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EVALUATION OF THE INTERACTION BETWEEN MELATONIN AND RAPAMYCIN FOR HEAD AND NECK CANCER THERAPY

Shen, Ying-Qiang

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Memoria que presenta el licenciado en Bioingeniería

D. Ying-Qiang Shen

Como aspirante al Grado de Doctor

Fdo: Ying-Qiang Shen

VºBº del Director de la Tesis Doctoral

Fdo: Dra. Germaine Escames
Doctora en Farmacia
Catedrática de Fisiología de la UGR

Universidad de Granada

CERTIFICACIONES

Dña. GERMAINE ESCAMES ROSA, Catedrática de la Universidad de Granada,

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Vº Bº Director

El interesado

Germaine Escames Rosa

Shen, Ying-Qiang

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To my family

献给我的父母和家人

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How can you look round at these august hills, look up at this divine sky, taste this finely tempered air, and then talk like a literary hack on a second floor in Bloomsbury?

George Bernard Shaw

He had destroyed his talent by not using it, by betrayals of himself and what he believed in, by drinking so much that he blunted the edge of his perceptions, by laziness, by sloth, and by snobbery, by pride and by prejudice, by hook and by crook. What was this? A catalogue of old books?

Ernest Miller Hemingway

ABBREVIATION

AADC: Aromatic amino acid decarboxylase

AANAT: Arylalkylamine N-acetyltransferase

ADP: Adenosine diphosphate

AEs: Adverse events

AFMK: N-acetyl-N-formyl-5-methoxykynuramine

AGC: Protein kinase A, G, and C

Akt: Protein kinase B

AMK: N-acetyl-5-ethoxykynuramine

AMPK: Adenosine monophosphate (AMP)-activated protein kinase

aMT: Melatonin

ASMT: N-acetylserotonin methyltransferase

ATCC: American Type Culture Collection

Atg: Autophagy-related protein

ATM: Ataxia-telangiectasia mutated

ATP: Adenosine triphosphate

BSP: Bone sialoprotein

C/EBP: CCAAT /enhancer-binding protein

c3OHM: Cyclic-3-hydroxy melatonin

cAMP: Cyclic AMP

CIP1: Cyclin-dependent kinase (CDK)-interacting protein 1

CRF1: Corepressor with FHL1 protein1

DCF: 2', 7' –dichlorofluorescein

DCFH-DA: 2′, 7′-dichlorofluorescin diacetate

DEP domain: Dishevelled, Egl-10 and Pleckstrin domain

DEPTOR: DEP domain-containing mTOR-interacting protein

DMEM: Dulbecco's modified Eagle medium

E-BP1: E-binding protein 1

ECAR: Extracellular acidification rate

EGFR: Epidermal growth factor receptor

eIF3: Eukaryotic initiation factor 3

eIF4F: Eukaryotic initiation factor 4F

eIF4G: Eukaryotic initiation factor 4 G

EMT: Epithelial–mesenchymal transition

ERK: Extracellular signal–regulated kinases

ETC: Electron transport chain

FAT: FRAP, ATM, TRRAP

FATC: C-terminal FAT

FBS: Fetal bovine serum

FCCP: Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone

FDA: Food and Drug Administration

FHL1: Forkhead-like 1

FIP200: Family interacting protein of 200 kD

FKBP12: FK506-binding protein 12

FOXO: Forkhead box O

FRAP: FKBP12-rapamycin associated protein

FRB: FK506-binding protein 12–rapamycin binding

GAP: GTPase-activating protein

GDP: Guanosine diphosphate

GLUT: Glucose transporters

GPx: Glutathione peroxidase

GRd: Glutathione reductase

GSH: Glutathione

GSK3: Glycogen synthase kinase 3

GSSG: Glutathione disulfide

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

HE: Hematoxylin-Eosin

Her: Human epidermal growth factor receptor

HIF: Hypoxia-inducible factor

HIOMT: Hydroxyindole-O-methyl transferase

HNC: Neck and neck cancer

HNE: hydroxy-2-nonenal

HNSCC: Head and neck squamous cell carcinomas

HODE: hydroxyoctadecadienoic acid

HPV: Human papillomavirus

