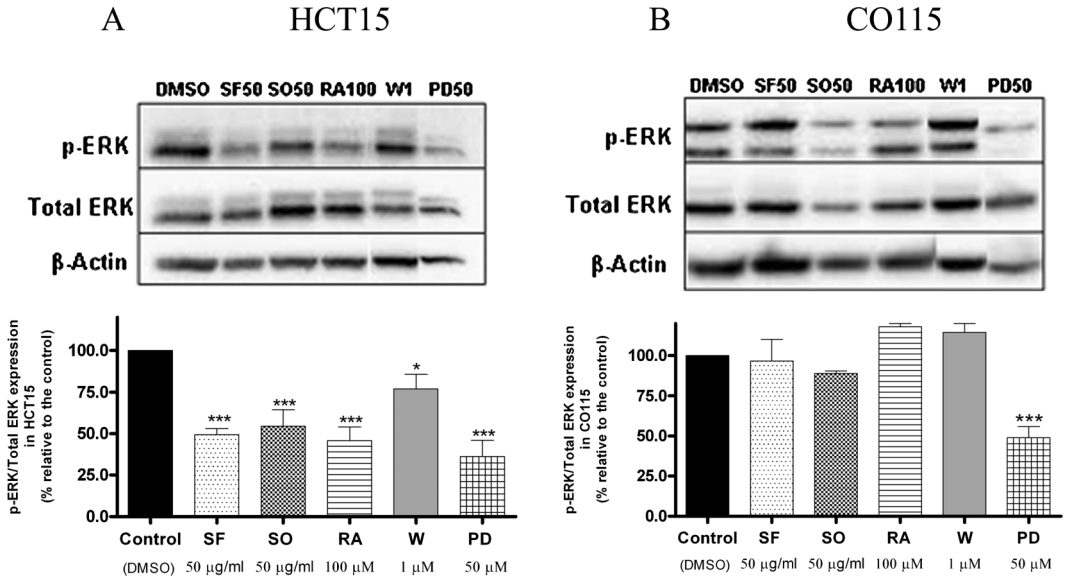

FIG. 4. Effects of *Salvia fruticosa* 50 μg/ml (SF50), *Salvia officinalis* 50 μg/ml (SO50), and rosmarinic acid 100 μM (RA100) for 24 h on the expression of phospho-extracellular-regulated kinase (p-ERK) in A: HCT15 cells and B: CO115 cells. PD-98059 50 μM (PD50) was used as a reference inhibitor of p-ERK. Values are mean ± standard error of the mean of at least 3 independent experiments. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ when compared to control. DMSO, dimethyl sulfoxide; W, wortmannin.

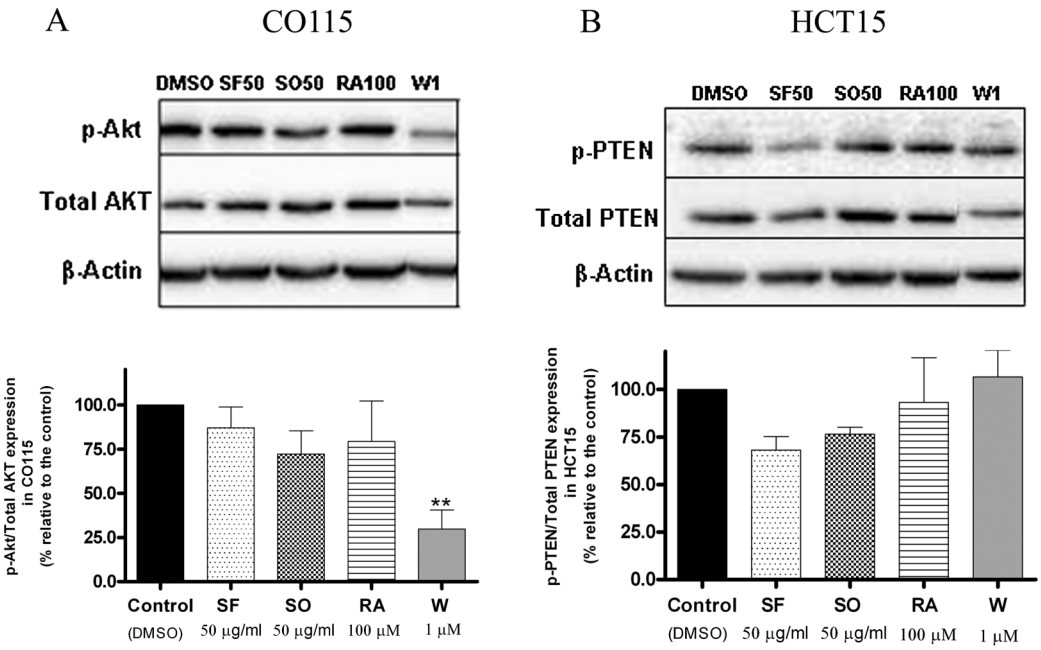

FIG. 5. Effects of *Salvia fruticosa* 50 μg/ml (SF50), *Salvia officinalis* 50 μg/ml (SO50), and rosmarinic acid 100 μM (RA100) for 24 h on the expression of phospho-v-akt murine thymoma viral oncogene homolog (p-Akt) in A: CO115 cells and phosphophosphatase and tensin homolog (p-PTEN) in B: HCT15 cells. Wortmannin 1 μM (W1) was used as a reference inhibitor of phosphatidylinositol 3 kinase. No p-Akt expression was observed in HCT15 cells, and no PTEN expression was observed in CO115 cells. Values are mean ± standard error of the mean of at least 3 independent experiments. $^{**}P \leq 0.01$ when compared to control. DMSO, dimethyl sulfoxide; W, wortmannin.

the RAS oncogene, did not express phospho-Akt possibly as a consequence of the high levels of the strong negative regulator of this pathway, phospho-PTEN, found in this cell line. In these cells, the antiproliferative effects of SF and SO correlate with an inhibition of phospho-ERK. However, RA showed a significant inhibition of phospho-ERK without inhibiting HCT15 cell proliferation. Inhibition of phospho-ERK seems, therefore, not to be the only factor involved in inhibition of cell proliferation in this cell line. Our findings are in agreement with previous studies (6,31), which have shown that an inhibition of MAPK/ERK pathway in KRAS mutated cell lines is not sufficient to inhibit cell proliferation. Therefore, the KRAS mutated HCT15 cells do not depend exclusively on MAPK/ERK pathway to proliferate, and as a result, SF and SO seem also to be inhibiting other proliferation pathways, which in these cells do not include Akt phosphorylation (Fig. 6).

In CO115 cells, where SF and SO did not have antiproliferative effect, there was no inhibition of phospho-ERK or phospho-Akt. RA also did not inhibit proliferation of CO115 cells. However, in contrast to the effects on the other cell line, RA was without effect on phospho-ERK. Inhibition of MAPK/ERK pathway by sage extracts and RA in HCT15 and not CO115 indicates that the effect may be upstream of BRAF and could be on KRAS (Fig. 6). In CO115 cells, a potential inhibition of RAS by sage extracts would not result in antiproliferative effects due to the downstream activating mutation of BRAF (Fig. 6). An inhibition of RAS oncogene has also been recently shown for quercetin, a common, natural-occurring, phenolic compound (32,33). It seems that the effects of RA depend on cell type and/or genetic background because others have also shown that RA decreases ERK phosphorylation in cardiac muscle cells, but it is without effect on Akt and ERK in melanoma cells (34,35).

SF, SO, and RA induced apoptosis in both cell lines. It seems, however, that under these conditions, apoptosis is not dependent on the cleavage of either caspase-9 or caspase-3 in both cell lines. Nevertheless, some authors have shown that RA promotes apoptosis in human Jurkat cells and HepG2 cells via the mitochondrial pathway and Bcl-2 suppression in which caspases are involved (36–38). Also, the mitochondrial pathway was induced by RA in activated T cells from rheumatoid arthritis patients (39). It seems, therefore, that the induction of caspase pathways by RA is cell type specific and/or dependent on concentration and time of exposure, which may explain the discrepancy between these and our results. The inhibition of MAPK/ERK pathway may contribute, at least in part, to the effects on apoptosis in HCT15 cells.

Besides a possible interaction with KRAS, sage extracts may act as antiproliferative and proapoptotic in these cancer cell lines through their antioxidant activity. It is known that cancer cells produce increased amounts of ROS, in particular hydrogen peroxide, which could inhibit protein fosfatases and also be associated with signalling events in MAPK pathways that lead to activation of redox-sensitive transcription factors, mediating cancer cell proliferation and survival (23,24). Therefore, the radical scavenging activity of the phenolic compounds present in the sage extracts may be reducing the ROS levels in these cancer cells contributing also to a decreased activity of redox-sensitive proliferative pathways through RAS signalling. Based on RA results, the effects described in this study seem, however, not to be totally explained by the antioxidant properties of the sage extracts.

In conclusion, our results show that SF and SO water extracts inhibit proliferation and induce apoptosis in CRC-derived cell lines, whereas RA was only effective on the induction of

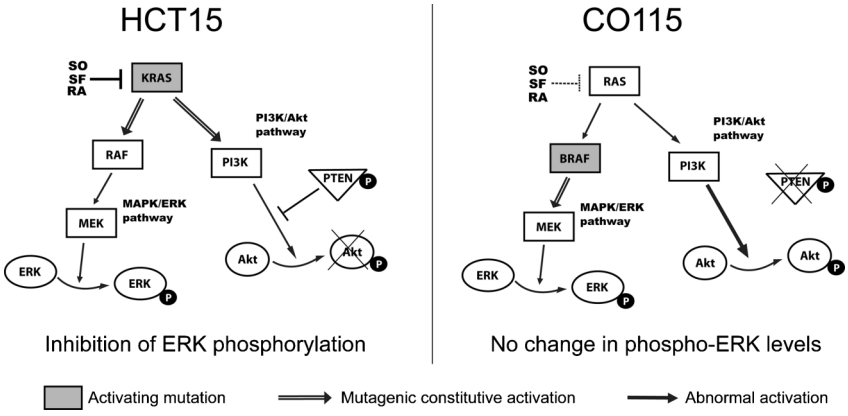

FIG. 6. Model for the inhibition of extracellular-regulated kinase (ERK) phosphorylation by *Salvia fruticosa* (SF), *Salvia officinalis* (SO), and rosmarinic acid (RA) in HCT15 but not in CO115 cells. SF, SO, and RA inhibit mutant V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) leading to a decrease on the levels of phospho-ERK in HCT15 cell line. In CO115 cells, SF, SO, and RA do not change ERK phosphorylation levels due to a V-raf murine sarcoma viral oncogene homolog B1 (BRAF) activating mutation downstream of RAS oncogene. The missing phosphatase and tensin homolog (PTEN) in CO115 cells and phospho-v-akt murine thymoma viral oncogene homolog (Akt) in HCT15 cells were also observed in this study. MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3 kinase.

apoptosis. Sage extracts and RA did not affect the PI3K/Akt pathway but inhibited the MAPK/ERK pathway in the KRAS mutated HCT15 cell line. The inhibitory effects of sage extracts on phospho-ERK seem to result from an inhibition of KRAS, upstream to BRAF, because it was not observed in CO115 cells. The inhibition of MAPK/ERK by sage extracts seems, however, not to completely explain the inhibition of cell proliferation in HCT15 because RA inhibits phospho-ERK without affecting cell proliferation. These data add S. fruticosa and S. officinalis to the list of potential sources of new active anticancer compounds useful in particular in tumors with a mutagenic KRAS activation and also suggest their possible use in dietary strategies for the control of CRC progression.

ACKNOWLEDGMENTS

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CHAPTER III

ANTICARCINOGENIC EFFECTS OF *HYPERICUM* EXTRACTS

Hypericum androsaemum water extract inhibits mutant BRAF with inhibition of human colorectal cancer cells proliferation

The work presented in this chapter is submitted to *Journal of Biomedicine and Biotechnology*:

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Hypericum androsaemum water extract inhibits mutant BRAF with inhibition of human colorectal cancer cells proliferation

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Abstract

MAP kinase and PI3K/Akt signalling pathways are commonly altered in colorectal carcinoma (CRC) leading to increased proliferation and inhibition of apoptosis. Several species of the genus *Hypericum* are medicinal plants to which digestive tract effects have been attributed. In the present study, the antiproliferative effects of the water extracts of *H. androsaemum* (HA), *H. perforatum* (HP) and *H. undulatum* (HU) were investigated in two human colon carcinoma-derived cell lines, HCT15 and CO115, which harbour activating mutations on KRAS and BRAF, respectively. Contrarily to HU and HP, HA significantly inhibited cell proliferation and induced apoptosis in both cell lines. HA decreased BRAF and phospho-ERK expressions in CO115 cells, but not in HCT15. HA also decreased Akt phosphorylation in CO115, suggesting an inhibition of PI3K/Akt pathway. Furthermore, an induction of p38 and JNK stress-activated kinases were observed in both cell lines. Chlorogenic acid, the main phenolic compound present in the HA extract and less represented in the other two species, did, however, not show any of those effects. In conclusion, HA controlled CRC proliferation and specifically acted on mutant BRAF.

Keywords: Chlorogenic acid, Colorectal Carcinoma, *Hypericum androsaemum*, MAP kinases, PI3K/Akt Pathway

Introduction

Environmental factors, many of which diet related, are responsible for 70-80% of total cases of colorectal carcinoma (CRC), an important health problem worldwide [1, 2]. Activating mutations of KRAS, BRAF and/or PI3K have been found in more than 50% of CRC cases and constitutively activate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and/or the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathways [3, 4]. The constitutive activation of these pathways plays an important role during CRC progression and results in a higher cell proliferation rates and in inhibition of apoptosis [3, 5-7]. Since MAPK/ERK and PI3K/Akt pathways are involved in CRC progression and drug resistance, proteins associated with these pathways are good molecular therapeutic targets for drug discovery [8, 9].

The stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 are two other major MAPK pathways also frequently deregulated in cancers, including CRC [9, 10]. These pathways are activated by diverse cellular stresses including UV light, X-rays, hydrogen peroxide, heat and osmotic shock and have been implicated in the control of cell proliferation, differentiation, migration, apoptosis and survival and their effects appear to be largely dependent on cell type and/or cellular context [10, 11]. In particular, JNK activation is involved in the control of cell growth and proliferation, as well as apoptosis, since JNK may induce activation of the mitochondrial pathway [11, 12]. Activation of the p38 pathway has also been shown to promote growth arrest, to induce apoptosis by activating p53 and to block tumor growth. On the other hand, p38 inactivation has been shown to induce cell proliferation and enhance cellular transformation [13, 14]. Therefore, p38 and JNK MAP kinases may also be considered as potential targets for cancer therapy [10].

Plants of the genus *Hypericum* (family Hypericaceae) have been attributed important medicinal properties. *Hypericum perforatum* (HP), also known as St. John's wort, is the species more studied and it is known for its high pharmacological activities, such as antidepressant, antiviral and antibacterial properties [15]. The anticarcinogenic activity of HP has also been reported in several cancer cell types, but not in CRC cells [16-19]. Its antitumor effects have been related with one of its main constituents, hypericin [20-22]. *Hypericum androsaemum* (HA) is less known and it is used as diuretic, hepatoprotector, cholagogue, and also in kidney failure and in the relief of

digestive tract disorders [23, 24]. Its anticarcinogenic activity has, to our knowledge, never been reported. Recent *in vitro* studies showed the antioxidant and hepatoprotective activities of HA water extract [25, 26]. The effects of HA have been attributed, at least in part, to the presence of several flavonoids, such as quercetin and its glycosides, and phenolic acids, such as chlorogenic acid [26]. *Hypericum undulatum* (HU) is the least known of the three species. Antioxidant activity and effects on memory have been found [27]. Plants containing a variety of phenolic compounds have been shown to play an important role as dietary antioxidants in cancer prevention [28, 29]. However, evidence is increasing that the anticarcinogenic properties of plant food constituents is not only the result of their antioxidant activity. In fact, many of these natural compounds have been demonstrated to act on multiple key elements in signalling transduction pathways related to cellular proliferation and apoptosis [30, 31].

Since *H. androsaemum*, *H. undulatum* and *H. perforatum* (HP) are popularly consumed as herbal tea (water extract) for the relief of digestive tract disorders and they contain quercetin (mainly as glycosides), which we have shown in a previous study to possess anticarcinogenic activity against colon cancer cells [32], the antiproliferative and proapoptotic effects of the water extracts of these three *Hypericum* plants were tested in two human colon cancer-derived cell lines, HCT15 and CO115. These cell lines harbour different activating mutations that affect both MAPK/ERK and/or PI3K/Akt pathways: HCT15 has a KRAS (G13D) mutation [33] while CO115 harbour a BRAF (V599E) mutation [34], being representative of many CRC cases. The involvement of stress induced kinases p38 and JNK, and apoptotic markers were also studied.

Materials and methods

Reagents and Antibodies

All reagents and chemicals used were of analytical grade. Wortmannin (W), LY-294,002 (LY), staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and chlorogenic acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); PD-98059 (PD) was from Calbiochem (San Diego, CA, USA). Stocks solutions of W, LY, PD and STS were made in dimethyl sulfoxide (DMSO) and aliquots were kept at -20 ºC. DMSO (0.5%, final concentration) was used in the other conditions (control and HA extract alone) to exclude any solvent effect.

The primary antibodies, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-PTEN (Ser380/Thr382/383), anti-PTEN, anti-p44/42 MAPK and anti-phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling (Danvers, MA, USA); the anti-phospho-ERK, anti-Raf-B, anti-K-Ras, anti-PKC total, anti-PARP-1, anti-p38, anti-phospho-JNK and anti-JNK were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); the anti-caspase-3 was from Calbiochem (San Diego, CA); and the anti-β-actin from Sigma-Aldrich. The secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were from GE Healthcare (Bucks, UK).

Cell culture and conditions

HCT15 and CO115 human colon carcinoma-derived cell lines were a gift from Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal) and were maintained in culture at 37 ºC in a humidified 5 % CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 0.1 mM pyruvate, 1 % antibiotic-antimycotic solution (Sigma-Aldrich) and 6 % fetal bovine serum (FBS; EU standard, Cambrex, Verviers, Belgium). Cells were seeded onto six (2 ml) and twelve (1 ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml. Incubations for 48 h with different concentrations of the water extracts were performed for MTT and TUNEL analysis, and for 24 h and 48 h for western blot.

Preparation of HA extract

Plant of *Hypericum androsaemum* (HA) was cultivated at Canidelo, Northern of Portugal, in a farm owned by Cantinho das Aromaticas Lda., whereas *Hypericum perforatum* (HP) and *Hypericum undulatum* (HU) were obtained from Mapprod Lda., Braga, Portugal; plants are kept in active bank under the responsibility of the respective companies. The aerial parts of the plants were collected in July 2008 for HA and HU, and in July 2009 for HP; then, they were air-dried before being subjected to the water extraction by infusion. Batches of dried plant material are maintained at -20 ºC under the responsibility of CITAB with the accession numbers HA102008, HP072009 and HU122008, for HA, HP and HU, respectively. The plant infusions were prepared by pouring 150 ml of boiling deionized water onto 2 g of air-dried plant material and allowing it to steep for 5 min. After filtering, the water extracts were lyophilized to dryness and yields in terms of initial crude plant material dry weight of 27.0% (w/w), 16.7% (w/w) and 13.0% (w/w), for HA, HP and HU, respectively, were obtained.

Phenolic compounds were analyzed by HPLC as previously performed [23] and, for HA, a similar composition with a previous report of a water extract was obtained [26]. The main phenolic compounds found in the plant water extracts differ in quantity between them. The following compounds are present. In HA: chlorogenic acid (CA) and isomer (3-*O* and 5-*O*-caffeoylquinic acid; 53.82 μg/mg), quercetin 3-galactoside (16.35 μg/mg), quercetin 3-glucoside (5.41 μg/mg), quercetin 3-rutinoside (2.73 μg/mg) and quercetin (1.32 μg/mg). In HU: quercetin 3-galactoside (34.12 μg/mg), quercetin 3-glucoside (21.70 μg/mg), chlorogenic acid (CA) and isomer (3-*O* and 5-*O*-caffeoylquinic acid; 14.85 μg/mg), quercetin (4.97 μg/mg), quercetin 3-rhamnoside (1.97 μg/mg), amenthoflavone (0.30 μg/mg) and hypericin (0.03 μg/mg). In HP: quercetin 3-rutinoside (38.07 μg/mg), quercetin 3-galactoside (16.00 μg/mg), quercetin 3-glucoside (6.47 μg/mg), chlorogenic acid (CA) and isomer (3-*O* and 5-*O*-caffeoylquinic acid; 5.75 μg/mg), quercetin (3.39 μg/mg), quercetin 3-rhamnoside (0.24 μg/mg), amenthoflavone (0.33 μg/mg) and hypericin (0.03 μg/mg). CA is much more abundant in HA water extract than in the other two species where quercetin and related compounds are the most representative.

Cell proliferation/viability assay

MTT reduction assay was used as previously described [32]. Cells were treated with different concentrations of water extracts and CA for 46 h followed by two hours in the presence of MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04 M in isopropanol was then used to dissolve the formazan crystals. The number of viable cells in each well was estimated by spectrophotometry. Results are presented as mean ± SEM of at least three independent experiments.

Assessment of apoptosis by TUNEL assay

Cells treated with different concentrations of HA for 48 h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for 15 min at room temperature and then attached into a polylysine treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA). Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from

the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Protein extraction and western blot

After incubation periods, cells were first washed with PBS and then lysed for 15 min at 4 ºC with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA), supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with BSA as a protein standard. Twenty micrograms of protein from each sample were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA, incubated with the primary antibody followed by the secondary antibody conjugated with IgG horseradish peroxidase. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as a loading control. Results are presented as mean ± SEM of at least three independent experiments.

Statistical analysis

Student's t-test or one-way ANOVA followed by the Student-Newman-Keuls test was used to perform statistical analysis for TUNEL and western blot data. GraphPad Prism 4.0 software (San Diego, CA, USA) was used and *P*-values ≤ 0.05 were considered statistically significant.

Results

Anticarcinogenic effects of three *Hypericum* species

The effects of water extracts from *H. undulatum*, *H. perforatum* and *H. androsaemum* on cell viability/proliferation, in HCT15 and CO115 human colon carcinoma-derived cell lines, were investigated using the MTT assay. As shown in

Figure 1A, HCT15 cells were more resistant to HU extract than CO115 cells, while having the same sensitivity to the HP extract. However, effects on cell viability of both cell lines incubated with HU and HP were only observed from concentrations above 200 µg/ml. The HA extract was the most efficient in inhibiting cell proliferation in a concentration-dependent manner in both cell lines, with an IC50 (the concentration that inhibited cell growth by 50%) of around 85 µg/ml in HCT15 and 65 µg/ml in CO115 cells. The higher concentration tested of HA in HCT15 cells induced cell death by necrosis (negative value in Fig. 1A). Incubation of CRC cells with HA also significantly induced apoptosis in a concentration-dependent manner in both cell lines (Figure 1B). HA was the only extract with anticarcinogenic potential in CRC.

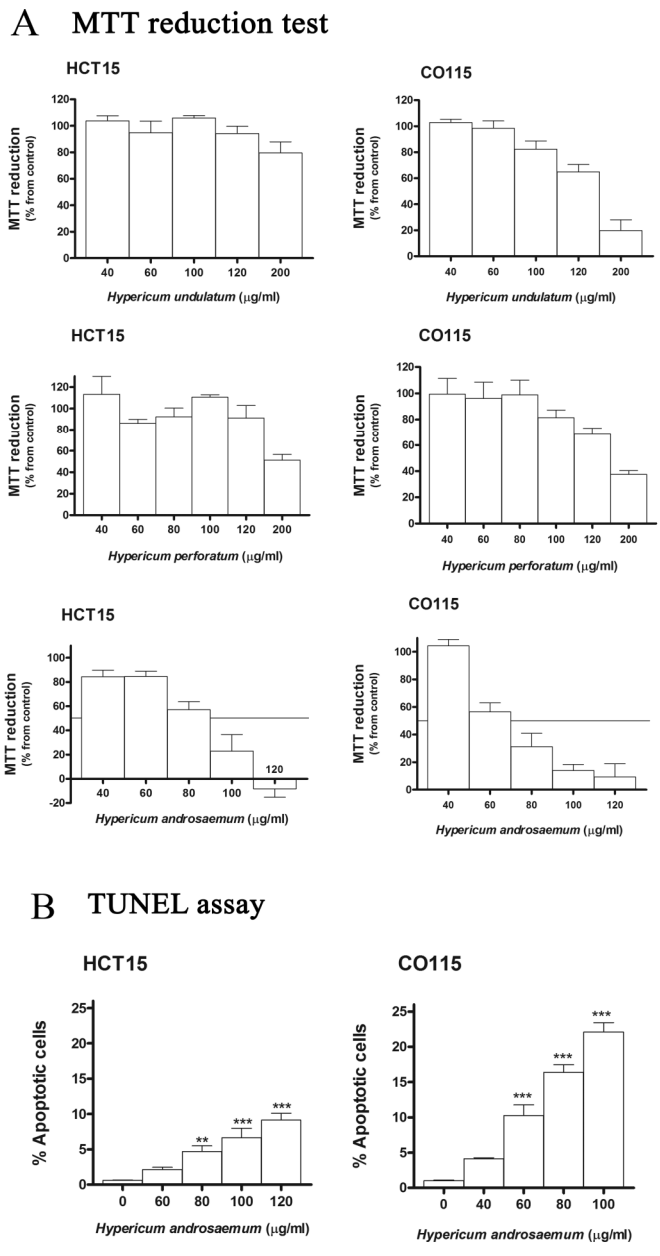

Figure 1. Effects of different concentrations of water extracts of *Hypericum undulatum*, *Hypericum perforatum* and *Hypericum androsaemum* on cell viability/proliferation assessed by MTT reduction (A). Effect of *Hypericum androsaemum* on apoptosis assessed by TUNEL assay (B), for 48 h, in HCT15 and CO115 cells. Values are mean ± SEM of at least 3 independent experiments. ** P≤ 0.01 and *** P≤ 0.001 when compared to control. In A: line represents the inhibition of 50% of cell proliferation (IC50); the negative value mean that the cells reduction capacity after 48 h in that condition was below than the one obtained in the control in the beginning of the treatment period (0 h), being a indirect indication of cell death by necrosis.