HT: Hydroxy-L-Tryptamine

HTP: hydroxy-tryptophan

IFH1: Interacts with Forkhead 1

IFN: Interferon

IGFs: Insulin-like growth factors

IKKβ: IκB kinaseβ

IL: Interleukin

iNOS: Inducible nitric oxide synthase

i-mtNOS: Mitochondrial iNOS

IRS: Insulin receptor substrate

IκB: Inhibitor of kappa B

JNK: Jun N-terminal kinases

LA: Linoleic acid

LKB1: Liver kinase B1

LPO: Lipid peroxidation

LO: lipoxygenase

MAP4K3: Mitogen-activated protein 4 kinase 3

MAPK: Mitogen-activated protein kinase

MAPKAP1: Mitogen-activated protein kinase-associated protein 1

MDA: Malondialdehyde

mLST8: mammalian lethal with SEC13 protein 8

MPTP: Mitochondrial permeability transition pore

MT: methoxytryptamine

MT1: Melatonin receptor type 1

MT2: Melatonin receptor type 2

MT3: Melatonin receptor type 3

mTOR: Mechanistic Target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

NADPH: Nicotinamide adenine dinucleotide phosphate

NAO: 10-n-Nonyl acridine orange

NEM: N-ethylmaleimide

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK: Natural killer

NLRP3: NOD-like receptor (NLR) family pyrin domain containing 3

OCR: Oxygen consumption rate

OPT: o-Phthalaldehyde

OXPHOS: Oxidative phosphorylation

PAS: Periodic acid-schiff

PDK1: Phosphoinositide-dependent kinase 1

PDPK1: Phosphoinositide-dependent kinase-1

PEPT1/2: Peptide transporters 1 and 2

PGC1α: PPARγ coactivator 1α

PI3K: Phosphoinositide 3-kinase

PIC: Preinitiation complex

PIKKs: Phosphoinositide 3-kinase-related kinases

PIP2: Phosphatidylinositol 4, 5 bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

Pol I: Polymerase I

Pol II: Polymerase II

PP2A: Protein phosphatase 2A

PPAR-γ: Peroxisome proliferator–activated receptor-γ

PRAS40: Proline-rich Akt substrate of 40 kDa

PRR5: Proline-rich protein 5, also known as PROTOR1

PS: Picrosirius

QR2: Quinone reductase II

Rap: Rapamycin

RAPTOR: Regulatory-associated protein of mTOR

Ras: Rat sarcoma

RCC: Renal cell carcinoma

Rheb: Ras homologue enriched in brain

RICTOR: Rapamycin-insensitive companion of mTOR

ROR: Retinoid orphan receptors

ROS: Reactive oxygen species

RP: Ribosomal protein

Rsk: Ribosomal s6 kinase

RTK: Receptor tyrosine kinases

RZR: Retinoid Z receptors

S6K: S6 kinases

SCN: Suprachiasmatic nucleus

SOD: Superoxideb dismutase

SQSTM1: Sequestosome 1

SREBP1: Sterol regulatory element binding protein 1

TCA: Trichloroacetic acid

TIF-IA: Transcription initiation factor-IA

TK: Tyrosine kinase

TNF α: Transforming growth factor α

TOP: Tract of oligopyrimidine

TPOH: Tryptophan-5-hydroxylase

TRRAP: Transcription domain-associated protein

TSC: Tuberous sclerosis complex

UADT: Upper aerodigestive tract

UlK1: Unc-51-Like Kinase 1

UPS: Ubiquitin–proteasome system

VEGF: Vascular endothelial growth factor

VPS34: Vacuolar protein sorting 34

YY1: Yin-yang 1

El doctorando / The *doctoral candidate* **[Yingqiang Shen]** y los directores de la tesis / and the thesis supervisor/s: **[Germaine Escames Rosa]**

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Granada, 22 diciembre 2017

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SUMMARY

Head and neck squamous cell carcinoma (HNSCC) is the most common malignant neoplasm arising in the upper aerodigestive tract (UADT), and has the sixth highest incidence among cancers worldwide. Despite therapeutic and diagnostic advances, survival rates are still only about 50%. This is because HNSCC often involves local recurrences, distant metastases, and second primary tumours. Chemoresistance development also constitutes a major obstacle in HNSCC management. There is clearly a need for alternative treatment strategies.

Cancer arises because of accumulation of genetic and/or epigenetic changes in cancer-associated genes. Among them, alterations in the PI3K /AKT/mTOR pathway are consistently observed in various cancers. Several studies suggest that mTOR signaling is important in HNSCC progression, metastasis, and therapy resistance. Unexpected Akt activation leads to activation of mTORC1, which contributes to cell growth, proliferation, and metabolism, and promotes tumorigenesis. Rapamycin, first isolated as a secondary metabolite production from Streptomyces hygroscopicus, is an allosteric inhibitor of mTORC1. Innumerable scientific and clinical studies have proved its anticancer activity, but only in a limited subset of cancer types. Rapamycin analogues, such as temsirolimus and everolimus, are under investigation for HNSCC treatment, but these drugs show poor tolerability and treatment leads to therapy resistance. Rapamycin-induced loss of the feedback inhibition of Akt may actually increase cancer cell survival, potentially resulting in more severe tumours. One means of overcoming this problem may be a combination therapeutic strategy in which the feedback inhibition of Akt is neutralized using other anticancer agents.

Melatonin (N-acetyl-5-methoxytryptamine) is a potent free radical scavenger with anti-oxidant and anti-inflammatory properties, which has well-known functions in immune system regulation, modulation of mitochondrial activity, and regulation of cell death and autophagy. Moreover, melatonin has attracted attention as a natural oncostatic agent that can suppress neoplastic growth in a variety of tumours. Possible mechanisms of melatonin oncostasis induce direct and indirect antioxidant effects, regulation of metabolism, anti-angiogenic and antimetastatic effects, the capacity to decrease telomerase activity and the ability to regulate the immune system. Notably, melatonin also induces downregulation of the phosphorylation of mTOR and Akt in various cancers. Melatonin shows synergistic anticancer activity and amelioration of the side effects of several chemotherapies and thus is a potential adjuvant in chemotherapy, with the ability to protect normal cells from a variety of insults. In light of these properties, melatonin seems potentially useful as a combined anti-cancer agent to enhance the therapeutic effect of rapamycin and to minimize the toxic side effects of this drug in HNSCC. mTOR inhibitor treatment is associated with a number of adverse events in cancer patients, being mucositis the most frequent adverse effect leading to dose reduction or interruption of therapy. We recently demonstrated that a melatonin gel prevents mucosal disruption and ulcer formation associated with oral mucositis.

In the present study, we aimed to elucidate the effects of melatonin on rapamycin-induced HNSCC cell death, and to identify potential cross-talk pathways. We analyzed the dose-dependent effects of melatonin in rapamycin-treated HNSCC

cell lines (Cal-27 and SCC-9). These cells were treated with 0.1, 0.5, or 1 mM melatonin combined with 20 nM rapamycin. We further examined the potential synergistic effects of melatonin with rapamycin in Cal-27 xenograft mice. Relationships between inhibition of the mTOR pathway, reactive oxygen species (ROS), and apoptosis and mitophagy reportedly increased the cytotoxic effects of rapamycin in HNSCC.

Our results demonstrated that rapamycin inhibited the downstream target of mTOR signaling effectively but also increased Akt expression. The combined treatment with rapamycin and melatonin blocked the negative feedback loop from S6K1 to Akt signaling. The combined treatment decreased cell viability, proliferation, and clonogenic capacity. Interestingly, combined treatment with rapamycin and melatonin induced changes in mitochondrial function. Melatonin enhanced the cytotoxic effects of rapamycin by augmenting the number of dysfunctional mitochondria. Combined treatment with rapamycin and melatonin elevated ROS generation compared with rapamycin alone in a dose dependent manner. Moreover, ROS accumulation resulted in severe oxidative damage of mitochondria and, therefore, in mitophagy. This led to increased cell death and cellular differentiation. Our data further indicated that melatonin administration reduced rapamycin-associated toxicity to healthy cells.