In order to characterize effects of HA on signaling pathways related to proliferation and/or apoptosis, the IC50 concentration for each cell line (85 µg/ml for HCT15 and 65 µg/ml for CO115) and a concentration below this were used and the effects on the levels of relevant molecular targets followed in subsequent experiments by western blot.

Effect of chlorogenic acid (CA) on cell proliferation

H. androsaemum was the most efficient plant water extract in inhibiting cell growth in HCT15 and CO115 CRC cell lines. In an attempt to find the compound responsible for these effects, the main phenolic present in this extract, chlorogenic acid (CA), which also distinguishes this extract from those of the other two plants, was evaluated individually at different concentrations, using MTT assay. CA is present at about 54 μg/mg in HA water extract, while in HU and HP CA is present in smaller amounts (about 15 and 6 μg/mg, respectively). No effects were observed on cell proliferation in both cell lines treated with CA up to 200 µM, which corresponds to a concentration 20 times higher than the one found in 100 µg/ml of HA (Figure 2). It seems therefore that the inhibition of cell proliferation produced by HA is not due to CA.

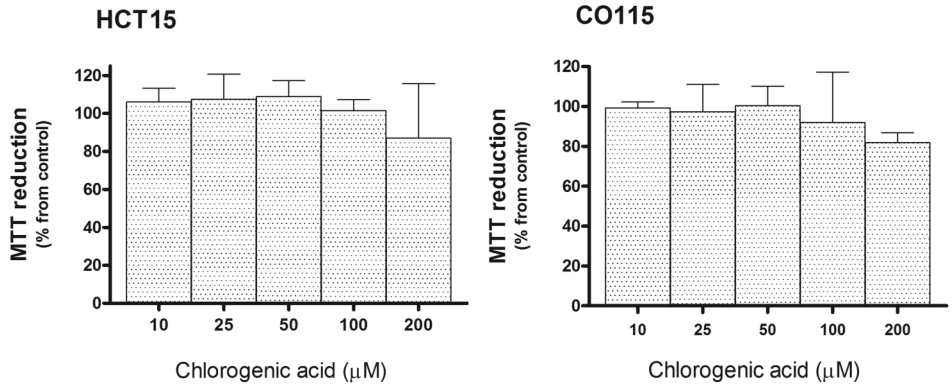

Figure 2. Effects of different concentrations of chlorogenic acid on cell viability/proliferation assessed by MTT reduction.

Effects of HA on PI3K/Akt and MAPK/ERK pathways

Constitutive activations of MAPK/ERK and PI3K/Akt pathways are present in a large number of CRC cases, leading to an increase of proliferation and an inhibition of apoptosis [3]. Phosphorylation of ERK and Akt, respectively, are indicators of their activation. As shown in Figure 3A, HA significantly decreased phospho-Akt protein

level in CO115 cells, in a concentration-dependent manner, after 24 h of incubation. This effect was not observed in HCT15 since there were no detectable amounts of phospho-Akt in these cells (data not shown), which is in agreement with previous observations [32]. As expected, reference inhibitors of PI3K, wortmannin (W) and LY-294,002 (LY), also significantly decreased phospho-Akt levels. The effect of HA on phospho-PTEN, a negative regulator of PI3K/Akt pathway, was also tested. As shown in Figure 3B, HA did not change phospho-PTEN levels in HCT15 cells. As previously reported [32], no detectable expression of PTEN was observed in CO115 cells (data not shown).

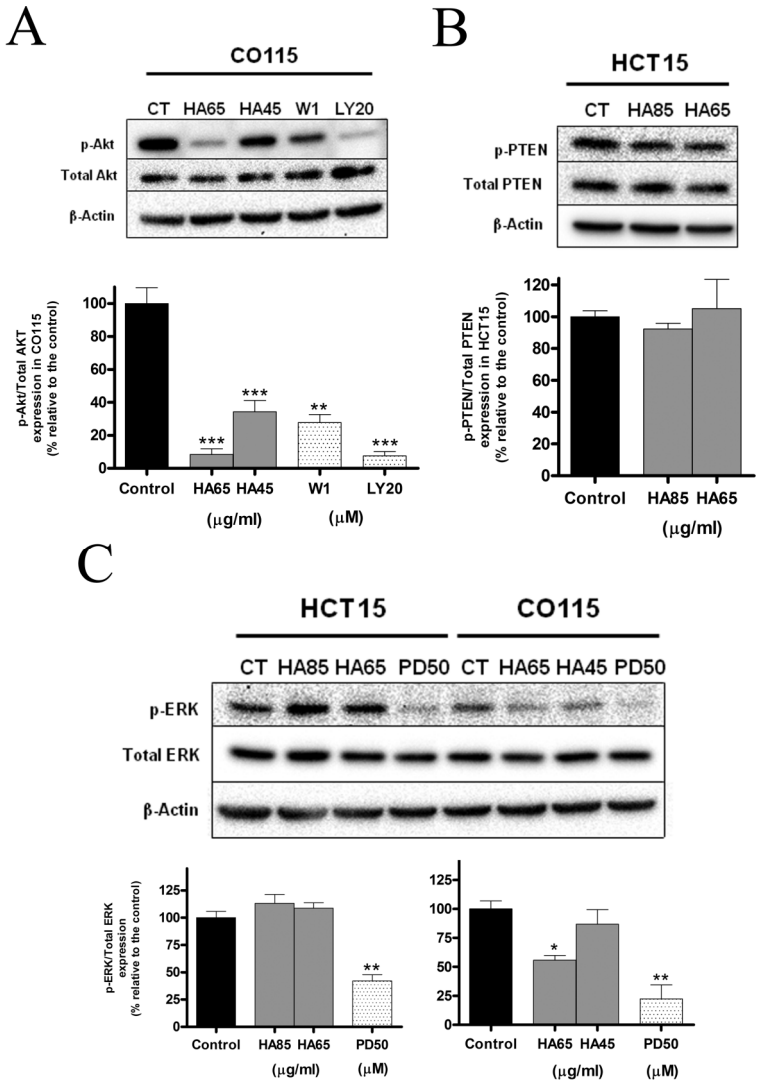

Figure 3. Effects of *Hypericum androsaemum* (HA) for 24 h on the levels of phospho-Akt in CO115 cells (A), phospho-PTEN in HCT15 cells (B) and phospho-ERK in HCT15 and CO115 cells (C) at 85 μg/ml (HA85), 65 μg/ml (HA65) and 45 μg/ml (HA45), using western blot. β-Actin was used as loading control. Wortmannin 1 μM (W1) and LY-294,002 20 μM (LY20) were used as a reference inhibitor of PI3K and PD-98059 50 μM (PD50) was used as a reference inhibitor of phospho-ERK. Values are mean ± SEM of at least 3 independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ when compared to control.

Concerning effects on the MAPK/ERK pathway, a significant decrease in phospho-ERK protein level was observed in CO115 cells, but not in HCT15, induced by the higher concentration of HA tested (Figure 3C). As expected, a significant reduction of phospho-ERK levels in both cell lines was also induced by PD-98059 (PD), a reference inhibitor of the MAPK/ERK pathway. In addition, we also treated both cell lines with CA. This compound did not decrease phospho-Akt or phospho-ERK protein levels at 10 and 100 µM in both cell lines (data not shown).

Effects of HA on BRAF and KRAS levels

Subsequently, since KRAS activates both MAPK/ERK and PI3K/Akt pathways and BRAF activates MAPK/ERK pathway, effects of HA on the protein expression of KRAS and BRAF oncogenes were studied. As shown in Figure 4A, the higher HA concentration tested was able to significantly decrease the levels of BRAF in CO115 cells (cells with mutant BRAF), after 24 h. In HCT15 cells, which express the wild type BRAF, no effect of HA on BRAF protein expression was observed. No significant changes were observed in wild type or mutant KRAS levels induced by HA (Figure 4B). CA did not change KRAS or BRAF levels at 10 and 100 µM in both cell lines (data not shown).

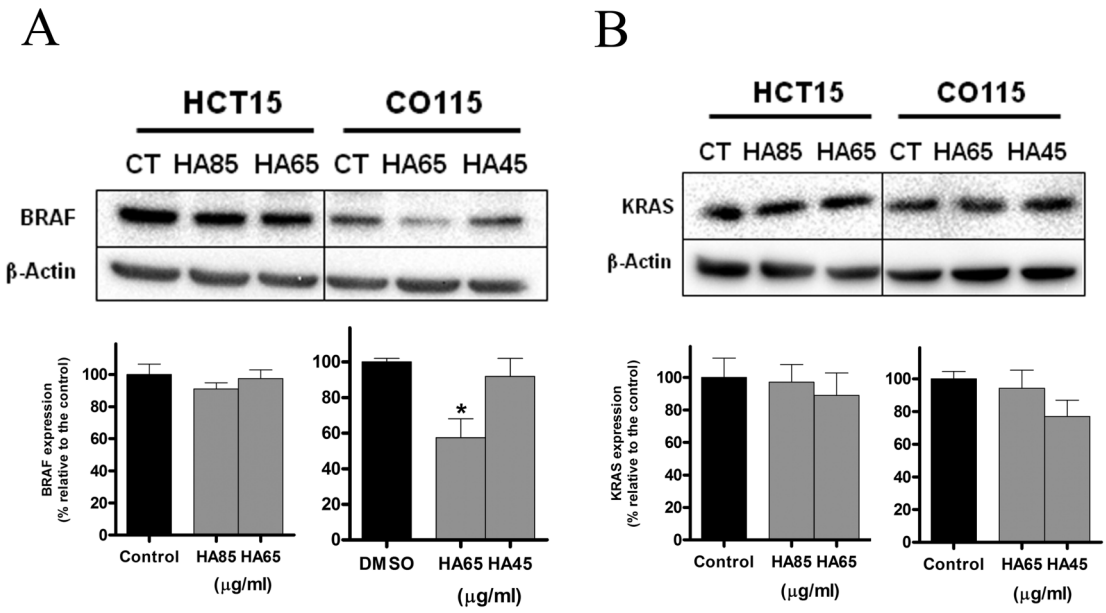

Figure 4. Effects of *Hypericum androsaemum* (HA) for 24 h on BRAF (A) and KRAS (B) levels in HCT15 and CO115 cells at 85 µg/ml (HA85), 65 µg/ml (HA65) and 45 µg/ml (HA45), using western blot. β-Actin was used as loading control. Values are mean ± SEM of at least 3 independent experiments. * P≤ 0.05 when compared to control.

Effects of HA on p38 and JNK pathways

The effect of HA on p38 and JNK signalling pathways, two stress-activated protein kinases that are involved in the control of proliferation and induction of apoptosis [10], were also studied. Our results show a remarkable induction of phospho-p38 expression at both concentrations tested and of phospho-JNK expression mainly at the higher concentration tested after 48 h in both cell lines (Figure 5A and 5B).

Effects of HA on Caspase-3 and PARP-1

In order to verify the role of caspase activation on the apoptotic effect of the extract HA, we studied caspase-3 and Poly (ADP-ribose) polymerase-1 (PARP-1) expressions by western blot. As shown in Figure 5C, HA water extract increased cleaved caspase-3 and cleaved PARP-1 in CO115 cells. In HCT15, we did not observe cleaved caspase-3 or cleaved PARP-1, although a decrease in total PARP-1 was detected. A higher expression level of total PARP-1 was observed in HCT15 as compared to CO115 cells. The cleavage of caspase-3 and PARP-1 were also induced by staurosporine, an apoptotic inducer used here as positive control, in both cell lines (data not shown).

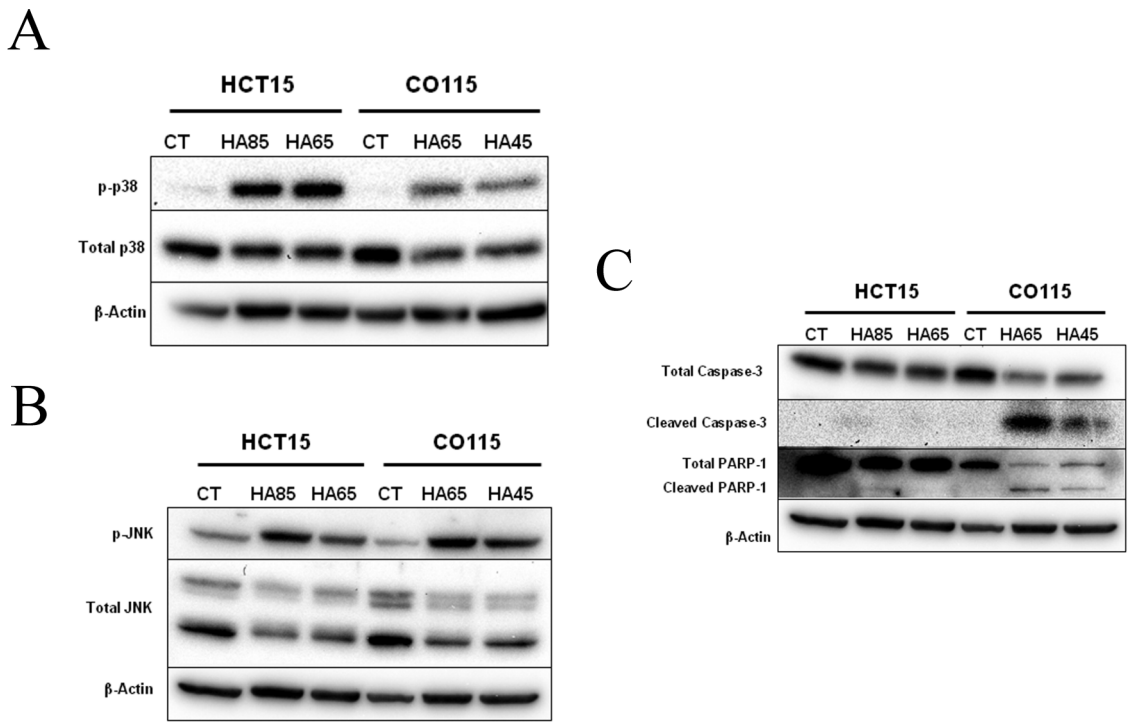

Figure 5. Effects of *Hypericum androsaemum* (HA) for 48 h on the levels of phospho-p38 (A), phospho-JNK (B) and caspase-3 and PARP-1 (C) in HCT15 and CO115 cells at 85 µg/ml (HA85), 65 µg/ml (HA65) and 45 µg/ml (HA45), using western blot. β-Actin was used as loading control. Images are representative of at least 3 independent experiments.

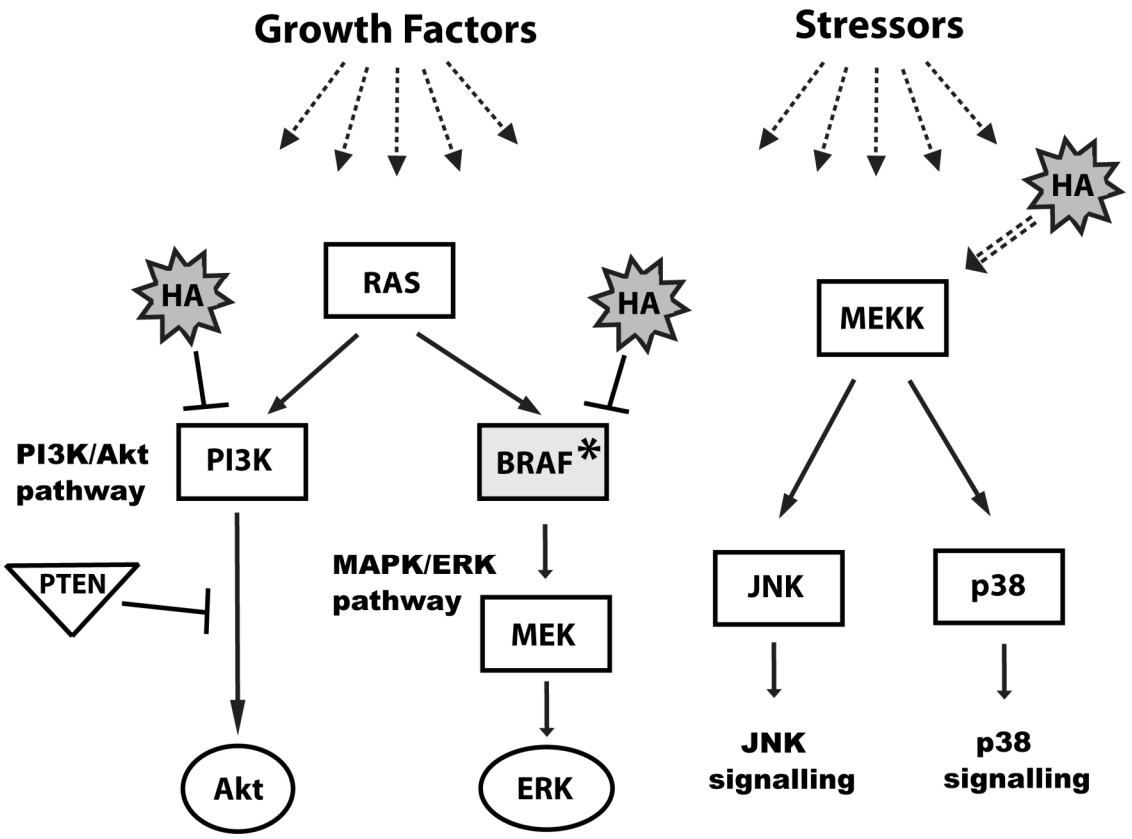

Figure 6. Proposed model for the inhibition of cell proliferation and induction of apoptosis in colon cancer cells by *Hypericum androsaemum* (HA), in particular with effects on PI3K/Akt, MAPK/ERK, JNK and p38 signaling pathways. The anticarcinogenic effect of HA could be due to an inhibition of PI3K/Akt pathway, a decrease on BRAF mutation leading to an inhibition of MAPK/ERK pathway and an induction of both p38 and JNK signalling.

Discussion

In the present study, the potential anticarcinogenic effects of water extracts of the medicinal plants *H. androsaemum*, *H. perforatum* and *H. undulatum*, as well as, the main phenolic constituent present in HA extract, chlorogenic acid (CA), were studied *in vitro* using HCT15 and CO115 human colorectal-derived cell lines. The extracts HU and HP did not show significant effects on cell viability in both cell lines. Previously we showed that quercetin has antiproliferative effects on these colorectal cancer cells [32], and since HU and HP water extracts are rich in quercetin and related compounds, these results were somewhat surprising. On the other hand, HA efficiently inhibited cell proliferation and induced apoptosis in a concentration-dependent manner in both cell lines. CO115 cells showed to be more sensitive to HA extract (IC50 ~65 μg/ml) when compared with HCT15 cells (IC50 ~85 μg/ml). As far as we know, this is the first report

of the anticarcinogenic effect of *H. androsaemum*, which is popularly used to treat problems of the gastrointestinal tract. Anticarcinogenic activities have been found for *H. perforatum* in other cell types [16-19] and related with one of its main constituents, hypericin [20-22], which is present in HP and HU water extracts (although at very small amounts) but not in the water extract of *H. androsaemum* [35]. These results suggest that the anticarcinogenic effects observed for HA reflect the presence of other compounds in this species. We, therefore, studied the antiproliferative effect of the CA, which is abundant in HA and present only in small amounts in HP and HU. However, this compound did not have any effect on cell proliferation in neither of the cell lines at the concentrations tested.

The differences on genetic background of the two cell lines used allowed the study of the relevance of KRAS mutation versus BRAF mutation for HA's effects. In CO115 cells (that harbour a BRAF mutation and overexpress Akt) a significant decrease of phospho-Akt expression was observed in a concentration-dependent manner. HA had no effect on PTEN, the endogenous inhibitor of PI3K, in HCT15 cells. These results show the ability of HA to decrease PI3K/Akt signalling probably by inhibiting PI3K activity, as happens with the inhibitors W and LY, and have also been shown for some flavonoids, such as quercetin [32, 36, 37]. HA was also able to decrease MAPK/ERK signalling in CO115 cells, as shown by a significant decrease in the phospho-ERK expression levels, an effect also observed with the inhibitor PD [38]. Importantly, HA also decreased BRAF expression in these cells. Since HA did not affect the expression of phospho-ERK or BRAF in HCT15, our results indicate that the HA water extract only affects mutant BRAF. Previous studies have shown that pharmacologic inhibition of RAF is highly effective at inhibiting the growth of BRAF mutant CRC cells [39]. Moreover, recent reports found that RAF inhibitors block MAPK signalling in tumor cells harbouring mutant BRAF but activate RAF-MEK-ERK pathway in cells harbouring wild-type BRAF, indicating the importance to inhibit specifically mutant BRAF to avoid secondary effects [40, 41]. It seems that the anticarcinogenic effects of HA in CO115 cells are, at least in part, related to an inhibition of PI3K/Akt and MAPK/ERK pathways, and the latter effect is associated with decreased levels of mutant BRAF (Figure 5). CA, the main phenolic compound present in HA water extract, has no effect on levels of phospho-Akt, phospho-ERK and BRAF in CO115 cells.

Regarding the RAS oncogene, no changes on the levels of KRAS induced by HA extract were observed in neither of the cell lines. Previously, we have shown that quercetin, at around 20µM, inhibits the proliferation of these cell lines in association with a decrease in KRAS levels [32]. Although the HA extract contains quercetin and glycosides of quercetin, their concentrations are low (less than 5µM), which may explain the lack of effect on KRAS. Mainly in the HCT15 cells that harbour the KRAS activating mutation, the HA-induced inhibition of cell proliferation and increased apoptosis seems to not result from effects on MAPK/ERK or PI3K/Akt pathways. Since no effect on cell proliferation was observed for CA when tested individually, other compounds or a synergism between the compounds present in the water extract of HA may be responsible for the inhibitory effects observed. Studies with CA in skin cancer have shown that this phenolic acid has an anti-inflammatory effect, interfering with NF-kβ activation and COX-2 activity, and has an inhibitory effect on skin cancer promotion [42, 43]. However, studies in CRC agree with our results where no effect on colonic cell proliferation has been observed for CA [43, 44].

The effect of HA on two other major MAPK pathways, the p38 and JNK, were also studied, since these stress-activated kinases are also involved in the control of proliferation and/or apoptosis. HA significantly induced the phosphorylation of p38 and JNK in both cell lines (Figure 5). HA could act on an upstream regulator of both pathways which could explain, at least in part, the anticarcinogenic activity [10, 45] of HA in these cells, and in particular in HCT15 cells. An induction of caspase-3 and Poly (ADP-ribose) polymerase-1 (PARP-1) cleavage was also observed in CO115 cells when incubated with HA. The induction of JNK by the extract may contribute to the activation of the mitochondrial caspase cascade [12] and lead to the high levels of apoptosis observed in these cells. On other hand, in HCT15 cleavage of caspase-3 or PARP-1 were not observed despite the induction of JNK. In fact, these cells showed to be more resistant to apoptosis. Also in a previous studied [32], the ability of quercetin to induce apoptosis in HCT15 cells was weak, and independent of caspase induction. The p53 mutation in HCT15 cells [33] may explain the high apoptosis resistance of these cells [46, 47]. In HCT15, the antiproliferative effect of HA is much more relevant then the effect on apoptosis, which suggests that effects on cell cycle could be involved through induction of p38 [10].

Conclusion

Our study shows that the water extract of *H. androsaemum* inhibits cell proliferation and induces apoptosis in CRC-derived cell lines more efficiently than *H. perforatum*, the most well known *Hypericum* species. The effects of HA may partially result from a specific inhibition of mutant BRAF, which leads to an inhibition of MAPK/ERK pathway only in BRAF mutant cells. Inhibition of PI3K/Akt pathway, as well as, an induction of both p38 and JNK pathways may also contribute to the anticarcinogenic activity of HA. Chlorogenic acid, the main phenolic compound present in the HA extract, seems not to be responsible for the anticarcinogenic effects observed. These data add *H. androsaemum* to the list of potential plants used in dietary strategies for the control of CRC progression and, as source of anticarcinogenic compounds.

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Competing Interests

The authors declare that they have no competing interests.

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CHAPTER IV

ANTICARCINOGENIC EFFECTS OF QUERCETIN, LUTEOLIN AND URSOLIC ACID

Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells

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Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells

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ABSTRACT

KRAS and BRAF mutations are frequent in colorectal carcinoma (CRC) and have the potential to activate proliferation and survival through MAPK/ERK and/or PI3K signalling pathways. Because diet is one of the most important determinants of CRC incidence and progression, we studied the effects of the dietary phytochemicals quercetin (Q), luteolin (L) and ursolic acid (UA) on cell proliferation and apoptosis in two human CRC derived cell lines, HCT15 and CO115, harboring KRAS and BRAF activating mutations, respectively. In KRAS mutated HCT15 cells, Q and L significantly decreased ERK phosphorylation, whereas in BRAF mutated CO115 cells the three compounds decreased Akt phosphorylation but had no effect on phospho-ERK. Our findings show that these natural compounds have antiproliferative and proapoptotic effects and simultaneously seem to act on KRAS and PI3K but not on BRAF. These results shed light on the molecular mechanisms of action of Q, L and UA and emphasize the potential of dietary choices for the control of CRC progression.

Keywords
Flavonois
Ursolic acid
Colorectal carcinoma
MAPK/ERK and PI3K pathways
Molecular nutrition

1. Introduction

Colorectal carcinoma (CRC) is the third most common cancer worldwide. In particular sporadic CRC corresponding to 70–80% of total cases [1] is influenced by environmental factors, many of which diet related [2]. The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt are signalling pathways that have been implicated in oncogenic transformation in CRC. They confer a proliferative phenotype and resistance to therapy which is reflected in low patient survival [3–7]. Components of MAPK/ERK and PI3K/Akt pathways constitute, therefore, molecular targets for anticancer strategies [8–10]. Mutations of either KRAS (32%) or BRAF (14%) genes occur alternatively [11] in CRC, causing activation of either MAPK/ERK and PI3K pathways or MAPK/ERK pathway, respectively. Activation of MAPK/ERK pathway regulates the expression of a large number of proteins involved in the control of cell proliferation, differentiation and apoptosis [4,12]. Activation of PI3K gene and inactivation of PTEN, common in CRC, result in overexpression of downstream targets, including Akt and PKC, which promote cell growth and rescue from apoptosis [13–17].

Epidemiological studies show that cancer incidence is inversely correlated with the consumption of diets rich in fruits and vegetables [18]. Natural compounds present in the diet, such as resveratrol and curcumin have been shown to be protective against cancer, contributing to decrease cancer risk and progression rate through their effects on signalling pathways related to proliferation and

apoptosis [18,19]. Studies in cell lines and animal models have shown that flavonoids inhibit cell proliferation and induce apoptosis in many types of cancer cells through different signalling pathways, which corroborate the suggestion that dietary choices may limit cancer progression [20–22]. Quercetin (Q) and luteolin (L) (Fig. 1) are two flavonoids found in fruits, vegetables and aromatic plants with high antioxidant activity [23] to which anticancer properties in CRC are attributed [24–27]. In addition, a recent study showed that quercetin reduces the formation of aberrant crypt foci in a rat colon cancer induction model, suggesting the importance of this compound also in the prevention of colon cancer by decreasing cancer initiating events [28]. Although structurally related, the absence of the hydroxyl group at position 3 of L renders it more lipophilic than Q which may confer better access to intracellular targets. In agreement with this, we have previously shown that L is a more potent intracellular antioxidant than Q, and that this was related with its higher lipophilicity [23]. Ursolic acid (UA; Fig. 1), a natural pentacyclic triterpenoid carboxylic acid, present ubiquitously in plant foods and also a major constituent in some medicinal plants possesses a wide range of biological activities, such as hepatoprotective and anti-inflammatory combined with low toxicity [29–31]. However, contrarily to Q and L, UA is not an antioxidant at relevant cellular redox conditions [32]. Antitumor properties have also been attributed to UA and in colon cancer cells UA has been shown to induce apoptosis and inhibit proliferation [33–35]. Although potential effects on proliferation have been described for these three compounds their effects on MAPK/ERK and PI3K pathways have not been established.

In spite of the general benefit of plant rich diets, variation in cancer incidence among individuals with similar dietary habits suggests interactions of food constituents with genetic factors [2,18]. In the present study we report on the effects of Q, L and UA on two human derived cell lines which harbor different oncogene activating mutations, representative of a large number of CRC: HCT15 has a KRAS (G13D) mutation [36] whereas CO115 has a BRAF (V599E) mutation [37]. These mutations impact on MAPK/ERK and PI3K pathways. The relevance of effects on both these pathways for successful cancer treatment has recently been emphasized [38]. CRC remains a human malignant tumor often resistant to available treatment and knowledge of anticancer properties of dietary constituents may guide dietary choices for cancer patients with particular genetic backgrounds and possibly also suggest their use in combination with conventional therapy in order to enhance therapeutic effects [39,40].

2. Material and methods

2.1. Reagents and antibodies

Quercetin (Q), ursolic acid (UA), wortmannin (W) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Luteolin (L) was from Extrasynthese (Genay, France) and PD-98059 (PD) from Calbiochem (San Diego, CA). All other reagents and chemicals used were of analytical grade. Stock solutions of Q, L and UA were made in dimethyl sulfoxide (DMSO) and aliquots kept at −20 °C.

Primary antibodies were purchased from the following sources: Cell Signaling (Danvers, MA, USA) the anti-p44/42 MAPK (ERK1/2) total, anti-phospho-Akt (Ser473), anti-Akt total, anti-phospho-PTEN (Ser380/Thr382/383) and anti-PTEN total; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) the anti-phospho-ERK1/2, Raf-B and K-Ras; and Sigma–Aldrich the anti-b-actin. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

2.2. Cell culture

HCT15 and CO115 human colon carcinoma-derived cell lines were maintained at 37 °C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma–Aldrich) supplemented with 10 mM HEPES, 0.1 mM pyruvate, 1% antibiotic/antimycotic solution (Sigma–Aldrich) and 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2 ml) and twelve (1 ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml. Incubations with different concentrations of test compounds were made in serum free medium for 48 h for MTT test, BrdU incorporation and TUNEL assay, and for 24 h (Akt, ERK and PTEN) or 6 h (BRAF and KRAS) for western blot analysis.

2.3. Assessment of cell toxicity/proliferation by MTT reduction test

A MTT reduction assay was performed in order to select concentrations of the test compounds that were not cytotoxic and significantly inhibited cell proliferation. Cells were treated with test compounds for 46 h before the 2 h incubation with MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04 M in isopropanol was then added to dissolve the formazan crystals. The number of viable cells in each well was estimated by the cell capacity to reduce MTT. The results were expressed as percentage rela-

Fig. 1. Chemical structures of quercetin (Q), luteolin (L) and ursolic acid (UA).

tive to the control (cells without any test compound). MTT reduction at the beginning of incubation (t = 0 h) was subtracted from all the experimental conditions used above, including the control. Since the effects of the compounds were studied after 48 h of incubation and cells grow significantly within this time period, the point at the beginning of the incubation allows to distinguish between cell death and inhibition of proliferation. MTT negative values indicate necrotic cell death due to cytotoxicity. Values between 0% and 100% indicate inhibition of cell proliferation.

2.4. Assessment of proliferation by BrdU incorporation

After 45 h of treatment with test compounds at the chosen concentrations, cells were incubated with bromodeoxyuridine 10 μM (BrdU; Sigma–Aldrich) for another 3 h. Both adherent and non-adherent cells were collected from each sample, fixed with 4% paraformaldehyde for 15 min at room temperature and attached into a polylysine treated slide using a Shandon Cytospin (Thermo Fisher Scientific Inc, Waltham MA, USA).

Cells were incubated with HCl 2 M for 20 min, washed in PBS containing 0.5% Tween-20 and 0.05% BSA (TPBS-B) and then incubated with monoclonal mouse anti-BrdU antibody (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature. After washing in TPBS-B, cells were incubated with anti-mouse IgG FITC conjugated secondary antibody (Sigma–Aldrich) for 1 h at room temperature, washed again and then incubated with Hoechst for nuclei staining. The percentage of proliferating cells was calculated as the ratio between BrdU positive cells and total cell number (nuclei staining with Hoechst) from a count higher than 500 cells per slide under a fluorescence microscope. Results are presented as mean ± SEM of at least three independent experiments.

2.5. Assessment of apoptosis by TUNEL assay

Cells treated with the test compounds at chosen concentrations for 48 h were collected (both floating and attached cells) and fixed with 4% paraformaldehyde for 15 min at room temperature and attached to a polylysine treated slide using a Shandon Cytospin. Cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed using a kit from Roche (Mannheim, Germany), following the manufacture's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescence microscope. Results are presented as mean ± SEM of at least three independent experiments.

2.6. Protein extraction and western blotting

After treatment with the chosen concentration of test compounds, cells were washed with PBS and lysed for 15 min at 4 °C with ice cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA), supplemented with 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as a protein standard. Twenty micrograms of total protein from each cell lysate were separated by SDS gel electrophoresis and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA (bovine serum albumin), washed in TPBS and then incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. b-actin was used as loading control. Results are presented as mean ± SEM of at least three independent experiments.

2.7. Statistical analysis

One-way ANOVA followed by the Student-Newman–Keuls test was used to perform statistical analysis, using GraphPad Prism 4.0 software (San Diego, CA, USA), and P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of Q, L and UA on cell proliferation

In order to choose doses that do not cause significant toxicity (necrosis) and have antiproliferative effects on the two human CRC derived cell lines, HCT15 and CO115, a MTT assay was performed with different concentrations of the test compounds. The results showed that necrotic cell death (negative values in Fig. 2) occurred in HCT15 cells with L at 20 μM and in CO115 with UA at 15 μM. All the test compounds inhibited cell proliferation in a concentration-dependent manner in both cell lines as shown by MTT assay (Fig. 2). Both reference inhibitors, wortmannin (W) and PD-98059 (PD), at the higher tested concentration did not induce cell toxicity. Based on MTT results, three concentrations of Q, L and UA (2 in case of CO115 cells) that inhibited cell proliferation without significant toxic effects, were selected and used in the following BrdU and TUNEL assays.

For the BrdU incorporation assay, cells were treated with compounds for 48 h. As shown in Fig. 3, a significant inhibition of proliferation indicated by lower levels of BrdU incorporation was observed for Q, L and UA in both cell lines, in a dose dependent manner. In HCT15 cells, the levels of BrdU incorporation decreased from 22.0% in the control to 5.2%, 3.4% and 9.8% in cells treated with Q 20 μM, L 15 μM and UA 4 μM, respectively (Fig. 3a). In CO115 cells, the percentage of cell proliferation significantly decreased from 19.1% in the control to 3.8%, 11.1% and 10.2% in cells treated with Q 15 μM, L 12 μM and UA 10 μM, respectively (Fig. 3b). The structurally-related flavonoids, Q and L, showed different responses in the two cell lines: L was a stronger proliferation inhibitor in HCT15 than in CO115, whereas Q was more effective in CO115 than in HCT15. The reference inhibitors, W, a PI3K inhibitor, and PD, a phospho-ERK inhibitor, significantly inhibited cell proliferation in HCT15 and CO115 cells, respectively (Fig. 3).

3.2. Effects of Q, L and UA on apoptosis

The ability of the test compounds to induce apoptosis was addressed by the TUNEL assay. As shown in Fig. 4, all compounds significantly induced apoptosis in both HCT15 and CO115 cells. Apoptotic cells in

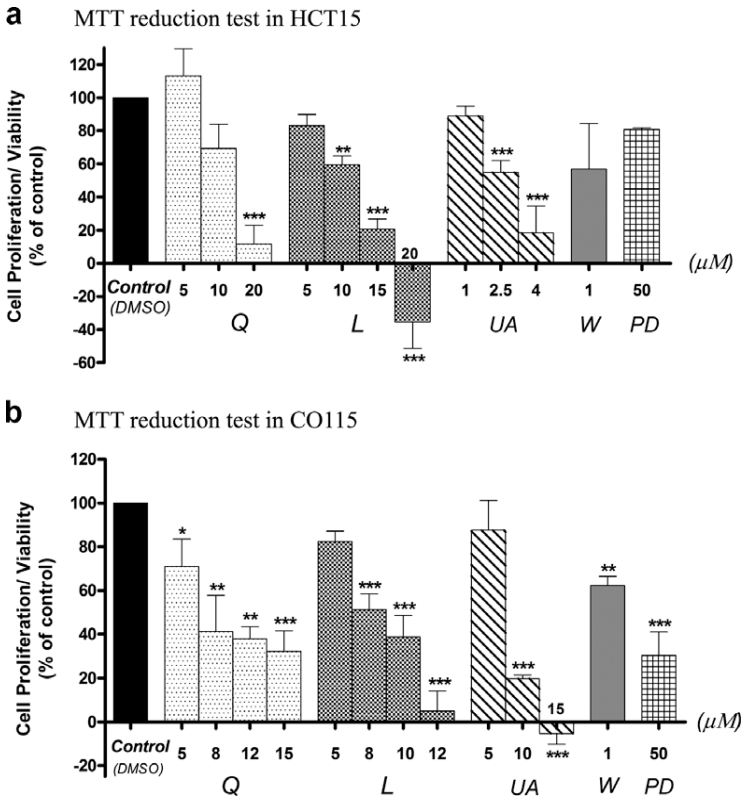

Fig. 2. Effect of treatment with different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA) for 48 h on MTT reduction in HCT15 (a) and CO115 (b) cells. Wortmannin (W) and PD-98059 (PD) were used as reference inhibitors of PI3K/Akt and MAPK/ERK pathways, respectively. Values are mean ± SEM of at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared to control.

HCT15 increased from 0.3% in the control to 4.4%, 3.9% and 6.6% in cells treated with the higher concentrations of Q, L and UA, respectively (Fig. 4a). In CO115 cells, apoptotic cells increased from 1.9% in the control to 36.2%, 15.1% and 12.4% in cells treated with the higher concentrations of Q, L and UA, respectively (Fig. 4b). Between the two flavonoids, Q seems to be a more potent inducer of apoptosis in both cell lines compared to L. In addition, UA showed to be the most effective in HCT15, where it induced 20 times more apoptosis at 4 μM when compared with control.

3.3. Effects of Q, L and UA on ERK phosphorylation

Activation of MAPK/ERK pathway is representative of a large number of CRC cases and the phosphorylation of ERK is an indicator of this activation. We observed high levels of phospho-ERK in both cell lines (Fig. 5). Incubations with L (15 μM) and Q (20 μM) significantly decreased phospho-ERK protein level in HCT15 cells (Fig. 5a), but not in CO115 cells, while UA did not have any effect on either of the cell lines (Fig. 5a and b). A significant reduction of phospho-ERK by PD, a reference inhibitor of MAPK/ERK pathway, was observed in both cell lines. Interestingly, L was a stronger inhibitor of ERK in HCT15 cells than the reference inhibitor PD and than the structure-related compound Q.

3.4. Effects of Q, L and UA on Akt phosphorylation

Because MAPK/ERK and PI3K/Akt pathways are both activated by RAS, we also checked if the PI3K/Akt pathway was affected by the test compounds, measuring phospho-Akt and phospho-PTEN expression levels. In HCT15 cells, there were no detectable amounts of phospho-Akt, in incubations with and without serum (Fig. 6a). High expression levels of phospho-PTEN were detected in HCT15 cells but were not altered by the test compounds (data not shown). In CO115 cells, phospho-Akt expression was observed and significantly decreased by Q (15 μM), L (12 μM) and UA (10 μM), as shown in Fig. 6b. Wortmannin, a reference PI3K inhibitor, also significantly decreased Akt phosphorylation, contrarily to PD that did not alter phospho-Akt levels in CO115 cells. Phospho-PTEN and total PTEN expression were not observed in CO115 cells, in medium with and without serum, in contrast with what was observed with HCT15 cells (Fig. 6c). The lack of phospho-Akt in HCT15 and PTEN signal in CO115 cells was reproducible and checked in the presence of a positive reactive sample (Fig. 6a and c). These observations seem, therefore, also not to be the result of protein degradation during protein extraction or sample preparation.

3.5. Effects of Q, L and UA on KRAS and BRAF expression

To check if the effects of the test compounds reflect direct effects on KRAS or BRAF, expression of these proteins was also monitored by western blot. As shown in Fig. 7, Q and L remarkably decreased the expression of KRAS but not BRAF in both cell lines. UA significantly changed the expression of KRAS only in HCT15 cells, but not as efficiently as the flavonoids. No significant changes were induced by UA in BRAF expression.

4. Discussion

The effects of quercetin (Q), luteolin (L) and ursolic acid (UA), natural compounds common in diets rich in fruits and vegetables, were studied in two different human colon carcinoma-derived cell lines representative of common CRC cases. We observed that the three test compounds, at concentrations that did not induce significant cell toxicity (necrosis), inhibited proliferation and induced apoptosis in both cell lines in a concentration-dependent

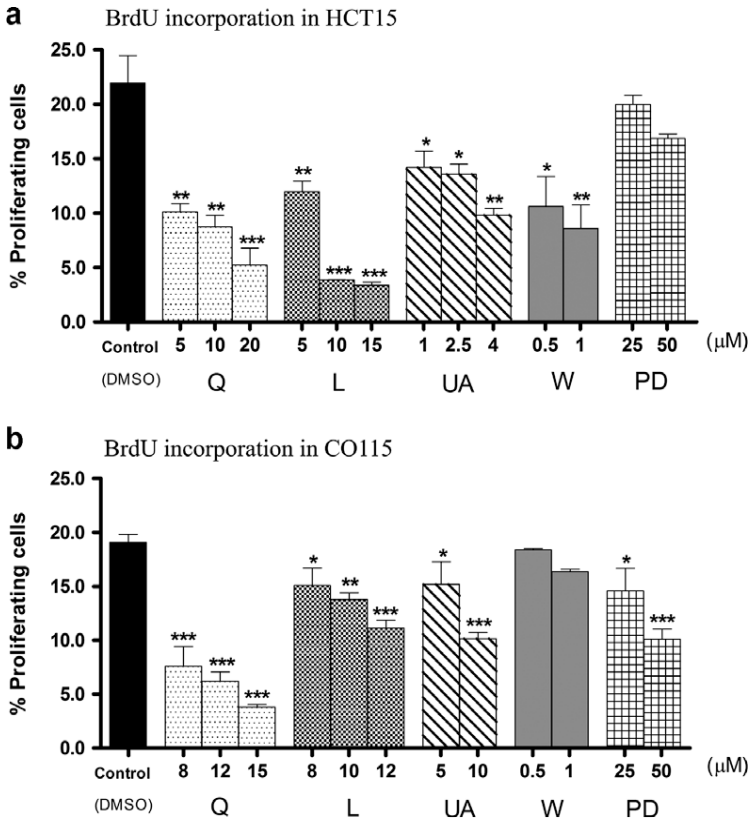

Fig. 3. Effect on cell proliferation of different concentrations of quercetin (Q), luteolin (L), ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48 h in HCT15 (a) and CO115 (b) cells, using the BrdU incorporation assay. Values are mean ± SEM of at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared to control.

manner. The purpose of the present study was to identify in HCT15 and CO115 cells molecular targets for Q, L and UA related with their antiproliferative and proapoptotic effects. HCT15 and CO115 have activating mutations of KRAS and BRAF, respectively.

Q and L decreased the expression of phospho-ERK in the KRAS mutated HCT15 cell line but not in the BRAF mutated CO115 cell line. These results suggest that the BRAF mutation in CO115 cells overrides any inhibitory effect of Q and L on phospho-ERK, indicating that these flavonoids act on KRAS upstream of BRAF (Fig. 8). This was further confirmed by a decrease in the expression of KRAS but not BRAF induced by both flavonoids. Our findings corroborate recent reports where quercetin treatment resulted in a reduction of Ras protein levels in colon cell lines expressing oncogenic Ras [41,42]. A recent study in skin epidermal cell line, showed a different effect of quercetin, which in these cells inhibited both Raf and MEK activity [43]. In addition, we observed in HCT15 cells that Q and L decreased phospho-ERK levels as efficiently as PD-98059 (PD), a specific inhibitor of MEK downstream of RAF [44]. Inhibition of proliferation and induction of apoptosis by Q and L in HCT15 cells does however not seem to be due to phospho-ERK inhibition alone since PD inhibited phospho-ERK but was without effect on cell proliferation and induction of apoptosis. This finding is in agreement with other reports [10,45], which showed that tumor cells carrying KRAS mutation do not rely only on MAPK/ERK pathway to proliferate. Since HCT15 cell proliferation was inhibited by wortmannin (W), a PI3K inhibitor, it seems that inhibition of proliferation by Q and L treatment could be through inhibition of PI3K dependent pathways. Contrarily to a previous report [46], we did not detect phospho-Akt in HCT15 cells, which could be explained by the high levels of phospho-PTEN observed (Fig. 8). It is known that other downstream targets of PI3K besides Akt also contribute to cell proliferation and apoptosis, such as PKC, which is known to be inhibited by Q and L [47–51]. PKC isozymes have been shown to be commonly deregulated in colon cancer and other natural compounds, such as curcumin, have also shown to inhibit PKC in CRC cells [17]. Thus, inhibition of proliferation in HCT15 cells by Q and L seems to be due to effects on KRAS, affecting not only the MAPK/ERK pathway but also other alternative pathways, such as PI3K/PKC pathway. However, apoptosis induced by Q and L in HCT15 cells does not seem to be due to inhibition of PI3K, since W did not induce apoptosis in this cell line. Other apoptotic targets of these compounds should be considered.

Studies have shown that BRAF mutated cell lines rely more on MAPK/ERK pathway for proliferation than KRAS mutated cell lines [10,45]. However, our results show that the dependence of CO115 cells on MAPK/ERK pathway for

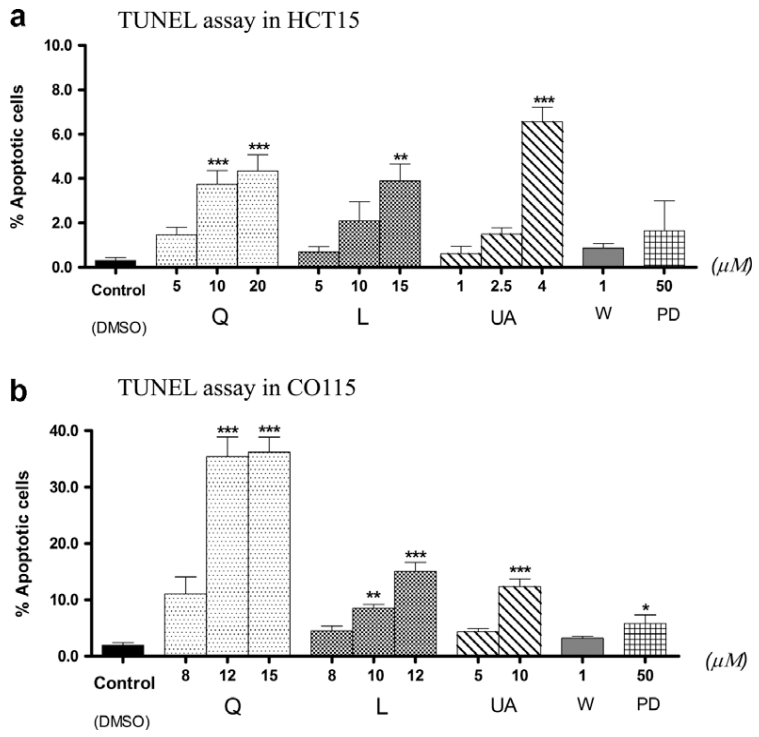

Fig. 4. Effect on apoptosis of different concentrations of quercetin (Q), luteolin (L) and ursolic acid (UA) and reference compounds, wortmannin (W) and PD-98059 (PD), for 48 h in HCT15 (a) and CO115 (b) cells, using the TUNEL assay. Values are mean ± SEM of at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared to control.

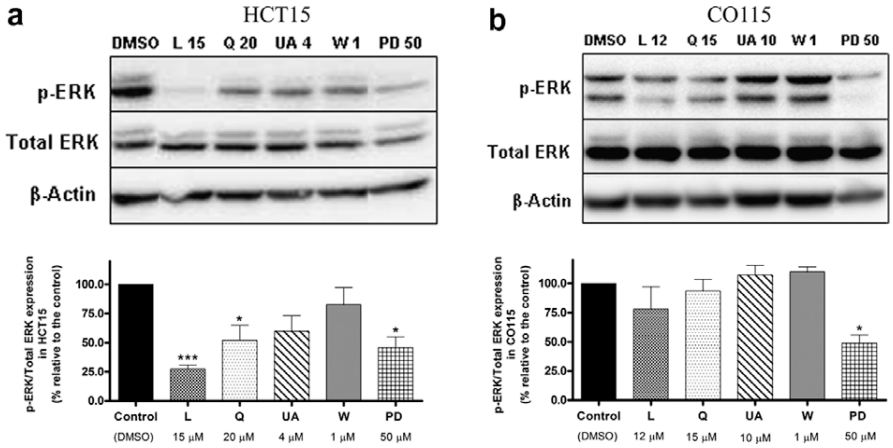

Fig. 5. Effects of treatment with quercetin (Q), luteolin (L) and ursolic acid (UA) for 24 h on phospho-ERK expression in HCT15 and CO115 cells, using western blot. β-Actin was used as loading control. (a) HCT15 cells were treated with L 15 μM (L15), Q 20 μM (Q20) and UA 4 μM (UA4) in serum free medium. (b) CO115 cells were treated with 12 μM L (L12), 15 μM Q (Q15) and 10 μM UA (UA10) in serum free medium. Wortmannin 1 μM (W1) and PD-98059 50 μM (PD50) were used as reference inhibitors of PI3K and MEK, respectively, in both cell lines. Values are mean ± SEM of at least three independent experiments. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ when compared to control.

proliferation is not exclusive, since all test compounds inhibit proliferation without affecting phospho-ERK levels. CO115 cells, besides harboring a BRAF mutation, also present high PI3K activity [52]. In agreement with this, a high expression of phospho-Akt was observed while PTEN was not detectable. Our results show a significant decrease in phospho-Akt expression by Q, L and UA. These suggest an inhibition of PI3K activity, in addition to an inhibition of KRAS (Fig. 8). Several studies have shown that quercetin and analogs are potent inhibitors of PI3K activity [53,54]. However, W although inhibiting phospho-Akt, did not inhibit proliferation or induce apoptosis. The effect of the

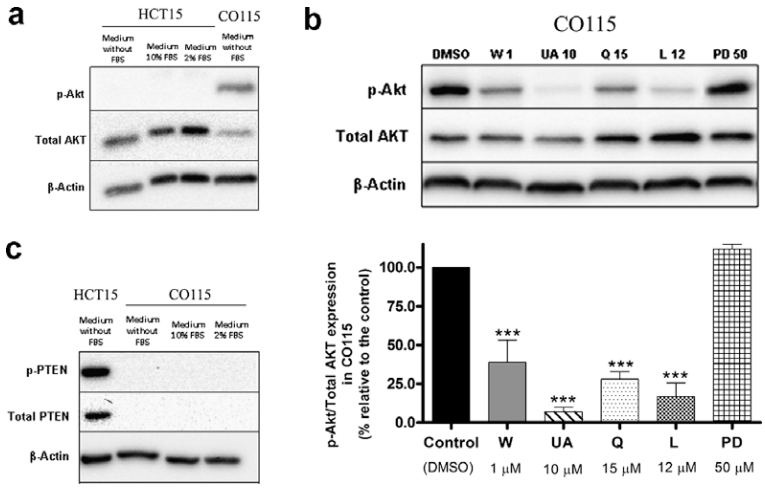

Fig. 6. Effects of treatment with quercetin (Q), luteolin (L) and ursolic acid (UA) for 24 h on phospho-Akt expression and phospho-PTEN levels in HCT15 and CO115 cells, using western blot. β-Actin was used as loading control. (a) HCT15 cells were cultured in medium without and containing 2% or 10% serum (FBS) and CO115 cells were cultured in serum free medium. (b) CO115 cells were treated with UA 10 μM (UA10), Q 15 μM (Q15) and L 12 μM (L12) in serum free medium. Wortmannin 1 μM (W1) and PD-98059 50 μM (PD50) were used as reference inhibitors of PI3K and MEK, respectively. (c) CO115 cells were cultured in medium without and containing 2% or 10% FBS and HCT15 cells were cultured in serum free medium. Values are mean ± SEM of at least three independent experiments. $^{***}P \leq 0.001$ when compared to control.

natural compounds on PI3K may, therefore, only partially explain their antiproliferative and proapoptotic activities in CO115 cells. The inhibition of phospho-Akt and phospho-ERK by Q and L has been reported in human hepatoma cell line (HepG2) and brain tumors [55–57].