Overall, our findings suggested that melatonin could be used as an adjuvant agent with rapamycin, improving effectiveness while minimizing its side effects.

RESUMEN

El carcinoma de células escamosas de cabeza y cuello (HNSCC) es la neoplasia maligna más común que se produce en el tracto aerodigestivo superior (UADT) y representa el sexto cáncer más común en todo el mundo. A pesar de los avances terapéuticos y de diagnóstico, la tasa de supervivencia sigue siendo tan solo de alrededor del 50%. Esto se debe a que el HNSCC a menudo incluye recurrencias locales, metástasis y segundos tumores primarios. El desarrollo de quimiorresistencia también constituye un obstáculo importante en el manejo de HNSCC. Por ello, es evidente la necesidad de tratamiento alternativos.

El cáncer surge debido a la acumulación de cambios genéticos y/o epigenéticos en los genes asociados a esta patología. Entre ellos, las alteraciones en la vía PI3K / AKT / mTOR se observan consistentemente en diversos cánceres. Varios estudios sugieren que la señalización de mTOR es importante en la progresión de HNSCC, de la metástasis y de la resistencia al tratamiento. La activación de Akt conduce a la activación de mTORC1, que contribuye al crecimiento celular, a la proliferación y a un cambio en el metabolismo, y promueve la tumorigénesis. La rapamicina, aislada por primera vez como una producción secundaria de metabolitos de *Streptomyces higroscópicas*, es un inhibidor alostérico de mTORC1. Innumerables estudios científicos y clínicos han demostrado su actividad oncostática, pero sólo en un subconjunto limitado de tipos de tumores. Los análogos de la rapamicina, como el temsirolimus y el everolimus, se encuentran en investigación para el tratamiento de HNSCC, pero estos fármacos muestran una tolerabilidad deficiente y el tratamiento conduce a generar resistencia. La pérdida de la inhibición de la retroalimentación de

Akt inducida por la rapamicina puede aumentar la supervivencia de las células cancerígenas, lo que puede resultar en tumores más graves. Una forma de superar este problema podría ser utilizar como estrategia una combinación terapéutica en la que la inhibición de la retroalimentación de Akt se neutralizase usando otros agentes anticancerosos.

La melatonina (N-acetil-5-metoxitriptamina) es un potente depurador de radicales libres con propiedades antioxidantes y antiinflamatorias, con funciones en la regulación del sistema inmunitario, en la modulación de la actividad mitocondrial y en la regulación de la muerte celular y autofagia. Además, la melatonina destaca como un agente oncostático natural que puede suprimir el crecimiento neoplásico en una gran variedad de tumores. Los posibles mecanismos oncostáticos de la melatonina incluyen efectos antioxidantes directos e indirectos, regulación del metabolismo, efectos antiangiogénicos y antimetastásicos, capacidad de disminuir la actividad de la telomerasa y capacidad de regular el sistema inmunológico. En particular, la melatonina también inhibe la fosforilación de mTOR y de Akt en diferentes tipos de tumores. La melatonina muestra actividad anticancerígena sinérgica con otros tratamientos de quimioterapia y mejora los efectos secundarios. Por tanto, es un posible coadyuvante en la quimioterapia, con la capacidad de proteger a las células normales de una gran variedad de daños. A la luz de estas propiedades, la melatonina parece ser potencialmente útil como agente antitumoral combinado con otros tratamientos para potenciar el efecto terapéutico de la rapamicina y minimizar los efectos secundarios tóxicos de esta droga en HNSCC. El tratamiento con un inhibidor

mTOR se asocia con una serie de efectos adversos en pacientes con cáncer, siendo la mucositis el efecto adverso más frecuente que conduce a la reducción de la dosis o la interrupción del tratamiento. Recientemente demostramos que un gel de melatonina previene la disrupción de la mucosa y la formación de úlceras asociadas con la mucositis oral.