Regarding UA, our results show that this compound does not affect phospho-ERK expression, being only effective in inhibiting phospho-Akt in CO115 cells. It seems that UA does not affect significantly KRAS (although it decreased expression levels in HCT15 cells) and has PI3K as one of its molecular targets. Interestingly, it was the most efficient proliferation inhibitor and inducer of apoptosis in HCT15 cells, which do not express phospho-Akt. Contrarily to the effects of the antioxidants Q and L, the antitumor properties of UA through redox-sensitive pathways are most likely not the result of its reactive oxygen species scavenging ability, since it was previously shown that UA is inactive as free radical scavenger [32]. Interestingly, all test compounds showed to be more efficient than the reference compounds, PD and W, in inhibiting cell proliferation and inducing apoptosis. Their wider range of molecular targets is therefore advantageous in the control of tumor progression [26] and the importance of modulating several signal transduction pathways associated with carcinogenesis is once again reinforced.

In summary, the antiproliferative and proapoptotic effects of Q and L seem to be, at least in part, due to effects on KRAS through regulation of both MAPK/ERK and PI3K pathways. The BRAF mutation overrides the compounds' inhibition of KRAS on the MAPK/ERK pathway but not on the PI3K pathway. UA seems to act on PI3K where Q and L may also act, independently of KRAS mutation. The results of this study suggest, therefore, the applicability of these phytochemicals in dietary strategies and as possible adjuvants in CRC therapy both in KRAS and BRAF gene mutation profiles.

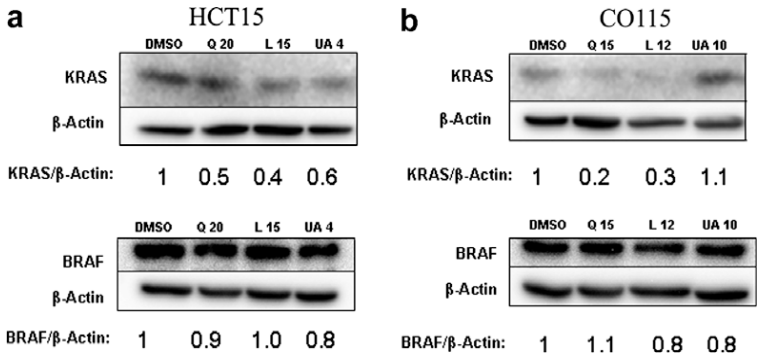

Fig. 7. Effects of treatment with quercetin (Q), luteolin (L) and ursolic acid (UA) for 6 h on KRAS and BRAF expression in HCT15 (a) and CO115 (b) cells, using western blot. β-Actin was used as loading control. Images and values are representative of three independent experiments.

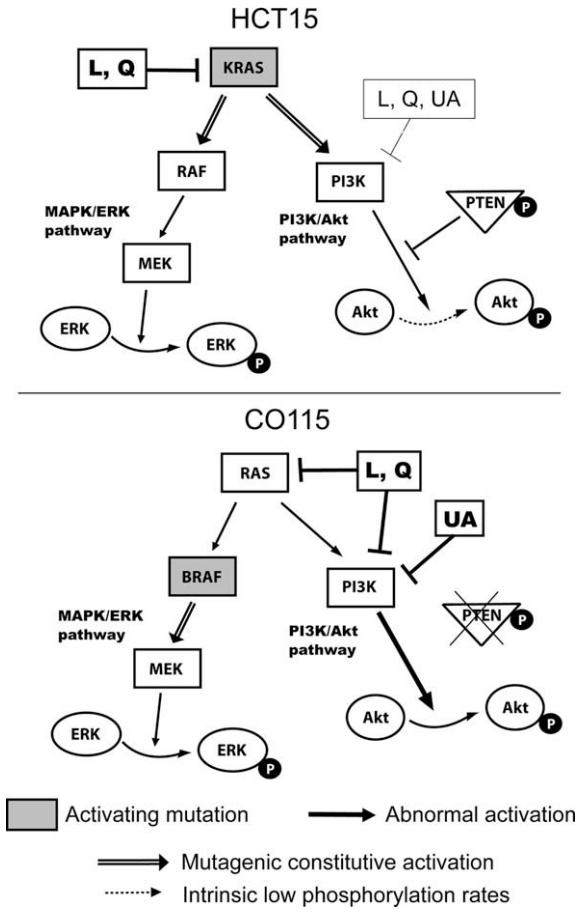

Fig. 8. Schematic representation of possible targets of quercetin (Q), luteolin (L) and ursolic acid (UA) on MAPK/ERK and PI3K/Akt pathways in HCT15 and CO115 cell lines, which may lead to inhibition of cell proliferation and induction of apoptosis.

Conflicts of interest statement

The authors do not have conflicts of interest.

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CHAPTER V

COMBINED EFFECTS OF QUERCETIN, LUTEOLIN AND URSOLIC ACID WITH 5-FLUOROURACIL

Manuscript 1

Quercetin synergistically enhances apoptosis induced by 5-Fluorouracil in p53 wild-type MSI colorectal cancer cells

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Quercetin synergistically enhances apoptosis induced by 5-Fluorouracil in p53 wild-type MSI colorectal cancer cells.

Quercetin synergistically enhances apoptosis induced by 5-Fluorouracil in p53 wild-type MSI colorectal cancer cells

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Abstract

Background: Colorectal carcinoma (CRC) is a common cause of cancer-related death. Tumors with microsatellite instability (MSI) have been shown to be resistant to chemotherapy with 5-fluorouracil (5-FU), the most widely used pharmacological drug for CRC treatment. It is therefore essential to find compounds that could contribute to treatment efficacy through increases in sensitivity to this drug.

Aim and Methods: In this study, we used two MSI human CRC derived cell lines, CO115 that is wild-type for p53 and HCT15 that harbors a p53 mutation. The sensitivity of these cells to 5-FU was evaluated and the effects on apoptosis induction of co-incubation of the flavonoids, quercetin (Q) or luteolin (L), with 5-FU were performed using TUNEL assay. The mechanisms of apoptotis induction of these flavonoids were assessed by western blot.

Results: Our results demonstrate that CO115 is more sensitive to 5-FU than the p53 mutant HCT15. The two cell lines also responded differently to the induction of apoptosis by the flavonoids. Apoptosis induction was higher and dependent on caspase activation in CO115 cells but not in HCT15 cells. In HCT15 cells, Q and L had an additive effect on apoptosis when combined with 5-FU. Q was the most efficient compound in enhancing the apoptotic effect of 5-FU in CO115 cells where a synergistic effect was observed. This effect seems to involve the mitochondrial caspase pathway since a remarkable increase in the expression of cleaved caspase 9, caspase 3 and PARP and a decrease in Bcl-2 expression were observed.

Conclusion: This study suggests the potential applicability of these phytochemicals, especially Q, for enhancement 5-FU efficiency in CRC therapy in resistant MSI p53 wild-type cells.

Keywords: Quercetin, Luteolin, Colorectal carcinoma, Apoptosis, 5-Fluorouracil

Introduction

Chemotherapy with 5-fluorouracil (5-FU) is the basis for treatment of colorectal carcinoma (CRC), which is the third most common form of cancer in developed countries [1]. However, significant resistance to this drug has been reported [2]. Drugs such as irinotecan and oxaliplatin are used in combination with 5-FU and have demonstrated increase treatment efficacy although not in all patients [3-5]. Genetic variability is one important factor that regulates the response and toxicity of a drug and should be taken in account when a therapy is chosen [5]. The enzyme thymidylate synthase (TS) is essential for the synthesis of deoxythymidine-5'-monophosphate, a precursor for DNA replication. An inhibition of this enzyme by the active metabolite of 5-FU (5-fluoro-2´-deoxyuridine-5´monophosphatate) is the main mechanism of 5-FU action, resulting in DNA strand breaks and inhibition of DNA synthesis, induction of apoptosis and cell cycle arrest [6]. A constitutive activation of TS contributes to 5-FU resistance in some tumors [5].

Mutations in mismatch repair (MMR) genes result in the inability of the MMR system to correct DNA replication errors leading to the accumulation of mutations and giving rise to microsatellite instability (MSI). Tumors presenting MSI occur approximately in 15% of patients with sporadic CRC and are generally associated with resistance to 5-FU [7-9] . *In vitro* studies have shown that DNA MMR deficiency may be responsible for tumor resistance to 5-FU and clinical evidence is suggestive of little or no benefit from 5-FU treatment in MSI patients [7,8]. Previous studies have also shown that mutations in the gene *P53* contribute to 5-FU resistance in CRC and have profound effects on drug responses [10] with reduced induction of apoptosis and inhibition of cell cycle [11,12]. In agreement with this, prognosis in patients having an MSI tumor with p53 mutation have been shown to be poor compared to the ones with wild-type p53 [13].

The induction of apoptosis by 5-FU occurs through both the intrinsic and extrinsic pathways with activation of caspases [14]. In the intrinsic pathway, Bcl-2 family proteins modulate mitochondrial membrane permeabilization, which leads to the release of cytochrome c and activation of caspase-9 that in turn activates the effector caspase-3. Activation of death receptors on the cell membrane (extrinsic pathway), which subsequently activates caspase-8 and caspase-3, may also be induced by 5-FU [14].

The c-Jun N-terminal kinase (JNK) and p38 are two stress-activated protein kinases of the family of the mitogen activated protein kinase (MAPK) that have key roles in inflammation, in controlling cell proliferation, differentiation and apoptosis, and their effects appear to be largely dependent on cell type and/or cellular context [15-18]. They are activated by diverse cellular stresses, including UV irradiation, oxidative stress, DNA damage, heat and osmotic shock [15]. Several studies in CRC cells show the ability of some phytochemicals, such as silibinin, curcumin and flavones to induce apoptosis through different signalling pathways [19-21]. Some of these dietary phytochemicals to which anticarcinogenic effects have been attributed are involved in the modulation of JNK and p38 signalling [22,23].

Compounds that alter the expression of apoptotic proteins may, therefore, contribute to decrease tumor malignance and chemoresistance [23]. In our previous study, we showed that quercetin (Q) and luteolin (L), two flavonoids found in fruits and vegetables, have antiproliferative effects in HCT15 and CO115 human CRC cells through regulation of KRAS and both MAPK/ERK and PI3K pathways [24]. HCT15 and CO115 are two cell lines derived from sporadic MSI CRC: HCT15 harbors a *P53* inactivating mutation whereas CO115 is wild-type for this gene [25,26]. In the present study, we investigate the ability of Q and L to induce apoptosis in these MSI CRC cell lines and their possible therapeutic enhancing effect when used in combination with the pharmaceutical drug 5-FU. Our data suggest a therapeutic potential of these phytochemicals in combination with 5-FU in CRC, particularly for Q, in the MSI p53 wild-type background.

Material and methods

Reagents and antibodies

Quercetin (Q), z-VAD-fmk (zVAD), staurosporine (STS), 5-Fluorouracil (5-FU) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin (L) was purchased from Extrasynthese (Genay, France). Stock solutions of test compounds were made in dimethyl sulfoxide (DMSO) and aliquots were kept at -20ºC. All other reagents and chemicals used were of analytical grade.

Primary antibodies were purchased to the following sources: anti-cleaved caspase-9 and anti-phospho-p38 MAPK (Thr180/Tyr182) to Cell Signaling (Danvers,

MA, USA); anti-caspase-3 to Calbiochem (San Diego, CA); anti-Bcl-2, anti-Bax, anti-PARP-1, anti-phospho-JNK, anti-JNK, anti-p38 total and anti-p53 to Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-β-actin to Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased to GE Healthcare (Bucks, UK).

Cell lines

HCT15 and CO115 human colon carcinoma-derived cell lines were maintained at 37ºC in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic/antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2ml) and twelve (1ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml.

Cell proliferation/viability assay

To investigate the effects of 5-FU on cell viability/proliferation in HCT15 and CO115 cells, the MTT reduction assay were used as described previously [24]. Cells were treated with different concentrations of 5-FU for 46h and then two more hours in the presence of MTT (final concentration 0.5 mg/ml). Hydrogen chloride 0.04M in isopropanol was then used to dissolve the formazan crystals. The number of viable cells in each well was estimated by the cell capacity to reduce MTT using a spectrophotometer. Results are presented as mean ± SEM of at least three independent experiments.

TUNEL assay

TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed to estimate the percentage of apoptotic cells in both cell lines treated for 48h with different concentrations of 5-FU alone and in combination with Q and L. The concentrations of Q and L used induce significant inhibition of cell proliferation without substantial necrotic death, as determined by BrdU assay and MTT test in our previous work using the same cells and conditions [24]. Both cell lines were also treated with Q and L in combination with 20μM z-VAD-fmk (zVAD), a general caspase inhibitor, for 48h, to assess the involvement of caspases activation in the apoptotic process induced by the test

compounds. Staurosporine (STS) 0.25µM, an apoptotic inducer, was also used as a positive control.

After treatments, cells were collected (both floating and attached cells), fixed with 4% paraformaldehyde for 15min at room temperature and attached into a polylysine treated slide using a Shandon Cytospin. Cells were then washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Hoechst was used for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells, from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Western blot analysis

Cells were treated with Q, L, 5-FU and STS alone and co-incubated with Q and 5-FU for 48h and total cell lysates were prepared to measure the expression of different proteins. The cells were washed with PBS and lysed for 15min at 4ºC with ice cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as a protein standard. To perform western blot analysis, 20µg of protein were resolved by SDS-polyacrylamide gel and then electroblotted onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA (bovine serum albumin), washed in TPBS and then incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control.

Statistical analysis

Statistical analyses were done using *t*-test, GraphPad Prism 4.0 software (San Diego, CA, USA). *P*-values ≤ 0.05 were considered statistically significant.

Results

Colon cancer cells` sensitivity to 5-FU

The effect of 5-FU on cell growth and apoptosis in HCT15 and CO115 cells were established by the MTT and TUNEL assays, respectively. As shown in Figure 1a, 5-FU was more effective in inhibiting cell growth in CO115 than HCT15. The concentrations that inhibit cell growth by 50% (IC50) are around 100µM in HCT15 and 1µM in CO115. The differences in susceptibility of the two cell lines to 5-FU were also observed for the induction of apoptosis (Figure 1b). The concentrations of 5-FU that inhibited cell proliferation by around 50% and significantly increased apoptosis were selected for the next experiments (100µM for HCT15 and 1µM for CO115).

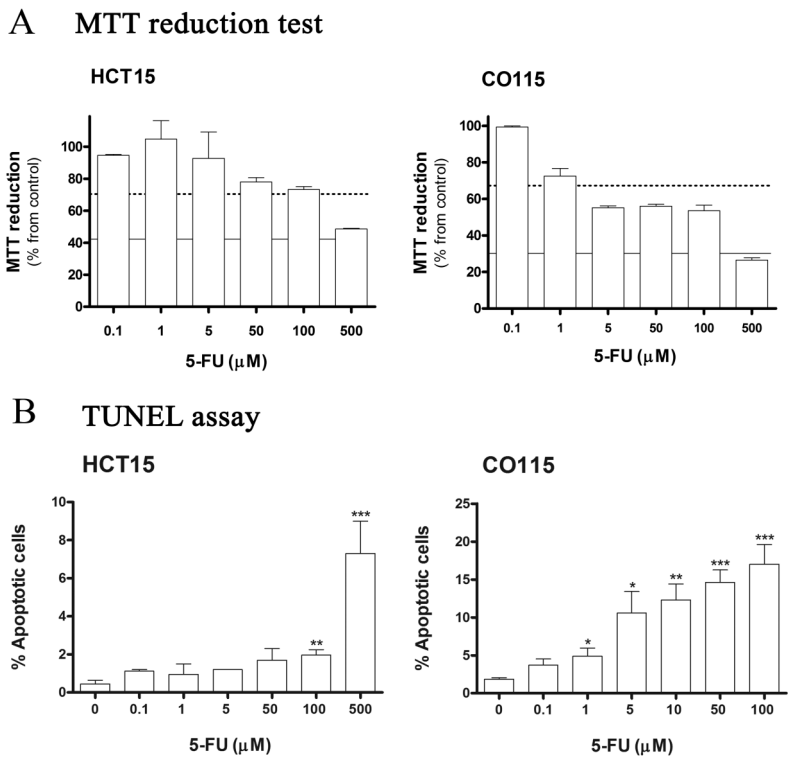

Fig.1. Effect on cell proliferation/viability (a) and apoptosis (b) of different concentrations of 5-fluorouracil (5-FU), for 48h, in HCT15 and CO115 colon cancer cells, using MTT and TUNEL assay, respectively. Results are presented as mean ± SEM of at least 3 independent experiments. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001. In A, full line represents quantity of cells in the beginning of the assay (t=0h) and values below this after 48h incubation with test compound mean cell toxicity by necrosis; dot line represents the concentrations that inhibit cell growth by around 50% (IC50).

Combined effect of 5-FU and test compounds on apoptosis

The induction of apoptosis in both cell lines treated with Q or L, at concentrations that induce significant inhibition of cell proliferation without substantial necrotic death, as tested previously [24], and 5-FU (IC50) was monitored by the TUNEL assay. As shown in Fig.2, flavonoids induced a high rate of apoptosis in CO115 cells when compared with HCT15. In HCT15 cells, L or Q in combination with 5-FU demonstrated an additive effect in the induction of apoptosis (Figure 2a). In CO115 cells, L in combination with 5-FU showed an additive effect in apoptosis induction while Q demonstrated to synergistically induce apoptotic cell death when combined with 5-FU (Figure 2b). In all cases, the effects on apoptosis of co-incubations were higher than 5-FU alone or test compound alone.

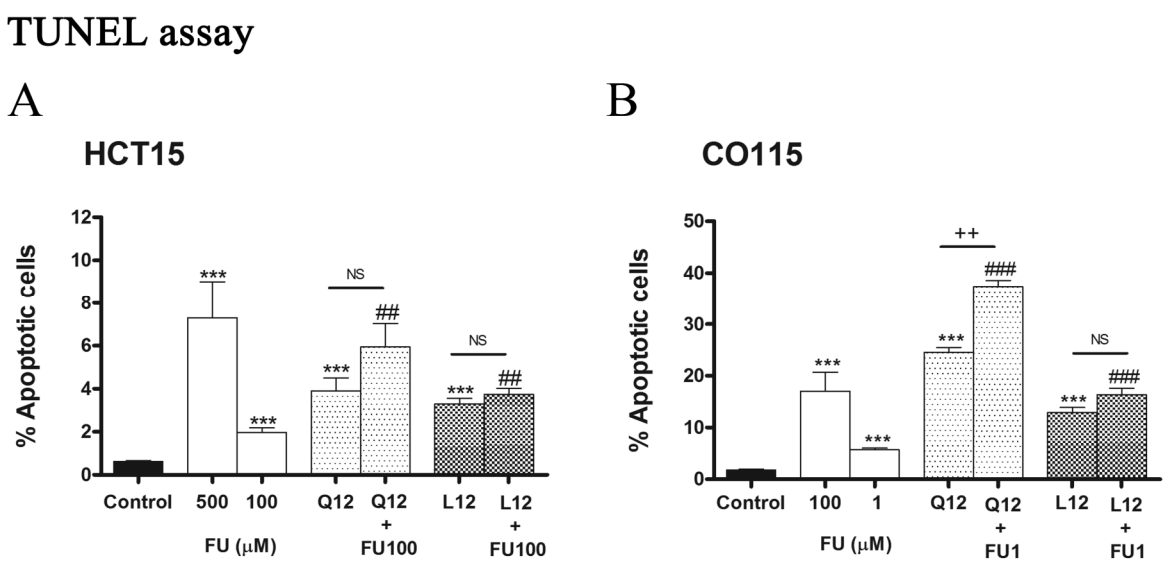

Fig.2. Effect on apoptosis by 5-fluorouracil (FU) 500, 100 and 1μM, quercetin 12μM (Q12) and luteolin 12μM (L12) alone, as well as the natural compounds co-incubated with FU for 48h, in HCT15 (a) and CO115 (b) cells, using TUNEL assay. Results are presented as mean ± SEM of at least 3 independent experiments. *** P≤ 0.001, when compared with control; ++ P≤ 0.01, when compared with the respective natural compound alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with FU alone; NS, not significant differences observed between each other.

Effects on caspases and other molecular markers of apoptosis

In order to determine the role of caspase activation on the apoptotic effects of the test compounds, the caspase inhibitor zVAD was used and apoptosis measured by TUNEL assay. As shown in Figure 3a, apoptosis induced by the test compounds, Q and L, and the apoptotic inducer, STS, were not inhibited by zVAD in HCT15 cells. In contrast, in CO115 cells apoptosis was totally suppressed when test compounds and

STS were co-incubated with zVAD (Figure 3b). In addition, effects on caspase-3, caspase-9 and PARP expressions were analysed using western blot. As shown in Figure 3c, in HCT15 cells, the test compounds, 5-FU and STS did not induce cleaved (active) caspase-9, and only STS induced cleavage of caspase-3 (active form) and cleavage PARP (inactive form). On the other hand, in CO115 cells cleaved caspase-9 and caspase-3 was observed with all test compounds as well as cleavage of PARP and/or a remarkable decreased of uncleaved PARP (active form). Interestingly, the expression levels of PARP are higher in HCT15 than in CO115.

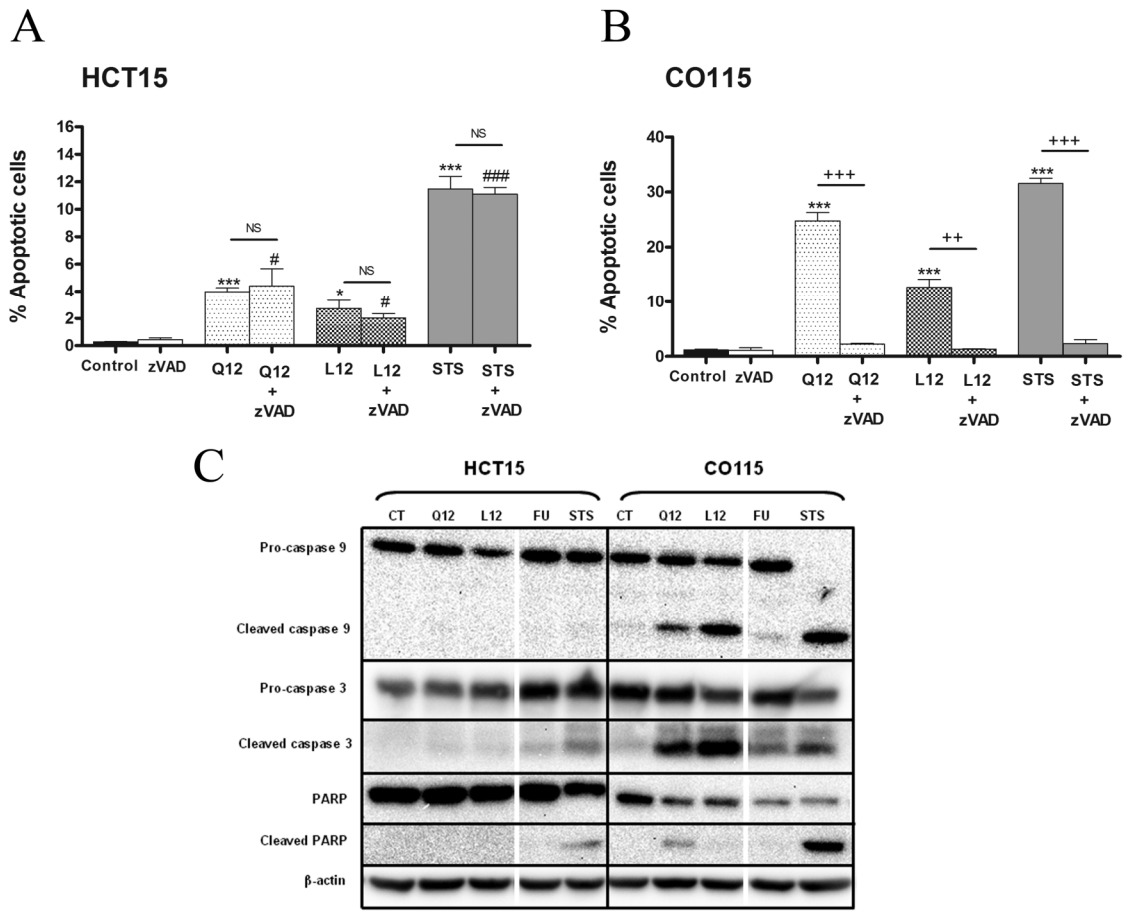

Fig.3. Effect of a caspase inhibitor zVAD-FMK (zVAD) 20µM on the apoptosis induction by quercetin 12µM (Q12), luteolin 12µM (L12) and staurosporine (STS) 0.25µM, for 48h, in HCT15 (a) and CO115 (b) cells, using TUNEL assay. Results are presented as mean ± SEM of at least 3 independent experiments. * P≤ 0.05, *** P≤ 0.001, when compared to control; ++ P≤ 0.01 and +++ P≤ 0.001, when compared with the respective compound alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with zVAD alone; NS, not significant differences observed between each other. (c) Effects on caspase-9, caspase-3 and PARP-1 expressions, for 48h, of Q, L, 5-FU (FU) and STS alone, in HCT15 and CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

To further elucidate the apoptotic effects of the test compounds and 5-FU, the expression of p53, Bax and Bcl-2 were also analysed by western blot (Figure 4). It was observed that Q, L and 5-FU induce p53 in both cell lines, this effect being more remarkable for Q in CO115 cells. Expression of Bcl-2 was notably decreased by all the compounds in both cell lines and Bax expression increased only in HCT15 since CO115 does not express Bax, as also shown by others [27].

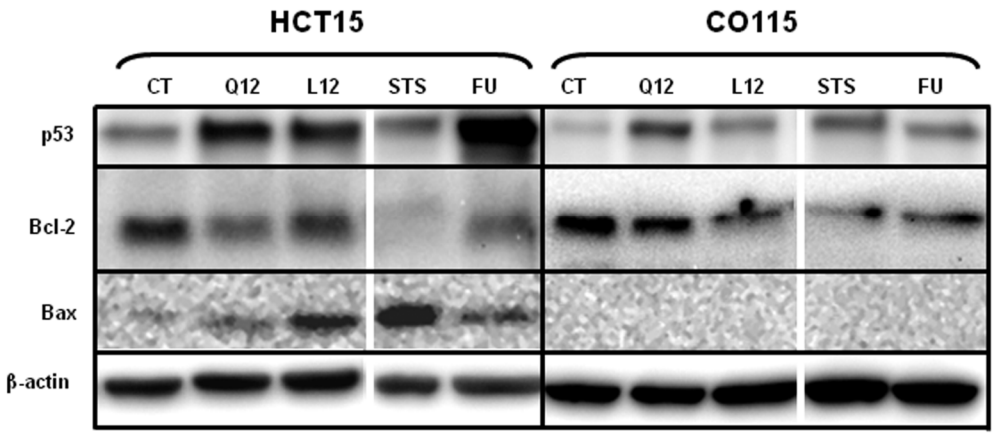

Fig.4. Effects on p53, Bax and Bcl-2 expressions, for 48h, of quercetin 12µM (Q12), luteolin 12µM (L12), staurosporine (STS) 0.25µM and 5-fluorouracil (FU) 1µM and 100µM, in HCT15 and CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

Effects of Q and 5-FU in co-incubation on molecular markers of apoptosis

To determine the possible causes of the synergistic effect of Q with 5-FU on the induction of apoptosis in CO115 cells, the expression of molecular markers was further evaluated. Co-incubation of Q and 5-FU synergistically increased the cleavage of caspase-3, caspase-9 and PARP and decreased Bcl-2 expression, but had no effect on p53 expression (Figure 5).

Effects on JNK and p38 pathways

The possible involvement of the JNK and p38 pathways in the induction of apoptosis by the test compounds and 5-FU was also evaluated. Our results show no effect on phospho-JNK expression by Q, L and 5-FU in neither of the cell lines (Figure 6a). In HCT15 cells no effect on phospho-p38 expression was observed by the flavonoids and 5-FU while in CO115 cells Q and L slightly increased the expression of phospho-p38 (Figure 6b). STS, an apoptotic inducer, significantly induced phospho-JNK expression and decreased the expression of phospho-p38 in both cell lines.

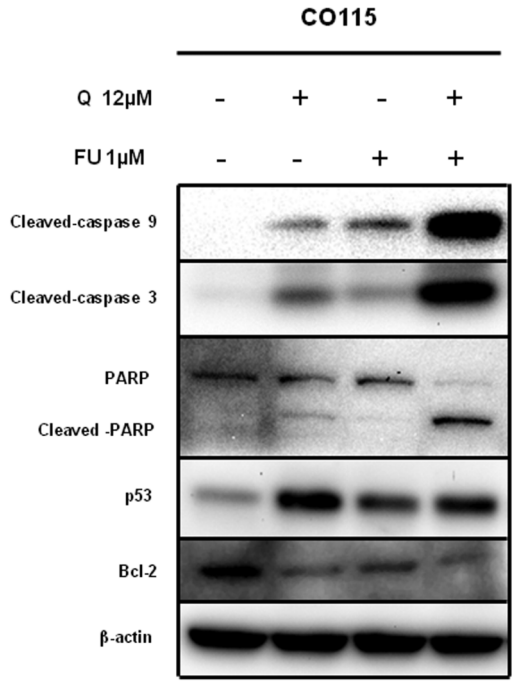

Fig.5. Effects on caspase-9, caspase-3, PARP-1, p53 and Bcl-2 expressions, for 48h, of co-incubation of quercetin 12µM (Q12) and 5-fluorouracil 1µM (FU1) in CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

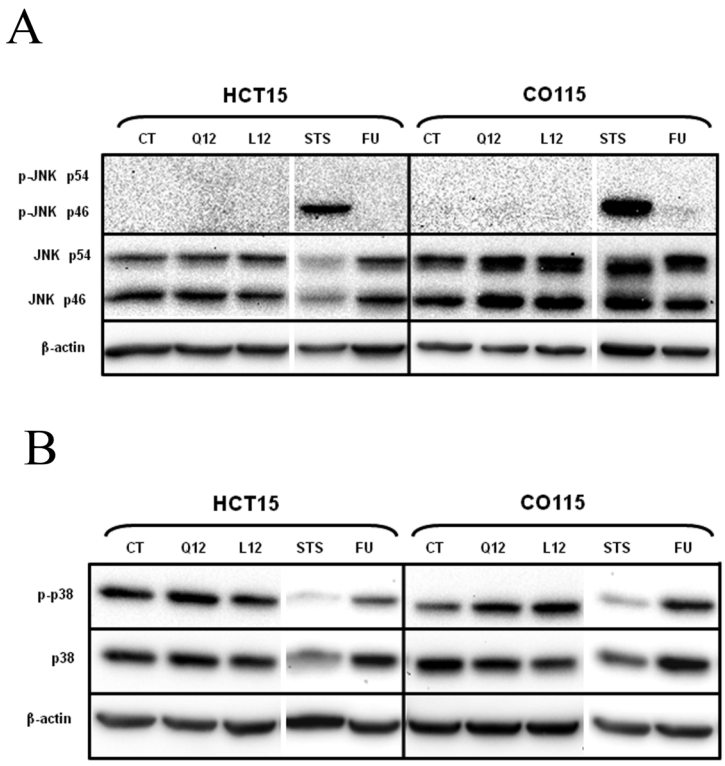

Fig.6. Effect on phospho-JNK and total JNK (a) and phospho-p38 and total P38 (b) expressions, for 48h, of quercetin 12µM (Q12), luteolin 12µM (L12), 5-fluorouracil 1µM (FU1) and 100µM (FU100) and staurosporine (STS) 0.25µM, in HCT15 and CO115 cells, by western blot. Images are representative of at least 3 independent experiments.

Discussion

5-Fluorouracil (5-FU) is the pharmacological drug most commonly used in CRC chemotherapy, however tumor cell resistance to this drug remains a significant concern. Several mechanisms of resistance involving failure to induce apoptosis have been reported to reduce the efficacy of 5-FU [6,12], such as MSI and mutant p53 [7,11,12]. Thus, new compounds are needed to use in combination with 5-FU to increase treatment efficacy. The potential of Q and L, two structurally related natural flavonoids, to enhance the apoptosis induction when used in combination with 5-FU on two MSI CRC cell lines, as well as their possible mechanisms of action, are presently reported. Inhibition of cell proliferation and induction of apoptosis by 5-FU were observed in a dose-dependent manner in both cell lines that showed, however, different susceptibilities to the drug. As expected, HCT15 cells harboring a p53 mutation were more resistant to 5-FU than CO115 cells (wild-type for p53). The anticancer effects of flavonoids are widely known, and we previously reported that both Q and L inhibited cell proliferation and induced apoptosis on CRC cells [24]. Here, we show that Q and L differently affect 5-FU cell death induction in two different genetic background CRC cell lines.

Our data show a significant ability of Q and L to increase 5-FU induced apoptosis in both MSI cell lines. Q (in HCT15 cells) and L (in both cell lines) additively enhanced apoptosis induced by 5-FU. A remarkable synergistic effect was detected when treating CO115 cells with Q and 5-FU. The effect of this combination was even more pronounced than that of a 100 times higher concentration of 5-FU when tested alone. However, this synergistic enhancement of apoptosis in Q combined with 5-FU was only obtained in the p53 wild-type background of CO115 MSI cells. Other natural compounds, such as triptolide and rosiglitazone, have also been studied in combination with 5-FU and shown to enhance the anticancer effect of this drug in the microsatellite stable (MSS) HT-29 CRC cell line [28,29]. In another study, notoginseng and its ginsenosides were also shown to enhance the antiproliferative and pro-apoptotic effects induced by 5-FU in the MSI p53 wild type HCT116 cell line [30] using, however, high concentrations of compounds.

In a previous study, we have shown that Q and L induce apoptosis in both CO115 and HCT15 cells [24], at the concentrations tested here. The mechanisms of apoptosis induction by these flavonoids have, however, not been studied previously in

these cells and, as shown here, appear to be cell line dependent. We observed that, in CO115 cells, the caspase inhibitor zVAD totally abrogated apoptosis induction by Q and L. This was in agreement with the expression of apoptotic-associated markers, such as induction of cleavage (activation) of caspase-9 and caspase-3 as well as a decrease in Bcl-2 expression. It seems, therefore, that Q and L induce apoptosis via caspase dependent pathways in CO115 cells with a contribution of the mitochondrial pathway, even in the absence of Bax expression. The test compounds also induced p53 expression, which indicate that they may induce apoptosis in p53 wild-type CO115 cells by modulating p53. The induction of apoptosis by 1μM 5-FU in CO115 cells was low, but caspase dependent. Although Q was the compound that more remarkably induced p53 expression, the synergism with 5-FU observed for Q in CO115 cells seems not be due to a further increase in p53 expression. A remarkable increase in the expression of cleaved caspase 9, caspase 3 and PARP as well as a decrease in Bcl-2 expression were observed when Q and 5-FU were combined, as compared with compounds alone. In addition, since Q and L inhibit the PI3K/Akt pathway in CO115 cells [24] and Akt is involved in the suppression of apoptosis contributing to the resistance of CRC cells to chemotherapy [31-33], the effect observed here may also have a contribution of an inhibition of Akt.

On the other hand, in HCT15 cells zVAD did not inhibit apoptosis induced by any of the compounds or the reference inducer STS. The lack of caspase-dependent apoptosis was corroborated by the absence of cleaved caspase-9 and caspase-3 when these cells were incubated with Q and L, as well as with 5-FU. Low levels of cleavage of caspase 3 and PARP were, however, observed when these cells were treated with STS. It seems, therefore, that in HCT15 the test compounds did not induce apoptosis through caspase activation although they decreased Bcl-2 and increased Bax expression. Q, L and 5-FU also induced p53 expression in these cells. However, since HCT15 harbors a *P53* inactivating mutation, alteration in the expression of this protein is not expected to be of functional significance for apoptosis in these cells. In a previous study [24], we showed that the Q and L in HCT15 have an inhibitory effect on MAPK/ERK pathway. Since this pathway is also associated with an inhibition of apoptosis and drug resistance [34,35], this effect may also contribute to the enhancement of apoptosis of Q and L with 5-FU.

Regarding the two stress-activated protein kinases, none of the flavonoids induced the JNK pathway in neither of the cell lines; they seem, however, to induce the

p38 pathway in CO115 cells. 5-FU also did not alter JNK or p38 expressions in neither of the cell lines. The induction of apoptosis by Q and L in combination with 5-FU could, therefore, also have a contribution of an induction of p38 pathway in CO115 cells. The lack effect of 5-FU on JNK pathway was also observed by others in HT-29 cells [36], and effects of 5-FU on p38 stimulation have been reported in KM12C CRC cells [37]. On the other hand, our results show that STS induces phosphorylation of JNK and decreases p38 expression in both cells. The activation of JNK pathway by STS associated with an induction of apoptosis has only been reported in breast cancer cells [38] and the effect of this compound on these two stress activated kinases in CRC is not well established. The effect of 5-FU and the two flavonoids on these MAP kinase pathways seems to be largely dependent on cell type and treatment conditions.

In conclusion, this study shows the potential applicability of Q and L in the enhancement of the apoptotic effects of 5-FU in MSI CRC cells. CRC MSI patients would gain from customized treatment modalities based on p53 status in order to enhance therapeutic efficacy. Although, the data provided here cannot be generalized to all CRC MSI cases, it suggests that treatment strategies, in particular with Q, may benefit selectively p53 wild-type MSI patients.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Manuscript 2

Ursolic acid synergistically enhances apoptosis induced by 5-Fluorouracil through JNK pathway and induces LC3 accumulation in colorectal cancer cells

The work presented in this chapter is in preparation to submit to *International Journal of Cancer*:

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Ursolic acid synergistically enhances apoptosis induced by 5-Fluorouracil through JNK pathway and induces LC3 accumulation in colorectal cancer cells.

Ursolic acid synergistically enhances apoptosis induced by 5-FU through JNK pathway and induces LC3 accumulation in colorectal cancer cells

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Abstract

Colorectal carcinoma (CRC) is a common cause of cancer-related death. Microsatellite instability (MSI) tumors with *P53* mutation and thymidylate synthase (TS) activation have been shown to be resistant to chemotherapy with 5-fluorouracil (5-FU), the most widely used pharmacological drug for CRC treatment. Here we tested effects of ursolic acid (UA), a natural triterpenoid, on cell death mechanisms in two MSI human CRC derived cell lines, one with mutant p53 and TS activation (HCT15) and the other wild-type for these two genes (CO115). In this study, we demonstrated that UA synergistically enhances apoptosis induced by 5-FU in HCT15 cells. This effect was associated with an activation of JNK by UA. A production of ROS levels by UA also showed to contribute to apoptosis induction. The increased apoptosis induced by UA in HCT15 and CO115 cell lines does, however, not explain all the cell death observed, which suggests the involvement of other types of cell death independent of caspases. The involvement of UA in the autophagic process was investigated. UA demonstrated to induce LC3 accumulation, an effect suppressed by inhibition of JNK. In conclusion, this study emphasises the potential of UA for enhancement apoptosis induced by 5-FU and suggest a role for UA on autophagy related cell death. Importantly, UA had a more pronounced effect on induction of cell death than 5-FU.

Keywords: Ursolic acid, Colorectal carcinoma, Apoptosis, LC3, JNK, 5-Fluorouracil

Introduction

Colorectal carcinoma (CRC) is the second cause of death for cancer worldwide and 5-fluorouracil (5-FU) is the main chemotherapeutic agent used in the treatment of this disease.[1] However, significant resistance to 5-FU has been reported, and drugs such as irinotecan and oxaliplatin are used in combination with 5-FU to increase treatment efficacy.[2, 3] The enzyme thymidylate synthase (TS), essential for the synthesis of a precursor (deoxythymidine-5'-monophosphate) for DNA replication, is inhibited by the active metabolite of 5-FU (5-fluoro-2´-deoxyuridine-5´monophosphatate) resulting in the induction of cell cycle arrest.[1, 4] Resistance to 5-FU has associated with TS activation.[1, 4] In addition, cells that harbour *P53* mutations when exposed to 5-FU have also showed reduced apoptosis.[5, 6] Tumors presenting microsatellite instability (MSI) status, which accounts for 15% of sporadic CRC, have demonstrated *in vitro* resistance to 5-FU,[7-9] suggesting, little or no benefit from 5-FU treatment in MSI patients, although clinical evidences are not always consistent.[9] These patients would clearly gain from new treatment modalities with enhanced efficacy.

Apoptotic cell death is a fundamental cellular process that plays an important role during development and tissue homeostasis, that also has profound effects on cancer growth and progression.[10] Apoptosis is one form of regulated cell death, also called programmed cell death. The apoptotic pathway is mediated by death receptors on the cell membrane (extrinsic pathway) or by the mitochondrial pathway (intrinsic pathway) and involves the activation of caspases.[10, 11] Death by necrosis occurs when the cellular contents are released in an uncontrolled manner into the cell´s environment, due to a rapid and drastic induction of death. Necrosis is often associated with an inflammatory response.[10] Other alternative cell death mechanisms have been proposed. Autophagy, or called type II cell death or autophagic cell death, although considered a mechanism of survival, has also been demonstrated to assume a role in cell death, especially when apoptosis is not functional.[10, 11] Some natural products have demonstrated the ability to modulated apoptosis and autophagy through different signalling pathways in CRC, thereby contributing to reduce cell growth and increase cell death.[12, 13]

The c-Jun N-terminal kinase (JNK), a stress-activated protein kinase of the family of the mitogen activated protein kinase (MAPK), has been implicated in many cellular events including apoptosis signalling.[14, 15] JNK is activated by diverse cellular

stresses, such as UV irradiation, DNA damage, heat and osmotic shock and oxidative stress, and its function has been shown to be dependent on cell type and stimulus.[15] JNK has been shown to induce apoptosis through TNF-α and via modulation of proapoptotic Bcl-2 family proteins.[14] More recently, JNK was found to be a mediator of autophagy, contributing for autophagic cell death in some types of cancer cells.[16-20] Studies have shown that an activation of JNK can mediate Beclin-1 expression[18], regulate damage-regulated autophagy modulator (DRAM),[17, 21] as well as, mediate p53 phosphorylation,[16] leading to autophagic cell death.

Ursolic acid (UA) is a naturally occurring triterpenoid that is found in fruits and medicinal herbs.[22] Several biological properties have been attributed to UA including anti-inflammatory and anticancer activities.[22] In our previous study[23] we demonstrated that UA has anticancer activity through effects on PI3K pathway, in two human derived-colorectal cancer cell lines, HCT15 and CO115. These are MSI CRC cell lines. HCT15 harbors additionally a *P53* inactivating mutation and a *TS* activating mutation while CO115 is wild-type for these two genes.[24, 25] Our data shows that UA induces cell death through apoptosis and other death mechanisms that seem to involve autophagy, being more efficient than 5-FU in inducing cell death. A therapeutic potential for UA in combination with 5-FU on the induction of cell death, in cells with a apoptosis resistant profile, was demonstrated.

Material and methods

Reagents and antibodies

Ursolic acid (UA), z-VAD-fmk (zVAD), staurosporine (STS), 5-Fluorouracil (5-FU), SP600125 (SP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the compounds were resuspended in dimethyl sulfoxide (DMSO).

Primary antibodies were purchased to the following sources: anti-phospho-JNK, anti-JNK, anti-p53 and anti-MAPLC3 to Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-phospho-mTOR and anti-mTOR to Cell Signaling (Danvers, MA, USA); and anti-β-actin to Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and sheep anti-mouse were purchased to GE Healthcare (Bucks, UK).

Cell lines

HCT15 and CO115human colon carcinoma-derived cell lines were maintained at 37ºC in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1% antibiotic/antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2ml) and twelve (1ml) well plates at a density of 0.75×10^5 (HCT15) and 1.0×10^5 (CO115) cells/ml.

Apoptosis analysis by TUNEL assay

TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed to estimate the percentage of apoptotic cells. After different treatments for 48h, cells were collected (both floating and attached cells), fixed with 4% paraformaldehyde for 15min at room temperature and attached into a polylysine treated slide using a Shandon Cytospin. Then, cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a count higher than 500 cells per slide under a fluorescent microscope. Results are presented as mean ± SEM of at least three independent experiments.

Cell death analysis by PI staining

After different treatments, cells were collected (both floating and attached cells) and washed in ice cold PBS containing 5% (v/v) FBS. Then, cells were resuspended in the previous ice cold buffer and propidium iodide (PI) solution added to a final concentration of 0.5 mg/ml. Cells were maintained on ice, protected from light, until microscope observation. Twenty microliters of the stained suspension were placed on clean microscope slides and overlaid carefully with coverslips. Immediately, cells were visualized on a fluorescent microscope and photos taken from different fields. The percentage of death cells (PI positive) was calculated from the ratio between PI positive cells and total number of cells (visualized under phase contrast), from a count higher than 500 cells per slide. Results are presented as mean ± SEM of at least three independent experiments.

Western blot analysis

Cells were subjected to different combinations for 48h, and total cell lysates were prepared to measure protein expressions. The cells were washed with PBS 1X and lysed for 15min at 4ºC with ice cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM $Na_2V_3O_4$ and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA used as a protein standard. To perform western blot analysis, 20µg of protein were resolved by SDS-polyacrylamide gel and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or BSA (bovine serum albumin), washed in TPBS and then incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control.

Statistical analysis

Statistical analyses were done using *t*-test, using GraphPad Prism 4.0 software (San Diego, CA, USA). *P*-values ≤ 0.05 were considered statistically significant. All results are presented as mean ± SEM of at least 3 independent experiments. Images are representative of three independent experiments.

Results

UA synergistically enhances apoptosis induced by 5-FU in HCT15

The cell lines HCT15 and CO115 were found to have different susceptibilities to UA in a previous work.[23] For this study, we choose a concentration of UA (based in MTT and BrdU assays) [23] and 5-FU (Xavier et al., unpublished data) that decreased cell growth by about 50% after 48h of incubation. In HCT15 cells, UA at 4µM and 5-FU at 100µM were used, while in CO115 UA at 10µM and 5-FU at 1µM were used. The

induction of apoptosis in the cells treated with UA and/or 5-FU was monitored by the TUNEL assay (Fig. 1A). A synergistic effect was observed when UA is combined with 5-FU in HCT15 cells, with an increase of about 6 times when compared with 5-FU alone. This synergistic effect was not observed for CO115 cells.

To study the involvement of caspase activation in UA-induced apoptosis, cells treated with UA were incubated with 0.250µM z-VAD-fmk (z-VAD), a general caspase inhibitor, for 48h (Fig. 1B). We observed that zVAD significantly inhibited apoptosis induced by UA and staurosporine (STS), a classical inductor of caspases used here as a control, in CO115 cells. Contrarily, no effect on apoptosis induced by UA and STS was observed for zVAD in HCT15 cells, suggesting a mechanism independent of caspase pathways in this resistant cell line.

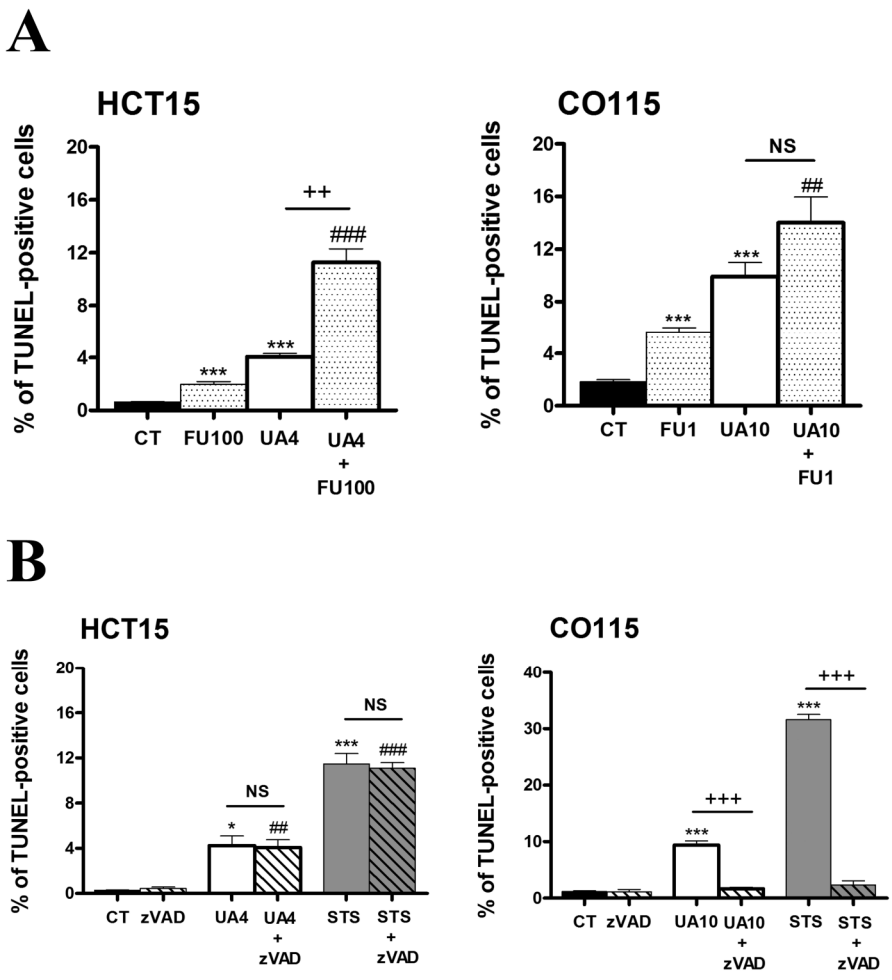

Figure 1 – Effect on apoptosis of 5-fluorouracil 100µM (FU100) and 1µM (FU1) co-incubated with ursolic acid 4µM (UA4) and 10µM (UA10), as well as, these compounds alone (**A**) and effect of a caspase inhibitor zVAD-FMK (zVAD) 20µM on the apoptosis induction by UA and staurosporine (STS) 0.25µM (**B**), in HCT15 and CO115, for 48h, using TUNEL assay. * P≤ 0.05 and *** P≤ 0.001, when compared with control; ++ P≤ 0.01 and +++ P≤ 0.001, when compared with UA alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with FU (A) or zVAD (B) alone; NS, not significant differences observed between each other.

Induction of apoptosis by UA is dependent on JNK activation in HCT15

Numerous evidences show that JNK contributes to apoptosis induced by various stresses.[14] Thus, the involvement of this kinase in the UA-induced apoptosis was evaluated. Firstly, using western blot analysis, we observed that UA induces phospho-JNK expression in both cell lines, with a remarkable effect in HCT15 (Figure 2A). An increase of phospho-JNK expression was also observed for STS in both cell lines, and no effect was detected for 5-FU.