En el presente estudio, el objetivo ha sido dilucidar los efectos de la melatonina en la muerte celular inducida por rapamicina en HNSCC, e identificar posibles vías de intercomunicación. Analizamos los efectos dosis dependientes de la melatonina en líneas celulares HNSCC tratadas con rapamicina (Cal-27 y SCC-9). Estas células se trataron con melatonina 0,1, 0,5 o 1 mM más rapamicina 20 nM. Además, examinamos los posibles efectos sinérgicos de la melatonina con la rapamicina en ratones con xenoinjerto de Cal-27. Las relaciones entre la inhibición de la vía mTOR, las especies reactivas de oxígeno (ROS) y la apoptosis y mitofagia aumentaron los efectos citotóxicos de la rapamicina en HNSCC.

Nuestros resultados demostraron que la rapamicina inhibió la diana de la señalización de mTOR de manera efectiva, pero también aumentó la expresión de Akt. El tratamiento combinado con rapamicina y melatonina bloqueó el circuito de retroalimentación negativa de S6K1 a la señalización de Akt. El tratamiento combinado disminuyó la viabilidad celular, la proliferación y la capacidad clonogénica. Curiosamente, el tratamiento combinado con rapamicina y melatonina indujo cambios en la función mitocondrial. La melatonina aumentó los efectos citotóxicos de la rapamicina al aumentar el número de mitocondrias disfuncionales. El

tratamiento combinado con rapamicina y melatonina elevó la generación de ROS en comparación con la rapamicina sola de una manera dosis dependiente. Además, la acumulación de ROS generó un daño oxidativo severo en las mitocondrias y, por tanto, en un aumento de la mitofagia. Esto condujo a un aumento de la diferenciación celular y, a su vez, de muerte celular. Nuestros datos indican además que la administración de melatonina reduce la toxicidad asociada a la rapamicina en células sanas.

En general, nuestros hallazgos sugieren que la melatonina podría usarse como un agente adyuvante con la rapamicina, mejorando la efectividad y minimizando sus efectos secundarios.

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I. INTRODUCTION

1. HEAD AND NECK CANCERS

Head and neck cancer is the most common malignant neoplasm arising in the upper aerodigestive tract (UADT), and has the sixth highest incidence among cancers worldwide (Ferlay et al., 2015). Head and neck cancer is a broad term that encompasses epithelial malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx. Almost all of these epithelial malignancies are squamous cell carcinoma of the head and neck (HNSCC) that develop in the upper aerodigestive epithelium after exposure to carcinogens such as tobacco and alcohol (Argiris and Eng, 2004). Actually, approximately three-quarters of head and neck cancers are attributable to tobacco or/and alcohol consumption. Infection with sexually transmitted human papillomavirus (HPV) also is a cause of a subgroup of squamous-cell carcinomas of the head and neck (Gillison et al., 2000). Surgery and radiotherapy have long been the major treatment approaches for head and neck cancer. Improved surgical and radiation treatment approaches and incorporation of systemic agents into curative therapy have improved clinical outcomes (Argiris et al., 2008).

1.1. RISK FACTOR OF HEAD NECK CANCER

The evidence is clearly sufficient and convincing to establish the fact that tobacco and alcohol consumptions are implicated in head and neck cancer formation. Alcohol- and aldehyde-metabolizing genes are associated with upper aerodigestive tract cancer (Hashibe et al., 2006). On the contrary, fruits and vegetables can reduce the risk of head and neck cancer (HNC) (Pavia et al., 2006).

Oral HPV infection is also strongly associated with HNC (D'Souza et al., 2007). Molecular evidence provides support for a role for HPV, particularly HPV-16, in the pathogenesis of a subgroup of HNSCC (Gillison et al., 2000). Genomic DNA of oncogenic HPV is detected in approximately 26% of all HNSCC worldwide (Kreimer et al., 2005). The association between HPV and HNC has potentially important implications for the cancer prevention. The vaccination against HPV may be a potential effective way in the prevention of HNC.