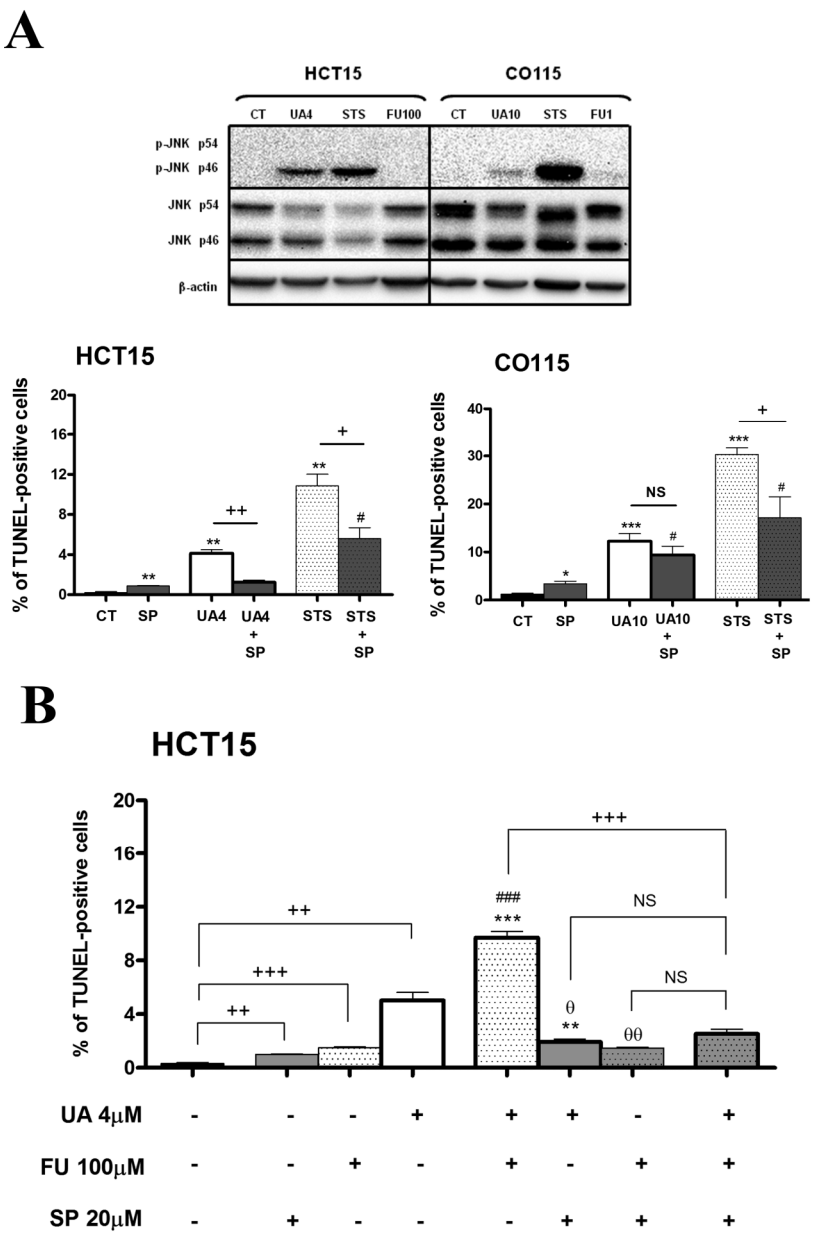

Figure 2 – Effect on phospho-JNK and total JNK expressions, for 48h, of ursolic acid 4µM (UA4) and 10µM (UA10), 5-fluorouracil 1µM (FU1) and 100µM (FU100) and staurosporine (STS) 0.25 µM, in HCT15 and CO115 cells, by western blot. Effect of SP600125 (SP), a JNK inhibitor at 20µM, on the apoptosis induced by UA and STS, for 48h, in HCT15 and CO115 cells, using TUNEL assay. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001, when compared to control; + P≤ 0.05 and ++ P≤ 0.01, when compared with

the respective compound alone; # P≤ 0.05, when compared with SP alone (**A**). Effect on cell death of FU100 and UA4 co-incubated with SP, for 48h in HCT15 cells, using PI staining. ++ P≤ 0.01 and +++ P≤ 0.001, when compared with the control and with the co-incubation FU+UA+SP; ** P≤ 0.01, *** P≤ 0.001, when compared with UA alone; θ P≤ 0.05 and θθ P≤ 0.01, when compared with SP alone; ### P≤ 0.001, when compared with FU alone (**B**). NS, not significant differences observed between each other.

To assess whether apoptosis induction was due to an activation of the JNK, TUNEL assay was performed in the presence of 25µM SP600125 (SP), a JNK inhibitor (Figure 3A). It was observed that SP totally inhibited apoptosis induced by UA in HCT15 cells and no effect was observed in CO115 cells. SP also inhibited the apoptosis induced by STS in both cell lines. Thus, it seems that the UA induction of apoptosis in HCT15 cells is dependent on JNK pathway.

In a further experiment, it was tested whether the activation of JNK by UA was the responsible for the synergistic effect of UA combined with 5-FU in HCT15 cells. We observed that SP did not inhibit the apoptosis induced by 5-FU, and, again, an inhibition of UA-induced apoptosis was observed in the presence of SP (Figure 2B). A total abrogation the synergistic effect on apoptosis of the combination of UA with 5-FU was observed in the presence of SP. These results suggest a dependence on JNK activation for UA-induced apoptosis in HCT15 cells, which seems to be the responsible of the synergistic effect with 5-FU.

Oxidative stress induced by UA contributes to apoptosis induction in HCT15

The role of oxidative stress on apoptosis induced by UA was tested using a common antioxidant N-acetylcysteine (NAC). When NAC was used in combination with UA for 48h, the induction of apoptosis was partially inhibited in HCT15 cells, but not in CO115 cells (Figure 3). This result suggests an implication of oxidative stress as a contributor for UA-induced apoptosis in HCT15.

UA induces cell death in colon cancer cells

The apoptosis induced by UA in HCT15 and CO115 cells at 48h, although significantly, only represents around 4% and 10% of total cell number, respectively, which does not correspond to the extensive morphological changes observed. Therefore, cell death was subsequently measured using PI staining, at 2h and 48h. As shown in Figure 4, UA did not increase remarkably cell death after 2h of incubation, suggesting there is no acute necrotic effect induced by UA. However, after 48h, UA induced cell

death to around 50% of cells, in both cell lines, as shown by the increase number of PI positive cells. On the other hand, 5-FU did not induce significant cell death in either of the cell lines after 48h. Cell death induced by the combined treatment of UA with 5-FU seems, therefore, to be due to UA. Therefore, UA induces cell death in HCT15 and CO115 cells, by mechanisms other than apoptosis or necrosis.

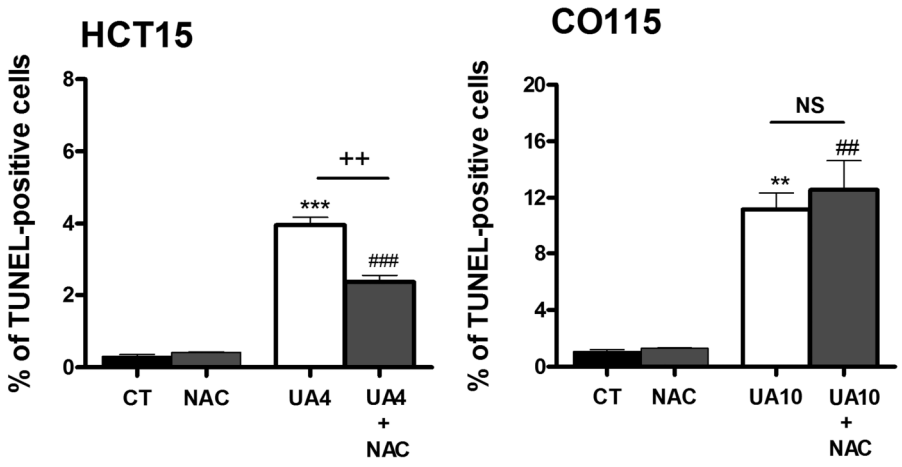

Figure 3 – Effect of N-Acetyl-L-cysteine (NAC) at 5mM, on the apoptosis induced by ursolic acid 4µM (UA4) and 10µM (UA10) in HCT15 and CO115 cells respectively, for 48h using TUNEL assay. ** P≤ 0.01 and *** P≤ 0.001, when compared to control; ## P≤ 0.01 and ### P≤ 0.001, when compared with NAC alone; ++ P≤ 0.01, when compared with the respective compound alone; NS, not significant differences observed between each other.

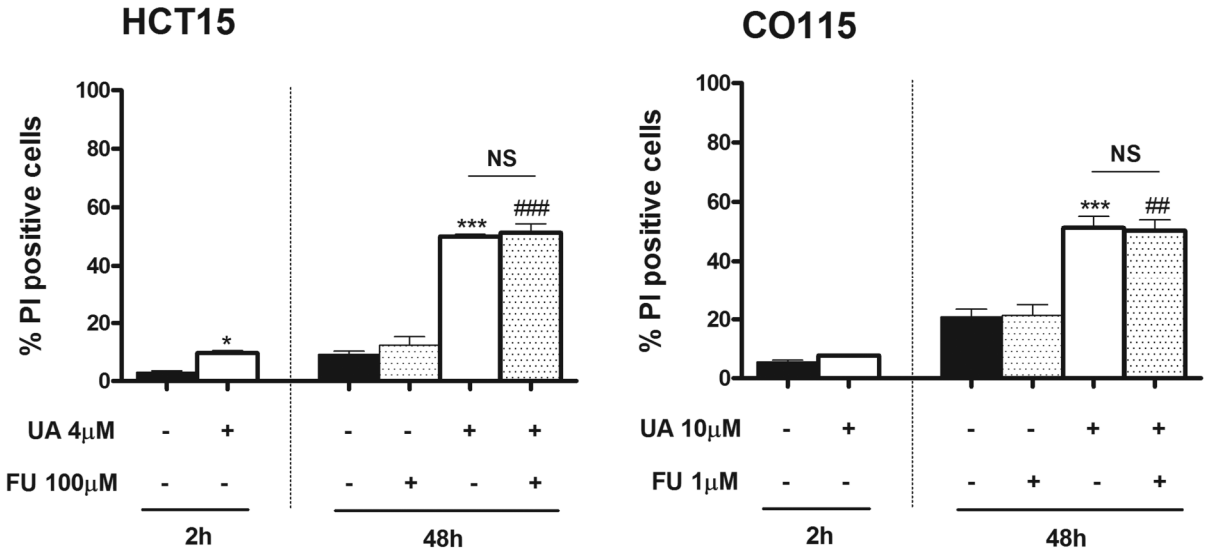

Figure 4 – Effect on cell death of 5-fluorouracil 100µM (FU100) and 1µM (FU1) co-incubated with ursolic acid 4µM (UA4) and 10µM (UA10), as well asthese compounds alone, for 2h and 48h, in HCT15 and CO115 cells, using PI staining. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001, when compared with control; ## P≤ 0.01 when compared with FU alone; NS, not significant differences observed between each other.

JNK is also implicated in other mechanisms of UA-induced cell death in HCT15

Previously, we demonstrated that JNK pathway was implicated in apoptosis induced by UA in HCT15 cells. Then, we tested whether this pathway was involved in total cell death, indicated by PI staining. As shown in Figure 5, SP partially inhibited the cell death induced by UA in HCT15 cells, as well as, the cell death induced by the combination of UA with 5-FU. No significant effect of SP on cell death induced by UA and UA combined with 5-FU was observed in CO115 cells. This data suggest that JNK activation by UA not only induces apoptosis, but also other forms of cell death seem to be partially dependent on this pathway in HCT15 cells. Contrarily, JNK seems not be involved in UA-induced cell death in CO115 cells.

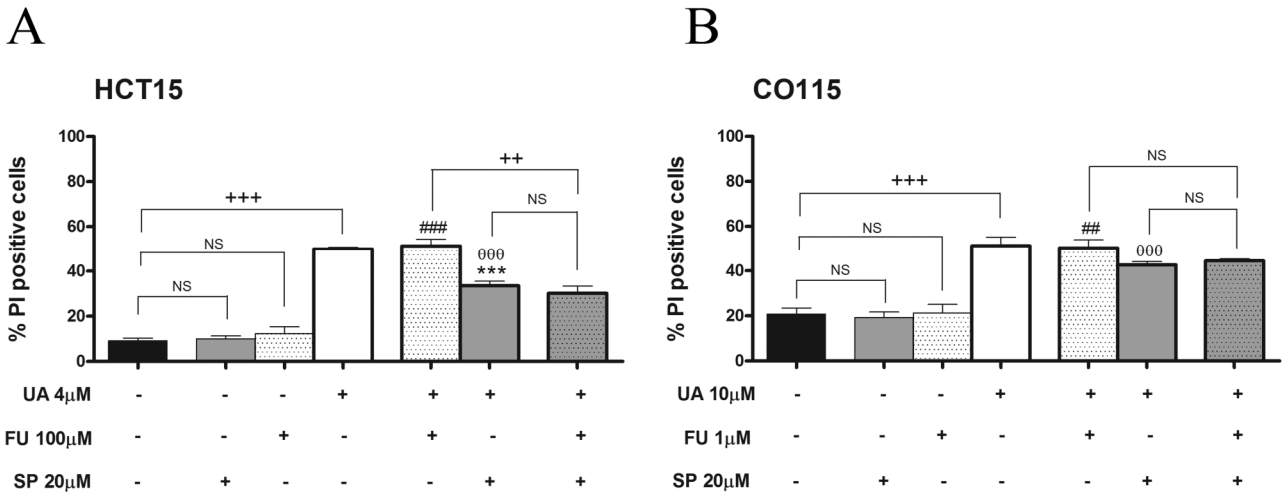

Figure 5 – Effect on cell death of 5-fluorouracil 100μM (FU100) and 1μM (FU1) co-incubated with ursolic acid 4μM (UA4) and 10μM (UA10) and SP600125 20μM (SP20), as well as, these compounds alone, for 48h in HCT15 and CO115 cells, using PI staining. ++ P≤ 0.01 and +++ P≤ 0.001, when compared with the control and with the co-incubation FU+UA+SP; *** P≤ 0.001, when compared with UA alone; θθθ P≤ 0.001, when compared with SP alone; ## P≤ 0.01 and ### P≤ 0.001, when compared with FU alone. NS, not significant differences observed between each other.

UA induces LC3 accumulation: involvement of JNK, mTOR and p53

It was observed that the percentage of apoptosis induced by UA, in both HCT15 and CO115 cells after 48h, does not explain all the cell death observed, suggesting an involvement of other types of cell death independent of caspase. Therefore, we investigated the possible role of UA on autophagy and in some regulators of autophagy, such as p53 and mTOR, as well as, the involvement of JNK, using immunoblotting analysis. One of the hallmarks of autophagy is the conversion of cytosolic LC3-I into

autophagosome-associated LC3-II, the amount of which reflects the abundance of autophagosomes. Our results showed that UA induces an accumulation of LC3-II expression levels in both cell lines, which was reduced in the presence of SP with a more remarkable effect in HCT15 cells (Figure 6A). In contrast, no effect on LC3-II expression was detected in cells treated with 5-FU or SP alone. The accumulation of LC3 observed in cells treated with UA and 5-FU seems to be due to UA, and this effect was inhibited in the presence of SP. The effect was more noticeable in HCT15 cells. These results suggest that UA interferes with autophagy, in both cell lines, and that JNK may be involved.

The autophagic process is modulated by a number of molecules such as p53 and mTOR.[26] The possible effect of UA on the levels of these regulators was also evaluated. UA decreased p53 levels in both cell lines, as well as, the p53 induced by 5-FU, suggesting a regulatory effect of UA on this protein. In addition, UA decreased phospho-mTOR levels in both cell lines, indicating a possible effect in this protein. These effects of p53 and mTOR are compatible with an induction of autophagy by UA.

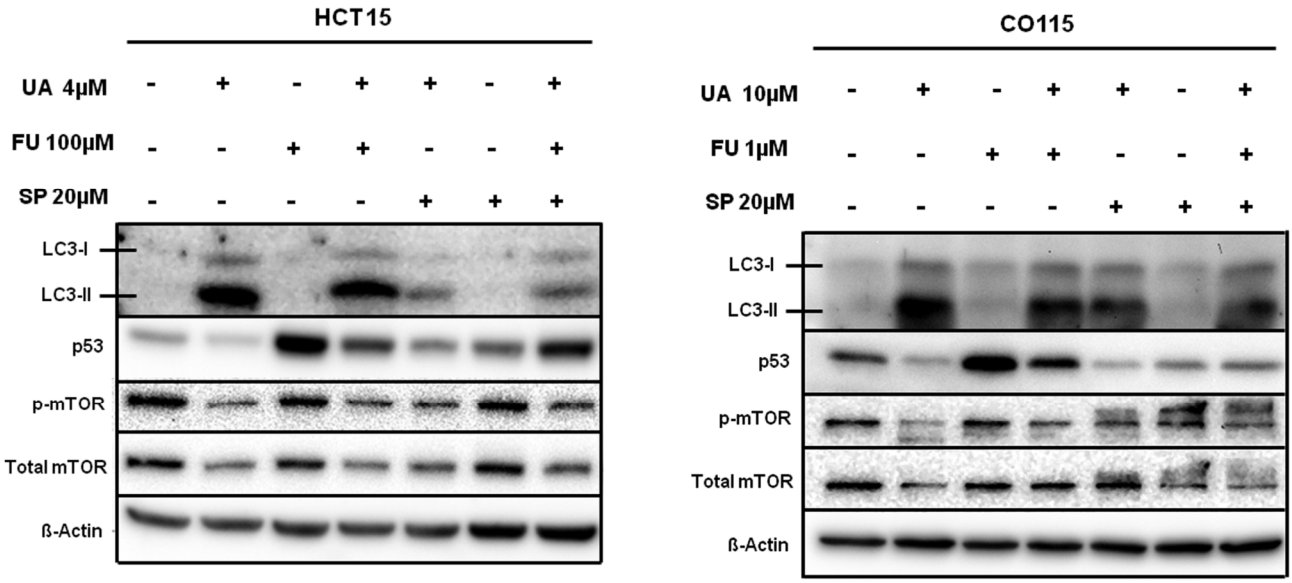

Figure 6 – Effect on the expression of LC3, p53, phospho-mTOR, total mTOR, phospho-JNK, and total JNK of ursolic acid (UA), 5-fluorouracil (FU) and SP600125 (SP) in HCT15 and CO115 cells, for 48h using western blot.

Discussion

The modulation of cell death, which includes apoptosis and autophagy, has demonstrated to be an important strategy in the fight against cancer. Defects in apoptosis play a central role in tumorigenesis and confer resistance to anticancer therapies.[27] 5-Flurouracil (5-FU) is the most commonly used pharmacological drug in CRC chemotherapy, however, several mechanisms of resistance have been reported to reduce its efficacy.[4, 5] CRC cells with defects in Bcl-2 family proteins and/or loss of p53 function have demonstrated to fail to respond to 5-FU treatment.[5, 6] Combinations of therapeutic agents with different modes of action have been suggested to increase treatment efficacy.

In a previous work[23], we demonstrated that UA, a naturally occurring triterpenoid, induces apoptosis in two human CRC cells (HCT15 and CO115). In the present study, the combined effect of UA with 5-FU on total cell death and in death by apoptosis were evaluated. UA showed to significantly induce total cell death and to a smaller extent death by apoptosis, in both cell lines, being more efficient than 5-FU alone. Furthermore, UA showed to induce apoptosis via caspase-independent pathway in HCT15 cells, contrarily to observed for CO115 cells. When UA and 5-FU were used in combination, UA synergistically enhanced apoptosis induced by 5-FU in HCT15 cells. Because this effect was observed in the MSI cells that harbour a *p53* mutation and a TS activation, suggest a relevant effect of UA in combination with 5-FU in a cell type resistant to apoptosis and 5-FU treatment.[1, 5, 8, 9, 28]

The JNK MAP kinase has been implicated in many cellular events including apoptosis [29-33] and autophagy.[16-20] In our study, we found that UA activates JNK in both cell lines, with a remarkable effect in HCT15 cells, whereas no effect on JNK induction was observed for 5-FU. In addition, through the use of the JNK inhibitor SP600125, JNK activation by UA showed to be involved in the apoptosis and total cell death induction in HCT15 but not in CO115 cells. The synergistic effect on apoptosis induced by the combination of UA with 5-FU in HCT15 cells was also shown to be dependent on JNK activation. Furthermore, the combination of UA with 5-FU was demonstrated to be partially dependent on JNK in HCT15 but not in CO115 cells. Therefore, both apoptosis and total cell death induced by UA alone or UA plus 5-FU in the resistant HCT15 cells demonstrated to be JNK-dependent, possible in response to oxidative stress produced by UA in these cells. Contrarily, this was not observed for apoptosis or

cell death induced by UA in CO115 cells, where a caspase-involvement was detected. The importance of JNK activation as one contributor mechanism to induce cell death in CRC is demonstrated by the drug atorvastatin. Atorvastatin, one of the chemotherapeutic drugs used in CRC, showed to induce apoptosis involving JNK activation.[34] This drug has a synergistic interaction with celecoxib, a selective cyclooxyhenase-2 inhibitor, in killing human CRC cancer cells.[34]

Nevertheless, apoptosis induced by UA comprises only a small percentage of death induction in both cell lines. PI results at 2 hours suggest that there was no necrotic cell death involved, indicating other types of cell death induced by UA. Autophagy, also called type II cell death or autophagic cell death is activated under stress conditions, such as nutrient and/or growth factor deprivation. This process represents a mechanism of survival but it may assume a cell death function when apoptosis is deregulated.[10, 35] Our results showed that UA induces accumulation of LC3-II in both cell lines, which is inhibited in the presence of SP (more remarkable in HCT15), suggesting UA's modulation of autophagy and a role for JNK also in this process. On the other hand, in our study, 5-FU seems not to affect the autophagic process because there were no changes in LC3-II. An accumulation of LC3-II, however, was recently found not be a certain indicative of autophagic induction.[36] LC3-II, a marker of autophagosomes, may increase when autophagic flux is activated, but it can also be accumulated when a blockage occurs downstream of autophagosomes formation.[36] Thus, our results indicate a role of UA in autophagy in both cell lines, an effect reported here for the first time, and suggest the autophagic cell death as a possible way of UA-induced cell death.

The proteins p53 and mTOR have been shown to be important regulators of the autophagic process.[26, 37] The cytosolic p53, both mutant and wild-type form, has been demonstrated to inhibit autophagy.[38, 39] On the other hand, an inhibition of mTOR is associated with an induction of autophagy.[26, 37] Our results showed that UA decreases mTOR, as well as, both wild-type and mutant p53 (in CO115 and HCT15 cells, respectively), corroborating a possible effect of UA on the induction of autophagy. Recent studies demonstrated the ability of an inhibitor of autophagy, 3-methyladenine, to enhance apoptosis induced by 5-FU in CRC cells[40, 41]. Thus, the effect of UA on autophagy should be further explored and the possibility that UA inhibits autophagy thereby increasing cell death should also be taken into consideration.

In conclusion, this study found that UA induces apoptosis, as well as, total cell death more efficiently than 5-FU alone. In addition, it suggests the potential

applicability of UA in the enhancement of the apoptotic effect of 5-FU in the resistant CRC cell line, where an activation of JNK seems to be responsible for this synergistic effect with a contribution of ROS. A role in the modulation of autophagy by UA and the role for JNK in this process were also found in both cell lines. The effect of UA on autophagy needs, however, to be better clarified in the future.

Acknowledgements

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CHAPTER VI

ROLE OF URSOLIC ACID ON AUTOPHAGY AND LYSOSOMES INTEGRITY

The natural triterpenoid ursolic acid inhibits autophagy and induces lysosomal membrane permeabilization in breast and colon cancer cells

The work presented in this chapter is in preparation to submit to *Autophagy*:

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The natural triterpenoid ursolic acid inhibits autophagy and induces lysosomal membrane permeabilization in breast and colon cancer cells

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Abstract

Autophagy is the lysosomal recycling of intracellular constituents that offers a survival mechanism to the cells under metabolic stresses. In cancer cells, autophagy has demonstrated a dual role through promotion of cell survival or induction of tumor suppression. Ursolic acid (UA), a natural triterpenoid, has various anticancer activities in autophagic-associated signalling pathways. However, its role in autophagy is still unknown.

In the present study, we characterized the effect of UA in the autophagic process, in MCF-7 and HCT15, breast and colon cancer cells, respectively. An accumulation of autophagosomal structures in both cell types without colocalization with LAMP-2 was observed, indicating that UA inhibits autophagy at the maturation step. This effect was further confirmed by the increased levels of p62 and by the LC3 turnover, using a luciferase-based real-time assay, showing UA-blockage of the autophagic flux. UA was also able to inhibit rapamycin-induced autophagy. These events were simultaneous with no effect on apoptosis induction, decreased cell survival and morphological changes, indicating an involvement of autophagy in UA induced cell death. Additionally, an effect of UA on lysosomal membrane permeabilization was observed, suggesting that UA destabilizes lysosomes probably also inducing lysosomal cell death pathway. Moreover, p38 and JNK MAP kinases seem not mediate UA-inhibition of autophagy in MCF-7 cells.

In conclusion, UA blocks autophagy at the maturation step probably through effect on lysosomes, suggesting this natural triterpenoid as a novel compound to be exploited in cancer therapy.

Keywords: Ursolic acid, colorectal and breast cancer cells, autophagy, LC3, lysosomes, signalling

Introduction

Macroautophagy (hereafter referred to as autophagy) is a lysosomal degradation pathway which, under basal conditions, maintains the metabolism of the cells providing them with nutrients by removing damage organelles.[1] Autophagy can be rapidly increased in response to numerous conditions of extracellular and/or intracellular stresses, such as nutrient and/or growth factor deprivation, hypoxia, hormones and DNA damage.[2] During autophagy initiation, organelles and parts of the cytoplasm are engulfed and double-membrane vacuoles, the autophagosomes, are formed. The autophagosomes fuse with lysosomes, forming the autolysosomes, where the cargoes are degraded by lysosomal hydrolases and recycled back to the cytoplasm (maturation step).[2] Autophagy has, therefore, been considered a survival mechanism when it is moderately activated, however, it can be implicated in autophagic cell death, when it is impaired[3, 4] or when it is massively activated.[5]

The autophagic process is commonly altered in cancer, at the levels of induction, regulation and lysosomal degradation. The reduced levels of oxygen and nutrients, genetic alterations in cancer-associated genes that modulate autophagy, and alterations of lysosomal activity and trafficking, were shown to influence the autophagic flow.[5] However, also in cancer cells, autophagy has been demonstrated to possess a dual role.[2, 6] Autophagy may contribute to tumor promotion providing the cells with a selective advantage for survival, but it can also function as a tumor suppression mechanism.[6]

Lysosomes are cytoplasmatic organelles that control cell death at several levels.[7, 8] These organelles take part of the autophagic cell death, since the fusion between autophagosomes and lysosomes and the lysosomal degradative capacity are crucial for the completion of the autophagic process.[5] Additionally, an induction of lysosomal membrane permeabilization (LMP) causes the release of lysosomal hydrolases, such as cathepsin proteases and other hydrolases, from the lysosomal lumen to the cytosol, triggering apoptosis through activation of mitochondrial and mitochondrial-independent cell death pathway.[7-10] A massive LMP, i.e., a complete disruption of lysosomes can also result in necrotic death.[8, 9] Compounds that directly target lysosomes by inducing LMP have recently been shown to be effective in killing cancer cells, especially apoptosis resistant cancer cells.[9, 10]

Ursolic acid (UA) is a natural triterpenoid found in some traditional medicinal herbs and in fruits, such as apples, blueberries, cranberries and guava.[11] A variety of

biological properties, such as anti-inflammatory, hepatoprotective and anticarcinogenic, combined with low toxicity, have been demonstrated for UA.[11] In breast cancer cells, UA has been reported to induce apoptosis by downregulating bcl-2 expression[12] and to suppress invasion by inhibiting phosphorylation of c-Jun NH-terminal kinase (JNK), Akt and mammalian target rapamycin (mTOR).[13] In colon cancer cells, UA showed anticancer effects by decreasing the level of phospho-Akt[14], bcl-2 expression, caspase cleavage and by inhibiting MAP kinases.[15, 16] The signalling pathways PI3K/Akt/mTOR and MAP Kinases have also been demonstrated to be implicated in the regulation of the autophagic process, in response to different intra- or extracellular stimuli.[1, 17]

In the present study, we attempted to identify and characterize the role of UA in the autophagic process, in colon and breast cancer cells, and its possible effect on lysosomes.