There are also few studies that have analyzed the role of anthropometric changes on laryngeal cancer risk (Garavello et al., 2006). They found that men with more abdominal fat had an increased risk of laryngeal cancer (Garavello et al., 2006). Additionally, some studies also focus on the genetic origin of HNC (Suárez et al., 2006). Furthermore, patients with cancer susceptibility syndromes, such as hereditary non-polyposis colorectal cancer, Li-Fraumeni syndrome, Fanconi's anaemia, and ataxia telangiectasia, have an increased risk of HNC (Argiris et al., 2008).

1.2. MOLECULAR SIGNALLING PATHWAYS RELATED WITH HNSCC

There is a considerable cross-talk between several signaling pathways. Epidermal growth factor receptor (EGFR) play a central role in cancer development and progression (Karamouzis et al., 2007). EGFR is a member of the epidermal growth factor receptor tyrosine kinase (TK) family. The binding of a ligand such as epidermal growth factor (EGF) or transforming growth factor α (TNF α) causes the EGFR to dimerize with itself or with another member of the ErbB family (Kalyankrishna and

Grandis, 2006). EGFR dimerization causes activation of the receptor-linked TK, recruitment of signaling complexes, and phosphorylation (activation) of multiple downstream cascades (Citri and Yarden, 2006) (Figure 1).

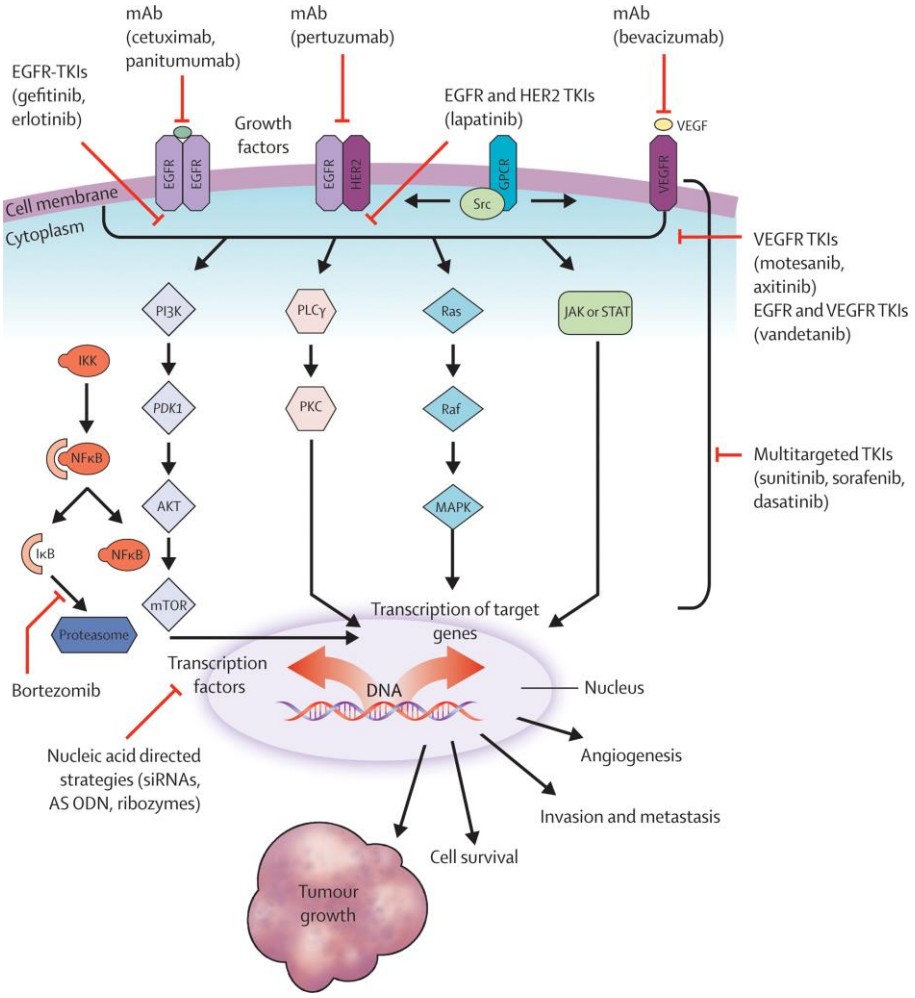

Figure 1. Molecular sinaling pathways related with HNC.