Results

UA induces cancer cell death

UA 8μM significantly decreased cell viability over time, in both MCF-7 and HCT15 cells, (Fig. 1A) with no significant effect on the survival of HBL-100 nontumorigenic breast-derived epithelial cells (data not shown). Additionally, no remarkable apoptosis (no more than 3% apoptosis of total cell number) induced by UA were observed over time, in both cell lines (Fig. 1B). Nevertheless, from 24 hours of UA treatment, cells were rounding up and detaching with increased effect over time, suggesting occurrence of cell death (Fig. 1C).

UA induces an accumulation of autophagosomes

To investigate the effect of UA on autophagy, we performed a LC3 puncta formation assay in MCF-7-eGFP-LC3 cells. In these cells that were treated with different concentrations of UA (5, 8, 10μM) for 24 hours, a significant increase of eGFP-LC3 translocation from a diffuse distribution to a puncta cytoplasmatic accumulation of LC3-II, the lipidated autophagosome-associated form of eGFPLC3, was observed (Fig. 2A,B). The accumulation of autophagosomes was confirmed by a remarkable accumulation of LC3-II expression levels, assessed by immunoblotting (Fig. 2C). In contrast, LC3 accumulation was not observed for oleanolic acid (OA) treatment,

a triterpenoid acid structurally related with UA, and wortmannin (W), a classical inhibitor of PI3K.

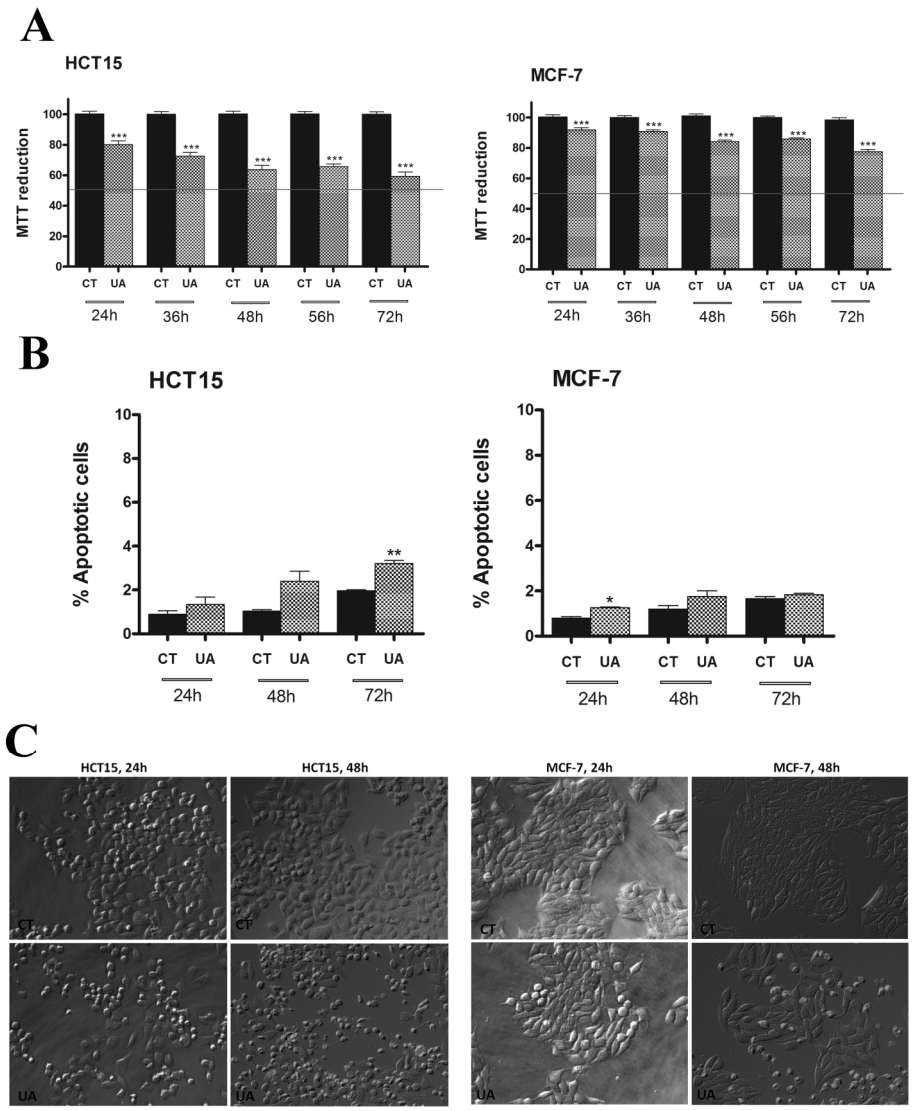

Figure 1 – Effect of ursolic acid (UA) over time on (**A**) MTT reduction, (**B**) apoptosis using TUNEL assay and (**C**) cell morphology, in HCT15 and MCF-7 cells treated with DMSO (control, CT) or UA 8μM. In A, full line represents the concentrations that inhibited cell growth by around 50%.* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, when compared to control.

UA blocks the fusion step in the autophagic process

LC3 accumulation, although an indicative of effects on autophagy, does not distinguish between induction or inhibition of autophagic flux.[18] Thus, to characterize the autophagic process, an immunofluorescence assay was performed on MCF-7 cells expressing the tandem fluorescent construct mRFP-GFP-LC3 in association with an antibody against the lysosomal-associated membrane protein 2 (LAMP-2). The tandem-tagged proteins GFP and mRFP have different sensitivities to the lysosomal pH

environment, GFP fluorescence signal decreasing after fusion between autophagosomes and lysosomes while mRFP signal remains unchanged (red fluorescence).[19] In MCF-7 cells, treatment with UA 8μM induces an accumulation of autophagosomal structures over time represented by the very large yellow dots (red plus green signal). Interestingly, these structures were not colocalized with LAMP-2, suggesting that no fusion events between lysosomes and autophagosomes take place and therefore, an impaired autophagosomal turnover (Fig. 3A). The same results were observed for HCT15 cells stained with LC3 and LAMP-2 antibodies (data not shown). Furthermore, MCF-7 cells were treated with 3-MA, an inhibitor of the initial steps of the autophagic process, and rapamycin, an inducer of autophagy, and these compounds were combined with UA during 24 hours. As expected, cells incubated with rapamycin alone presented purple colour (red plus LAMP-2 signal), whereas cells incubated with 3-MA alone had no LC3 accumulation (Fig. 3B). In cells incubated with 3-MA plus UA, yellow dots were not observed, which indicates that LC3 accumulation induced by UA is autophagy specific. On the other hand, cells incubated with rapamycin plus UA showed LC3 accumulation, demonstrating that UA really blocks the autophagic process at the maturation step.

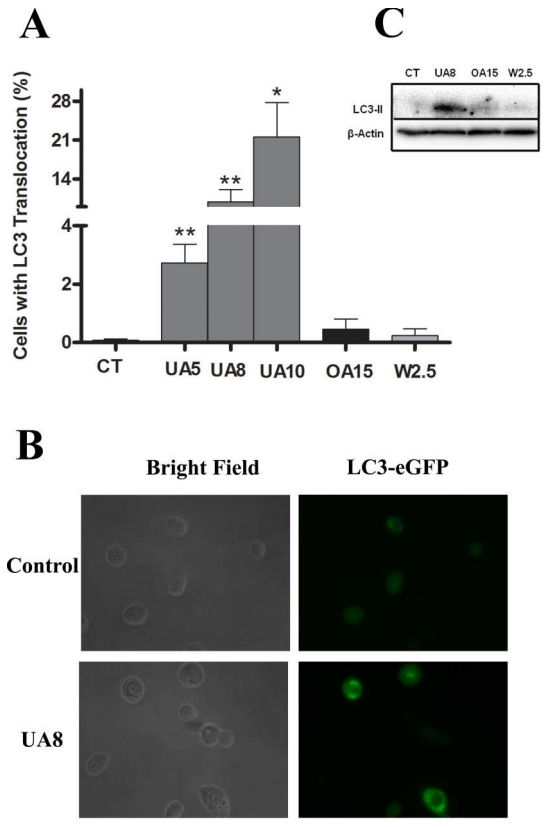

Figure 2 – (**A**) Effect of different concentrations of ursolic acid (UA) 5μM (UA5), 8μM (UA8) and 10μM (UA10), oleonolic acid 15μM (OA15) and wortmannin 2.5μM (W2.5) on LC3 translocation in MCF-7 cells, at 24 hours. The numbers of cells with more than five GFP-LC3 puncta were considered

positive for LC3 translocation. (**B**) Representative images of UA and control. * P≤ 0.05 and ** P≤ 0.01, when compared to control. (**C**) Effect of UA8, OA15 and W2.5 on LC3-II expression levels measured by western blot at 24 hours.

Additionally, the effect of UA on p62 expression was assessed to confirm the inhibition of autophagy. The protein p62 incorporates into autophagosomes through direct binding to LC3 being required for the formation and degradation of intracellular protein aggregates.[20] This protein has been shown to be efficiently degraded by autophagy, even more specifically than LC3, being, therefore, a reliable marker to monitor the autophagic flux.[18, 20] Our results showed an accumulation of p62 expression levels over time, in MCF-7 and HCT15 cell lines, confirming that UA blocks autophagy (Fig. 3C).

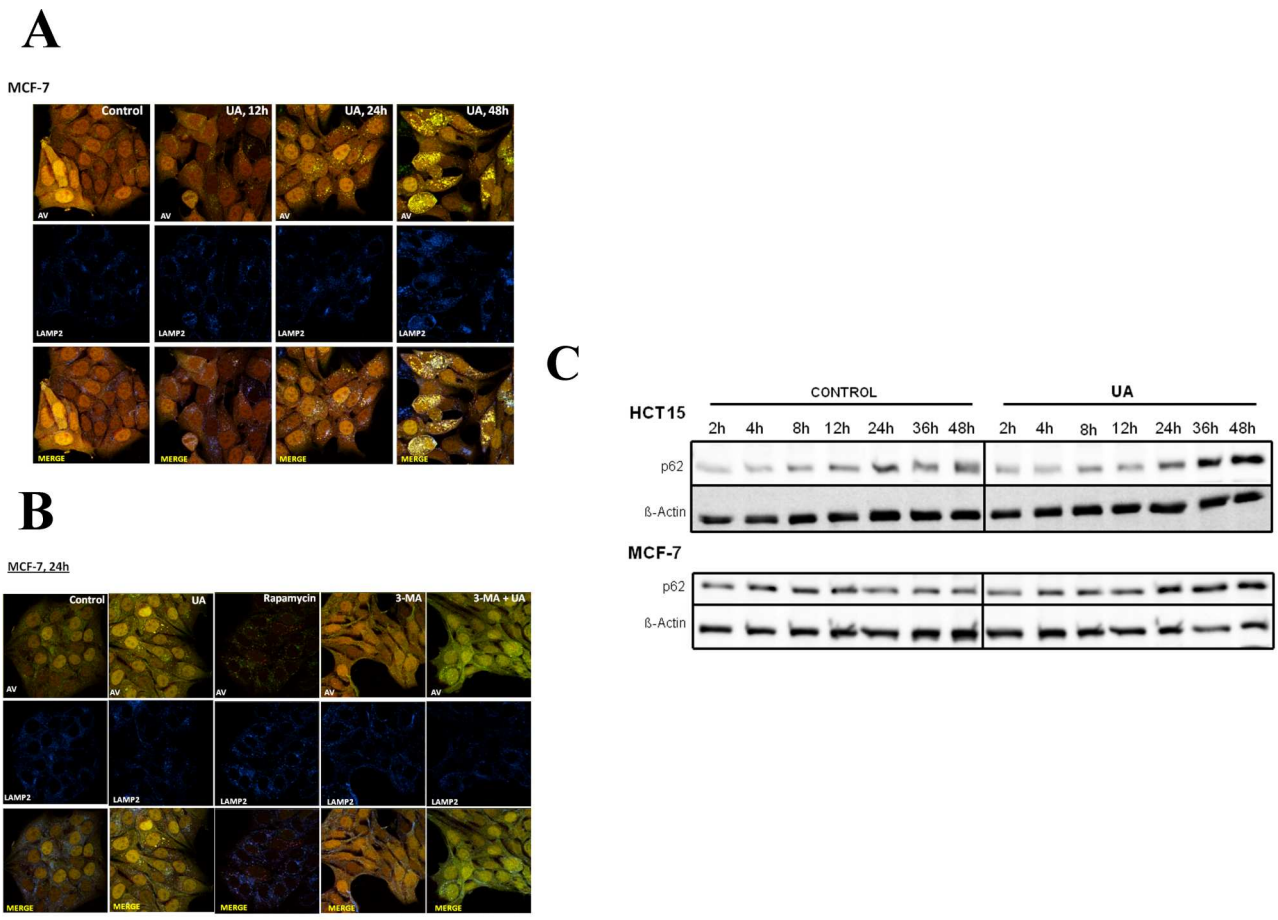

Figure 3 – (**A**) Representative images of different time incubations (12, 24 and 48 hours) with ursolic acid (UA) 8μM and (**B**) 24 hours of incubation with UA 8μM, rapamycin 100nM and 3-Metyladenine (3-MA) 10mM, in MCF-7 cells expressing mRFP-GFP-LC3 stained for anti-LAMP-2 (**C**) Effect of UA 8μM on p62 expression levels over time, in MCF-7 and HCT15 cells, using western blot.

UA inhibits autophagy flux and blocks the rapamycin-induction effect

A study in LC3 turnover using a luciferase-based real-time assay was then performed to corroborate the UA-inhibition of autophagy flux.[21] The cell kinetic assay was carried out for 20 hours with 2 hours intervals between measurements. In this assay, the ratio between the levels of RLucLC3wt (LC3 that is targeted for autophagy-dependent degradation) and RLucLC3G120A (mutated LC3 that is degraded at a much lower rate than RLucLC3wt) reflects the autophagic flux, where a decrease of the ratio corresponds to an induction of the autophagic flux. As observed in Fig. 4A, UA 8μM started gradually to inhibit the autophagic flux from 8 hours. In contrast, rapamycin-induced autophagy was blocked from 8 hours when the cells were co-incubated with UA.

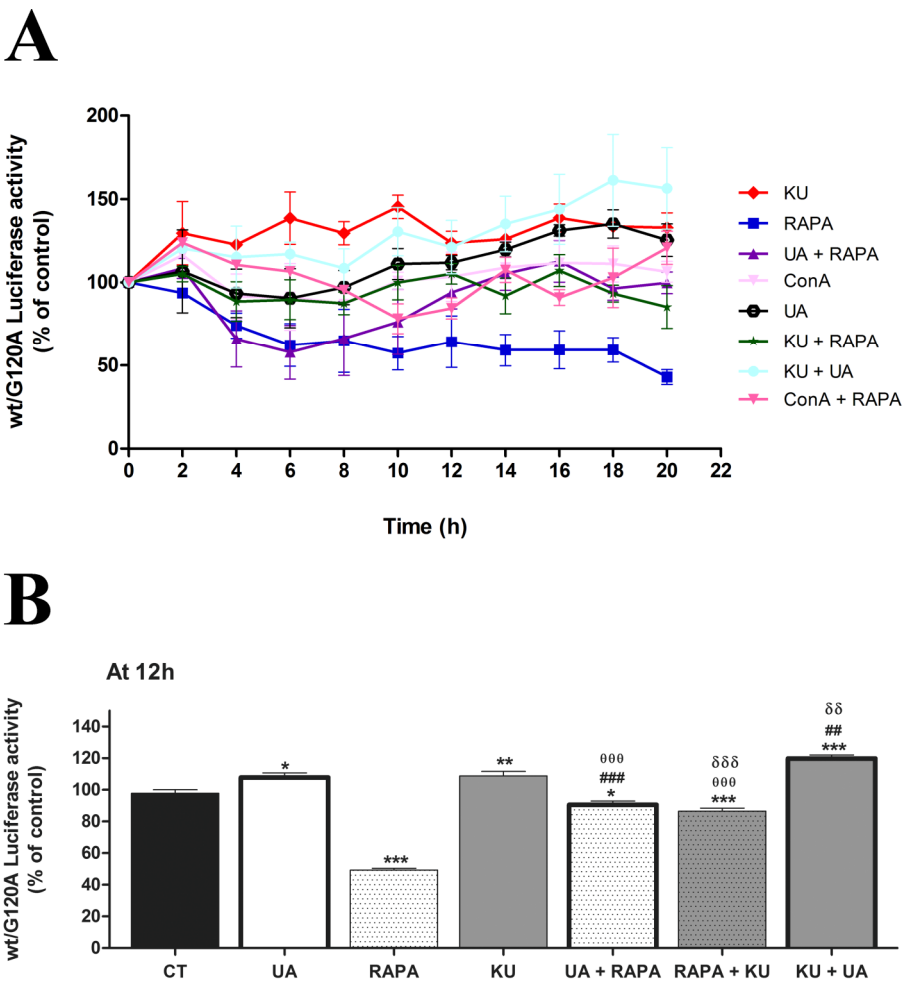

Figure 4 – Effect of ursolic acid (UA) 8μM, rapamycin (RAPA) 20nM, concamycin A (ConA) 2nM and KU 2μM on autophagic flux in MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3G120A. (**A**) Luciferase activity measured with 2 hours intervals during 20 hours and the ratio of the two cell lines were expressed as percentages of the corresponding ratio in untreated cells at T0. (**B**) Luciferase activity measured after 12 hours treatment. * P≤ 0.05, ** P≤ 0.01 and *** P≤ 0.001, when compared to control; ## P≤ 0.01 and ### P≤ 0.001, when compared with UA alone; θθθ P≤ 0.001, when compared to RAPA alone; δδ P≤ 0.01 and δδδ P≤ 0.001, when compared to KU alone.

Moreover, two inhibitors of autophagy, ConA (an inhibitor of V-ATPase and therefore of the lysosomal function, but with a minimal impact on autophagic flux[21]) and KU (an inhibitor of autophagy; unpublished observations) were also used. We observed that KU inhibited autophagy, as well as, inhibited the effect of rapamycin and its effect was further increased in presence of UA. ConA also showed to block the effect of rapamycin.

Furthermore, the RLucLC3wt/RLucLC3G120A was also measured at 12 hours. A significant inhibition of autophagy was observed for UA and KU while rapamycin showed a significant stimulatory effect (Fig. 4B). The inhibition of rapamycin-induced autophagy by UA was confirmed. Additionally, a cooperative effect between UA and KU in the inhibition of the autophagic flux was detected, suggesting different mechanisms of action for these two compounds. Overall, we validated our results that UA blocks autophagic flux and it is able to block the rapamycin-induction effect.

UA induces lysosome membrane permeabilization (LMP)

By immunocytofluorescence experiments, an induction of perinuclear clustering of LAMP-2 positive compartments was observed (Fig. 3). In addition, a decrease of acidic compartments, such as lysosomes, was visualized using acridine orange (data not shown). These results suggested that UA could interfere with lysosome integrity or functionality. Thus, effect on lysosomal membrane stability by UA was hypothesized. We observed that UA significantly decreased the total lysosomal hydrolases activities of cathepsins and NAG, indicating a possible impact on lysosomal biogenesis and/or a direct destabilizing effect on lysosomal membranes (Fig. 5A). Interestingly, UA significantly increased the release of cathepsins and NAG to the cytosol (Fig. 5B), which demonstrates an LMP induction. ConA and TNF were used as positive controls in the induction of LMP.

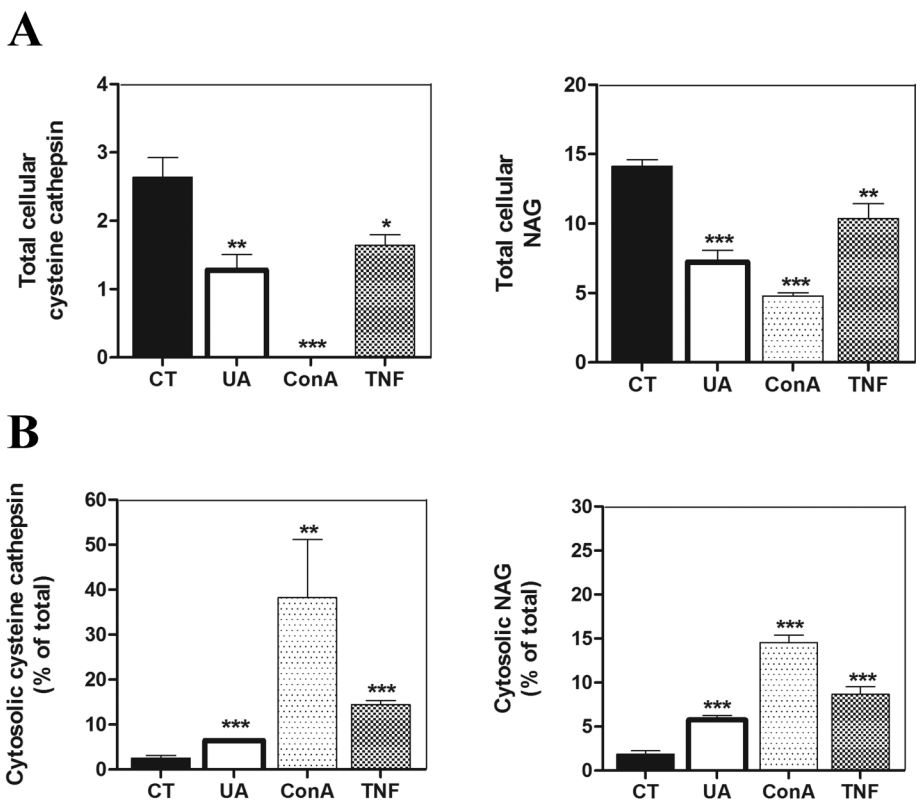

Figure 5 – Effect of ursolic acid (UA) 8μM, concamycin A (ConA) 2nM and TNF 10ng/ml on the release of (**A**) total and (**B**) cytoplasmatic cysteine cathepsin and β-N-acetyl-glucosaminidase (NAG) in MCF-7 cells at 24 hours. Lactate dehydrogenase (LDH) activity of the cytosol was used as an internal standard. * P≤ 0.05; ** P≤ 0.01 and *** P≤ 0.001, when compared to control.

Involvement of p38 and JNK signalling on UA-inhibits autophagy

In a previous study, we observed an activation of JNK pathway in HCT15 cells by UA, which seemed to play a role in autophagy in those cells (unpublished observations). Therefore, the involvement of p38 and JNK signalling on UA-inhibited autophagy in MCF-7 cells was tested, using the luciferase-based real-time assay. For that, SP600125 (SP), an inhibitor of JNK and other kinases, [22] a peptide that specifically inhibits JNK and SB203580 (SB), a specific inhibitor of p38, were used. Our results showed that both SP and SB inhibited the autophagic flux in MCF-7 cells and that this effect increased in the presence of UA, demonstrating that these inhibitors and UA may act through different mechanisms (Fig. 6). In contrast, the peptide alone had no effect on autophagic flux and when it was combined with UA, an inhibition of autophagy was observed to UA values, suggesting an inhibition of autophagy only mediated by UA. It seems that p38 interferes with the autophagic flux contrarily to JNK and neither JNK nor p38 mediated UA-effect on autophagic flow in MCF-7 cells, although JNK seems to have a role at least in some colon cancer cells (unpublished observations).

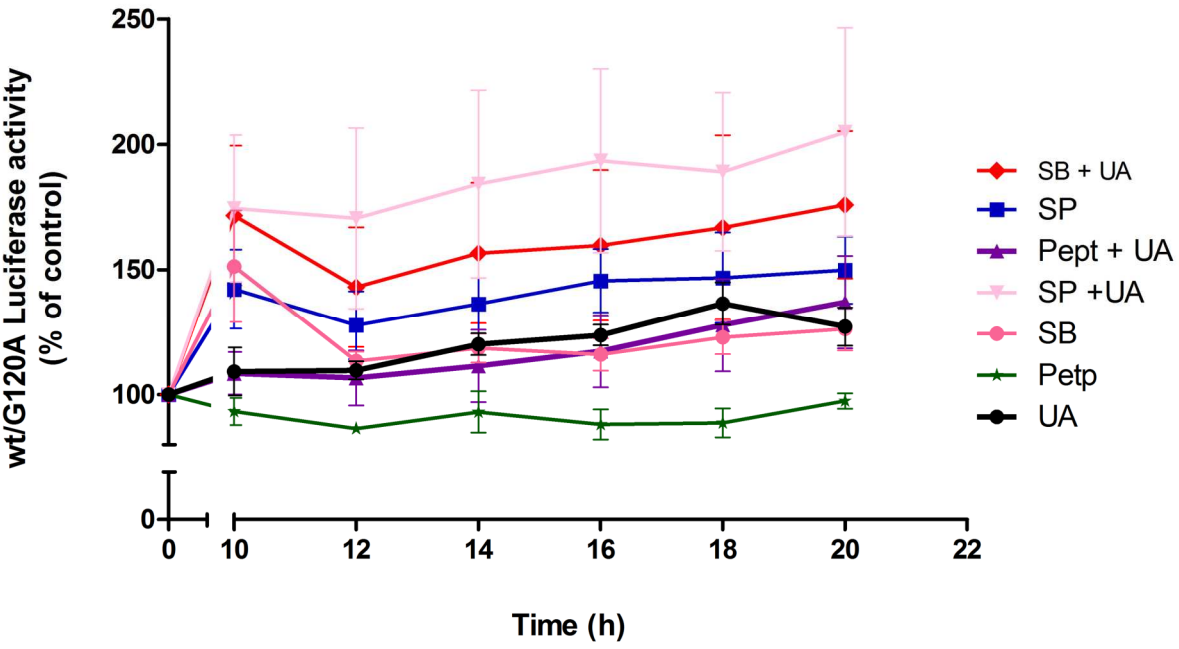

Figure 6 – Effect of ursolic acid (UA) 8µM, SP600125 (SP) 20µM, SB203580 (SB) 20µM and C-Jun N-terminal kinase peptide inhibitor 1 (Pept) 2µM on autophagic flux in MCF-7 cells stably expressing RLuc-LC3wt or RLuc-LC3G120A. Luciferase activity was measured with 2 hours intervals during 20 hours and the ratio of the two cell lines were expressed as percentages of the corresponding ratio in untreated cells at T0.

Discussion

The modulation of autophagy, as well as, effects on lysosome functions have been shown to trigger cancer cell death and suggested as therapeutic target to be addressed by new anticancer drugs.[5, 9] In the present study, we demonstrate that the natural triterpenoid ursolic acid (UA) is an inhibitor of autophagy through effects on lysosomes. This effect on autophagy induces death in colon and breast cancer cells without significant effects on nontumorogenic cells.

Our results show that UA induced accumulation of lipidated LC3-II to the autophagosomal membrane. These events were simultaneous with decreased cell survival and morphological changes in the cells, although without effect on apoptosis induction, suggesting the involvement of autophagy in cell death. Since an accumulation of autophagic structures is not always correlated with an induction of autophagy,[18] several approaches were used to identify and characterize the effect of UA on autophagic flux.

Effects on the autophagic process may occur at several levels: at the initiation step, in the control of autophagosome formation; at the maturation step, involving the

fusion between autophagosomes and lysosomes; or at the degradation process.[1, 5] Our data showed that UA inhibits autophagy at the maturation step since no fusion events between autophagosomes and lysosomes were detected, which results in an accumulation of autophagosomal structures, an observation consistent with an accumulation of LC3-II. The absence of colocalization between autophagosomes and lysosomes was evident at 24 hour UA incubations in both breast MCF-7 and colon HCT15 cancer cells. The UA-inhibitory effect on autophagy was corroborated by an increase in p62 levels from 24 hours in both cell lines. Furthermore, the luciferase-based real-time assay that measured the LC3 turnover by following the kinetics of the autophagic flux over time [21], showed that UA exerts a gradual inhibition of autophagy from 8 hours. Interestingly, UA also efficiently blocked the effect of rapamycin-induced autophagy. A cooperative effect between UA and KU in inhibiting autophagy was also observed, indicating that these two inhibitors may act through different mechanisms. These effects were further supported by the same assay at 12 hours.

In addition to the absence of fusion events, the induction of perinuclear clustering of lysosomes were indications that UA could affect lysosomal integrity. Thus, UA´s effects on lysosomal membrane permeabilization (LMP) were measured in MCF-7 cells. Our results show that, at 24 hours, UA induced the release of the lysosomal hydrolases cathepsins and *N*-acetyl-*β*-glucosaminidase to the cytosol, as well as, decreased their total activities. These data indicate a role of UA in lysosome destabilization and in disruption of the integrity of lysosomal membranes. The induction of LMP is, however, not a rapid and a strong effect since a decrease of cell viability and morphological changes in the cells were only detected from 24 hours, with a gradual effect over time. The release of lysosomal contents, especially cathepsins, to the cytosol is considered an important step for lysosomal death pathway activation,[7] suggesting that, besides its role on autophagy, UA can also be involved in the activation of this pathway without classical apoptosis induction.

Recently, changes in the intracellular lipid composition were reported to have pronounced effects on vesicular fusion efficiency, thus affecting autophagy.[23] The reduced levels of lipids, in particular cholesterol, in both autophagosomes and lysosomes, were associated with lower fusogenic capability and reduced rates of autophagy.[23] In fact, previous studies demonstrated that UA may have a role in lipid metabolism by stimulating lipolysis in primary-culture adipocytes,[24] as well as, increasing sphingomyelinase activity during initiation and progression of colon cancer

that lead to aberrant crypt foci inhibition.[25] Therefore, UA, a lipophylic molecule, may induce LMP due to effects on lysosomal membrane composition, causing inhibition of the maturation step in the autophagic process.

Several cell signalling pathways modulate autophagy at the initiation and maturation of autophagosomes.[1] Recently also the stress kinases, JNK and p38, were found to play a role in the control of autophagy. [1, 17, 26, 27] An activation of JNK in cancer cells showed to be involved in the induction of autophagic cell death through mediation of Beclin 1 expression[28] and/or activation of the protein Atg7.[27] In contrast, a p38 activation was associated with an inhibition of autophagy in colorectal cancer cells[29] and an inhibitory effect at the maturation step was found.[26] UA has recently been reported to inhibit JNK in colon[16] and breast[13] cancer cells. Our previous observations showed that UA induced activation of JNK in HCT15 cells with a possible involvement of this stress MAP Kinase in autophagy. Here, results demonstrated that neither JNK nor p38 pathways seem to be involved in the inhibition of autophagy induced by UA in MCF-7 cells.

Additionally, although UA demonstrated to modulate PI3K/Akt pathway through inhibition of phospho-Akt,[13, 14] the classical inhibitor of PI3K class I, wortmannin,[30] was not able to induce LC3 accumulation, suggesting that UA inhibits autophagy independently of effects on this pathway. Interestingly, oleanolic acid, a compound with a similar chemical structure of UA for which similar biological properties have been reported,[11] did not show effect on autophagy. UA and oleanolic acid differ in the position of one methyl group on their E ring, which could contribute to this difference.

In conclusion, the data presented here shows the ability of ursolic acid to block the autophagic process at the maturation step due to effects on lysosomal membrane stability in breast and colon cancer cells. This effect on the autophagic maturation probably triggers death in cancer cells, which seems to be independent of JNK and p38 pathways in MCF-7 cells. Hence, ursolic acid shows potential as a lysosome-destabilizing drug or as an autophagic inhibitor that could be used in combination therapy in order to increase treatment efficacy.

Material and Methods

Chemicals

Ursolic acid (UA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rapamycin (RAPA), 3-Methyladenine (3-MA), concanamycin A (ConA), wortmannin (W), etoposide and oleonolic acid (OA) were purchased from Sigma-Aldrich. C-Jun N-terminal kinase peptide inhibitor 1 (Pept) was purchased from Enzo Life Science. All the compounds were resuspended in dimethyl sulfoxide (DMSO).

Cell culture

HCT15 human colorectal carcinoma and MCF-7 human breast carcinoma cell lines were grown in RPMI-1640 (Gibco, 61870) supplemented with 6% fetal calf serum, penicillin and streptomycin and maintained at 37ºC in a humidified 5% CO_2 atmosphere. When plating for luciferase reported assays, RPMI-1640 without phenol red (Gibco, 11835) was used. The MCF-7-eGFP-LC3 cell line is a single cell clone of MCF-7 cells expressing a fusion protein consisting of enhanced green fluorescence protein (eGFP) and rat microtubule-associated protein light chain 3 (LC3). MCF-7-mRFP-GFP-LC3 are MCF-7 cells stably expressing the tandem fluorescent construct consisting in two fluorescent proteins, GFP and monomeric ref fluorescent protein (mRFP), tagged with LC3 (construct kindly provided by Dr T. Yoshimori[19]). MCF-7 cells stably expressing Renilla-luciferase (RLuc) tagged with LC3 wild type or with a C-terminal glycine mutant of LC3 that is defective in ubiquitin-like conjugation with phosphatidylethanolamine (GFP-LC3G120A[31]) were used to monitor autophagy capacity.[21]

Cell viability, Apoptosis and Morphological analysis

MTT reduction assay was used as described previously.[14] Briefly, MCF-7 and HCT15 cells were treated with UA 8μM for 22, 46 and 70 hours and then two more hours in the presence of MTT at final concentration 0.5 mg/ml. Hydrogen chloride 0.04M in isopropanol was then used to dissolve the formazan crystals. The viable cells in each well were estimated by the cell capacity to reduce MTT using a spectrophotometer.

To estimate the percentage of apoptotic cells, TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed. After incubation of HCT15 and MCF-7 cell lines with UA 8μM for 24, 48 and 72 hours, cells were collected (both floating and attached cells), fixed with 4% paraformaldehyde for 15 minutes at room temperature and attached into a polylysine treated slide using a Shandon Cytospin. Then, cells were washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany), following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei staining. The percentage of apoptotic cells was calculated from the ratio between TUNEL positive cells and total number of from a count higher than 500 cells per slide under a fluorescent microscope.

The morphology of the cells incubated with UA 8μM during 24 hours and 48 hours was observed and images were taken using Olympus fluorescence microscope.

LC3 puncta formation assay

MCF-7-eGFP-LC3 cells were incubated with different concentrations of UA (5μM, 8μM and 10μM), 15μM OA and 2.5μM W for 24 hours. Autophagosomes were detected in the cells fixed in 3,7% formaldehyde for 10 minutes at room temperature, by counting the percentage of cells with more than five eGFP-LC3 positive dots (a minimum of 100 cells/sample) applying Olympus fluorescence microscope.

Immunofluorescence

MCF-7-mRFP-GFP-LC3 cells on coverslides were treated with UA 8μM at different hours (12, 24 and 48 hours) and/or with rapamycin 100nM and/or 3-MA 10mM for 24 hours. Then cells were fixed using ice-cold methanol for 2 minutes or 3,7% formaldehyde for 10 minutes at room temperature. Cells were stained with followed primary antibodies at 4ºC, overnight: mouse anti-human LC3 (Nanotools), mouse anti-human LAMP-2 (clone H4B4 Developmental Studies Hybridoma Bank, University of Iowa) and p62 (Enzo Life Science). After washing, samples were incubated with the appropriated Alexa Fluor-488 and Alexa Fluor-647-coupled secondary antibodies (Invitrogen). Confocal images were taken using a Zeiss Axiovert 100 M Confocal Laser Scanning Microscope equipped with LSM 510 system (Carl zeiss MicroImaging).

Immunoblotting

MCF-7 and HCT15 cells after treatment with UA 8µM at different hours were extracted in SDS-lysis buffer. Extracts (20µg protein) were separated on SDS-PAGE and transferred to nitrocellulose membranes. Protein concentration was quantified using a BCA Protein assay kit (Bio-Rad Laboratories). The primary antibodies used were: p62 (Enzo Life Science) and LC3 (Nanotools), followed by appropriated peroxidase-conjugated secondary antibodies from DAKO (Glostrup, Denmark). Anti-β-actin (from Sigma-Aldrich) was used as loading control.

Reporter assays

For luciferase-based real-time assay[21] in living cells, MCF-7 cells stably expressing RLucLC3wt and RLuc-LC3G120A were plated in the uneven and even numbered columns of white 96 wells dishes (Nunc, 136101) respectively at $8x10^4$cells/ml. The following day, 60µl medium containing 50nM EnduRen™ (Promega) were added to the cells and incubated for 2 hours. The luminescence was measured (Enspire 2300 Multilabel reader, Perkin Elmer) after 2 hours corresponding to time zero (To) define as 100%. The readout was obtained by calculating the ratio in luminescence between RLucLC3wt and RLucLC3G120A, which gives the autophagic flux. Thereafter, the compounds dissolved in EnduRen™ containing medium were added in the volume of 30µl and luminescence measurements were performed in intervals of 2 hours during 20 hours.

For the reporter assay[21] at 12 hours, MCF-7 cells expressing RLuc-LC3wt and RLuc-LC3G120A were used and Renilla-luciferase detected using the reagents in the Dual-Luciferase® Reporter assay System (Promega), according to the manufacturer's instructions. Briefly, after 12 hours of incubation with compounds, cells were lysed in 40µl /well of 1x lysis buffer and subjected to a single freeze/thaw cycle. Renilla-Luciferase was further measured in white half area 96-well plates (Costar, 3694) by adding 80µl of lysate and Stop&Glo® Reagent to 6µl of sample.

Cathepsin and NAG activities

The cysteine cathepsin (zFR-AFC, Enzyme System Products) and *N*-acetyl-*β*-glucosaminidase (NAG) activities of MCF-7 cells treated with UA 8µM, ConA 2nM and TNF 10ng/ml were measured after 24 hours.[32] Briefly, the cytosolic fraction was extracted with 20µg/ml digitonin and the total cellular fraction with 200µg/ml digitonin.

The rate of the appropriate substrate hydrolysis Vmax was measured over 20 minutes at 30 ºC on a SpectraMax Gemini fluorometer (Molecular Devices, Sunnyvale). Lactate dehydrogenase (LDH) activity of the cytosol, determined by a cytotoxicity detection kit (Roche), was used as an internal standard.

Statistical analysis

All values represent the mean ration ± SEM of at least three independent experiments. Statistical analyses were done using a two-tailed unpaired *t*-test in GraphPad Prism 4.0 software. *P*-values ≤ 0.05 were considered statistically significant. Images are representative of at least three independent experiments.

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CHAPTER VII

FINAL CONSIDERATIONS

1. General Discussion and Conclusions

In recent years, natural products have received great attention for cancer research owing to their various health benefits, associated with low of toxicity and side effects. Colorectal cancer (CRC), a common cause of cancer-related death, has been increasing over time and the main reason for its high incidence has been associated to diet. The work developed in the scope of this thesis contributed to support the importance of dietary constituents in certain genetic profiles of CRC and to increase the value of potential dietary natural compounds as anticancer agents.

In the first part of this work (chapter II and III), the possible anticarcinogenic effects of *Salvia* sp. and *Hypericum* sp. water extracts, prepared as a tea, which is one of the most common forms of human consumption of these plants, and the main phenolic compounds rosmarinic acid and chlorogenic acid present in these extracts, were evaluated in CRC cell lines. These medicinal plants are popular for different reasons and they have been experimentally investigated and their benefits confirmed. Nevertheless, their anticarcinogenic effects in CRC have never been explored. The second part of the work (chapter IV, V and VI) focused on two phenolic compounds (quercetin and luteolin), which are active compounds present in the extracts tested above, as well as, the triterpenoid ursolic acid that is present in sage extracts. Although the anticancer effects, especially of the phenolic compounds, have been widely studied, their effects on particularly CRC mutation patterns and their possible combination with 5-fluorouracil (5-FU) is not known. The chemotherapeutic agent 5-FU is currently the most widely used drug, however some resistances have been arising, and thus combinatory drugs have been developed to improve CRC treatment. Therapies targeting pathways involved in CRC carcinogenesis have increased benefits when in combination with this traditional chemotherapeutic agent, overcoming current limitations of the agent.

Due to the high frequency of mutations in components of the PI3K/Akt and MAP Kinases pathways present in CRC humans the focus of this study was on these relevant molecular targets. Therefore, the experiments were performed using different CRC cell lines: HCT15 harbors a KRAS mutation and CO115 harbors a BRAF mutation and overexpresses Akt. Moreover, the effects of compounds on molecular targets of apoptotic and non-apoptotic pathways of cell death were studied. The antiproliferative and proapoptotic effects were evaluated using different *in vitro* tests,

such as MTT, BrdU and TUNEL assays. To assess the possible effects on different molecular targets western blot were performed.

Firstly, the potential effect of water extracts of S. *fruticosa* (SF) and S. *officinalis* (SO) was evaluated (**chapter II**). These sages showed to significantly inhibit cell proliferation and induce apoptosis (50µg/ml). Different effects were found for the two CRC cell lines. The results revealed that those effects may be associated, at least in part, with the inhibition of MAPK/ERK pathway through effects upstream of BRAF. The presence of the BRAF mutation showed to act as a barrier for the antiproliferative effect of these sages. The main phenolic compound present in the sages, rosmarinic acid, did, however, not show the same effects, even at higher concentrations (100µM), presenting only proapoptotic effects. Thus, other active compounds present in the extracts at lower concentrations, or the mixture between them, may be responsible for the antiproliferative effects of SF and SO.

Subsequently, the anticarcinogenic activities of three *Hypericum* water extracts sp., *Hypericum androsaemum* (HA), *Hypericum perforatum* (HP) and *Hypericum undulatum* (HU), were investigated in the same CRC cell lines **(chapter III**). The study demonstrated that HA efficiently inhibits cell proliferation and induces apoptosis in both genetic backgrounds. These effects seem to be related to suppression of the mutant BRAF and PI3K/Akt pathway (at 65µg/ml). Interestingly, this extract only interferes with mutant BRAF without affecting wild type BRAF, suggesting a more specific effect of this extract on CRC. Moreover, HA also increased p38 and JNK activation, which may explain the extracts' proapoptotic effects. In order to find the active compound of HA plant, the main phenolic compound present in HA, chlorogenic acid, was tested. This compound did, however, not show any of the observed effects, even at very higher concentrations. On the other hand, the study demonstrated that HU and HP species are different from HA, since no effects on CRC cells viability, at reasonable concentrations, were detected. The results demonstrated new beneficial effects of *Hypericum androsaemum* and, more significantly, it showed to be a source of compounds that specifically act in BRAF mutation, frequently found in CRC tumors.

All these observations showed the ability of these medicinal plants in decreasing CRC progression by affecting the expression of important molecular targets of proliferation and apoptosis. In addition, they suggest them as sources of potential anticarcinogenic compounds. Sage extracts are very rich in several derivates of luteolin, whereas *Hypericum* extract possess quercetin and a variety of glycosides in its

composition. Since these conjugated glycosides have been shown to be converted into their active aglycones quercetin (Q) and luteolin (L) in the intestine, we decided to study the aglycones as the most important players in the cell. In addition, we also studied a triterpenoid, ursolic acid (UA), which is also present in sage water extracts.

In **chapter IV**, the antiproliferative and proapoptotic effects of Q, L and UA were evaluated and the possible involvement of PI3K/Akt and MAPK signalling pathways on their mechanism of action were analysed. All the compounds showed significant inhibition of cell proliferation and induction of apoptosis in CRC cell lines. The effects of Q (around 20µM) and L (around 15µM) seem to be due, at least in part, to effects on KRAS through regulation of both MAPK/ERK and PI3K pathways. However, the presence of a BRAF mutation prevents these compounds from having effect on the MAPK/ERK pathway. On the other hand, UA (10µM) seems to be efficient in suppressing PI3K, at concentration below the other compounds. In addition, these natural compounds demonstrated to be even more efficient in inhibiting the pathways than the reference inhibitors commonly used to study the involvement of PI3K/Akt and MAPK/ERK pathways.

Taken together, sage extracts inhibit MAPK/ERK pathway through suppression of KRAS probably due to derivates of luteolin, since this compound alone showed the same effects. However, L additionally demonstrated to decrease PI3K/Akt pathway, thereby affecting other molecular targets. An effect on PI3K was also observed for UA. Regarding HA, the inhibition of PI3K/Akt pathway could probably be due to the presence of Q, one constituent of this extract that showed the same effect. Surprisingly, HA also suppresses mutant BRAF, effect that was not found for Q, which instead acts on KRAS, or for the main phenolic compound chlorogenic acid. To look for the active compound responsible to the effect on mutant BRAF should be considered in future experiments. These recent findings highlight the impact of compounds that specifically act on mutant BRAF like HA water extract. It must also be taken in account that possible applications of the flavonoids Q and L as inhibitors of MAPK/ERK pathway seem to be restricted to tumors without BRAF mutation. Effects of extracts are not due to main phenolic compounds present and may result from other classes of compounds and/or for synergism between constituents.

In **chapter V**, the effects of Q, L and UA in combination with 5-FU were tested, in order to find agents that could overcome 5-FU-resistance and improve treatment efficacy. One mechanism of action of this chemotherapeutic 5-FU is apoptosis

induction, which was the focus of this work. The results showed that Q and L (both at 12µM) efficiently enhance apoptosis induced by 5-FU and a synergistic effect was observed for Q in the p53 wild-type cell line. Q seems to increase the effect of 5-FU through induction of the mitochondrial pathway because an increased on cleaved caspase 3, caspase 9 and PARP and a decrease in Bcl-2 expression were detected when Q in co-incubated with 5-FU in the CO115 p53 wild type cell line. This suggest the possible applicability of this flavonoid in combination with 5-FU to improve cell death dependent on p53. UA (4µM) was also tested in combination with 5-FU and a synergistic enhancement of apoptosis was observed in the HCT15 mutant p53 cells. In this case, UA did not induce caspase activity, but was able to induce JNK activation, where the inhibitor of the JNK, SP600125, almost completely abrogated UA-induced apoptosis. These results suggest that UA induces apoptosis through JNK pathway when p53 is not functional, being the possible reason for the synergistic effect with 5-FU. Moreover, UA-induced cell death (other than by apoptosis) also seems to be partially dependent on JNK. Interestingly, this triterpenoid demonstrated to be more efficient in inducing cell death than 5-FU alone, suggesting UA as a potential chemotherapeutic candidate against CRC. Since apoptosis induced by UA did not explain all the cell death observed, effects on autophagy, another type of cell death, were elucidated. UA induced accumulation of LC3, an effect inhibited by SP, indicating an involvement of this compound in autophagy and a role of JNK in this process, in HCT15 cells.

Finally (**chapter VI**), a characterization of the role of UA in autophagy was made. Agents that have a role in the autophagic process have been appearing and seem promising as anticancer agents. An accumulation of LC3, as demonstrated in the previous study, may be an indication of both induction or inhibition of the autophagic process. The expression of p62, as well as, immunohistochemistry stained with autofagosomes and lysosomes markers were further analyzed. This study found that UA inhibits autophagy in HCT15 cells over time. Moreover, this inhibition appears to be at the maturation step since no fusion event between lysosomes and autophagosomes was observed. Taking advantage of some currently new techniques, MCF-7 breast cancer cell line was used to confirm the inhibitory effect of UA on autophagy process at the maturation step. Interestingly, UA also showed to revert the effect of rapamycin, a classical inducer of autophagy, and to cooperate with another inhibitor of autophagy, increasing its effect. Moreover, neither JNK nor p38 pathways seem to be involved in the inhibition of autophagy induced by UA in MCF-7 cells, contrarily to what was

previously observed for HCT15 cells. The role of JNK in autophagy seems be dual depending on the cell type. In order to understand more deeply how UA inhibits the maturations step, effects on lysosomal integrity was tested. The results showed that UA increased the release of the lysosomal hydrolases to the cytosol and decreased their total activities, suggesting an induction of lysosomal membrane permeabilization, as well as, a possible impact on lysosomal biogenesis and/or a direct destabilizing effect on lysosomal membranes. The effect of UA on the destabilization of lysosomes structure helps to explain the lack of fusion between them and autophagosomes. Taken together, this work found a new effect for UA in the inhibition of autophagy, which is probably due to effects on lysosome integrity.

In general, this work uses a new methodological approach by using CRC cell lines that harbor different mutations and characterizes mechanisms of action of extracts and individual compounds of medicinal plants of the genus *Salvia* and *Hypericum*, on particular molecular targets comparativelly. The sage water extracts demonstrated capacity to inhibit MAPK/ERK upstream of BRAF, while *Hypericum androsaemum* acted specifically on mutated BRAF, effects that explain, at least in part, the antiproliferative and proapoptotic effects of these plants. In addition, these effects were not due to the most representative antioxidant compounds present in the extracts (rosmarinic acid and chlorogenic acid, respectively), suggesting the effects are independent of reactive oxygen species (ROS)-mediated signaling. Concerning the isolated compounds, Q, L and UA, it was shown, for the first time, in this work the impact of their effects on molecular targets on different genetic patters in CRC and their possible use in combination with the chemotherapeutic drug 5-FU. Q and L demonstrated to act on KRAS, affecting both MAPK/ERK and PI3K/Akt pathways, although the effect on MAPK/ERK inhibition is no longer observed in the presence of BRAF mutation. The combination of Q with 5-FU showed a synergistic induction of apoptosis probably through via mitochondrial pathway in the p53 wild-type background of CO115. Finally, UA demonstrated to inhibit PI3K pathway and to synergistically enhance apoptosis induced by 5-FU via JNK. For the first time, UA was found to inhibit autophagy at the maturation step of autophagosomes lysosomes fusion, possibly by affecting the lysosomal membrane, contributing to cell death.

In conclusion, the plant extracts and the isolated natural compounds used in this thesis demonstrated to be promising agents against CRC in different molecular targets by decreasing proliferation and in sensitizing cancer cells to death apoptosis. This study

contributes to add *Salvia fruticosa, Salvia officinalis,* and *Hypericum androsaemum* to the list of plants with potential anticarcinogenic effects. Moreover, the isolated compounds quercetin and luteolin demonstrated to be effective on a variety of molecular targets and to be promising agents in combination with 5-FU. During this work, UA was the most promissory compound since it was able to target the lysosomes at lower concentrations inhibiting the autophagic process and thus leading CRC and breast cancer cells to death. More research is needed, however, to safely add these dietary natural compounds as therapeutic tool against cancer and especially we should be aware that *in vivo* studies are needed to confirm our data.

2. Future Perspectives

The studies enclosed in this thesis were carried out to find potential anticarcinogenic effects of *Salvia* and *Hypericum* species and, if possible, to identify active principles, characterizing the mechanism of action. In spite of the advances obtained in this work, there are still many questions unanswered.

Presently, we found that *Salvia fruticosa, Salvia officinalis* and *Hypericum androsaemum* possess anticarcinogenic in CRC. In an attempt to find which compounds are responsible the effects observed, we tested the main phenolic compounds present in the water extracts. However, neither of the compounds, rosmarinic acid and chlorogenic acid, respectively, showed the ability to suppress KRAS or BRAF, the active principle of these extracts being still unknown. Since the *Hypericum* water extract was only effective in inhibiting mutant BRAF without affecting the wild-type BRAF, to find in the plant the possible responsible for this effect constitute a good challenge. As future work, a screening of compounds present in the *Hypericum* water extract should be performed. Although phenolic compounds have been demonstrated to have a wide range of effects against several cancer cell types, other classes of compounds present in these extracts must be taken into account.

The isolated compounds quercetin and luteolin showed to inhibit MAPK/ERK and PI3K/Akt pathways through inhibition of KRAS. However, more studies should be performed in order to clarify how these compounds interact and decrease the expression of this protein. Both flavonoids also demonstrated to enhance apoptosis induced by 5-FU and, moreover, a synergistic effect was observed with quercetin in the p53 wild type

cells. Nevertheless, to confirm and validate these results, studies *in vivo* should be carried out.

The triterpenoid ursolic acid induced cell death at small concentrations and had effect on lysosome integrity, which leads to a blockage of the maturation step during the autophagic process. According to some recent studies, UA affects the metabolism of lipids, which could explain the observed effects on lysosomal membrane. It would be interesting to explore more in detail the interference of UA with lysosomes and cell membranes in general.

Finally, since an ideal anticancer agent should be toxic to malignant cells with minimum toxicity towards normal cells, it is important to evaluate the effects of these plant water extracts and isolated compounds in cells considered “normal”. In fact, UA was tested in HBL-100 nontumorogenic breast-derived epithelial cells and no significant effect on its survival, at the concentration used, was observed. The same should be investigated for quercetin and luteolin. Moreover, to further reveal the impact of the applicability of these natural compounds in CRC therapy, effects in animal models are essential.