One of these cascades is the PI3K (phosphoinositide 3-kinase) /Akt (Protein kinase B, also known as PKB)/mTOR (Mechanistic Target of rapamycin) pathway, which could contribute to the malignant growth and metastatic potential of HNC. Activation of mTOR is an early and one of the most frequent events in HNC (Nathan et al., 2004).

1.3. TREATMENT FOR HNC

The treatment for HNC depends on the stage. Either radiation therapy or surgery will be succeeding for early stage. Stage III and stage IV cancers are best treated with surgery followed by radiation therapy. Unresectable lesions are treated with combined chemotherapy and radiation therapy (McMahon and Chen, 2003).

Surgery is the standard treatment for HNSCC but is frequently limited by the anatomical extent of tumour and the desire to achieve organ preservation (Argiris et al., 2008). Microsurgical treatment, which uses endoscopic laser or robotic techniques and high resolution magnified optics, has become technically feasible and could be cost saving compared with open surgical procedures or radiotherapy for early stage laryngeal cancer with acceptable voice outcomes (Argiris et al., 2008).

Radiotherapy is an integral part of primary or adjuvant treatment of HNC (Eom et al., 2010). Radiation therapy for treatment of HNSCC is typically given in daily fractions of 2·0 Gy, 5 days a week, up to a total dose of 70 Gy over 7 weeks.

Furthermore, some agents such as platinum compounds, antimetabolites, and taxanes have shown activity against HNC. The platinum compound cisplatin is regarded as a standard agent in combination with radiation or with other agents. EGFR inhibitor are now regard as a new kind of treatment for HNC, considered its important role in the tumorigenesis. Its combination also with other molecularly targeted agents (eg, angiogenesis inhibitors) has surfaced as a novel strategy, whereas the combination of these novel agents with chemotherapy and radiotherapy is also

under investigations (Argiris et al., 2008).

2. mTOR AND RAPAMYCIN IN CANCER

In the 1970s, rapamycin was first isolated as a secondary metabolite production from *Streptomyces hygroscopicus*. In 1965, this soil bacterium was first separated from a soil sample collected on Easter Island (Rapa Nui), which named the molecule rapamycin (Vezina et al., 1975). Few years later, the scientist disclosed the spectacular properties of rapamycin: immunosuppressive and antitumor activity. More thorough investigations brought the discovery of target of rapamycin, which entailed the mechanisms of action of rapamycin. The study of rapamycin as well as the TOR dependent processes has been revealing many new signaling molecules and a network increasingly viewed as important in cancer during the past decades years (Guertin and Sabatini, 2007)

2.1. THE mTOR STRUCTURE AND MODEL OF RAPAMYCIN ACTION

mTOR is one Ser/Thr protein kinase belonging to the phosphoinositide kinase-related kinase (PIKK) family and it is highly conversed from yeast to human. As a large (~ 290KDa) protein, mTOR contains multiple domains. As shown in Figure 2, the amino terminus of mTOR contains tandem HEAT repeats (anti-parallel α-helices found in huntingtin, **e**longation factor 3, protein phosphatase 2A (PP2A) and TOR) that are important for protein–protein interactions. The FAT and C-terminal FATC domains modulate the activity of the kinase domain. FAT stands for **F**RAP

(FKBP12-rapamycin associated protein), **A**TM (ataxia-telangiectasia mutated), **T**RRAP (transcription domain-associated protein) (which are all members of the same phosphoinositide 3 kinase-related kinase (PIKK) family as TOR). The FRB (FK506-binding protein 12 (FKBP12) – rapamycin binding) domain is the docking site of the FKBP12–rapamycin complex. Upon activation, mTOR autophosphorylates on Ser^{2481}, and upon activation by mTORC1, S6K1 phosphorylates mTOR Ser^{2448} via a feedback loop. mTOR Ser^{1261}, Ser^{2159} and Thr^{2164} phosphorylation promotes mTORC1 signaling (Benjamin et al., 2011; Magnuson et al., 2012).

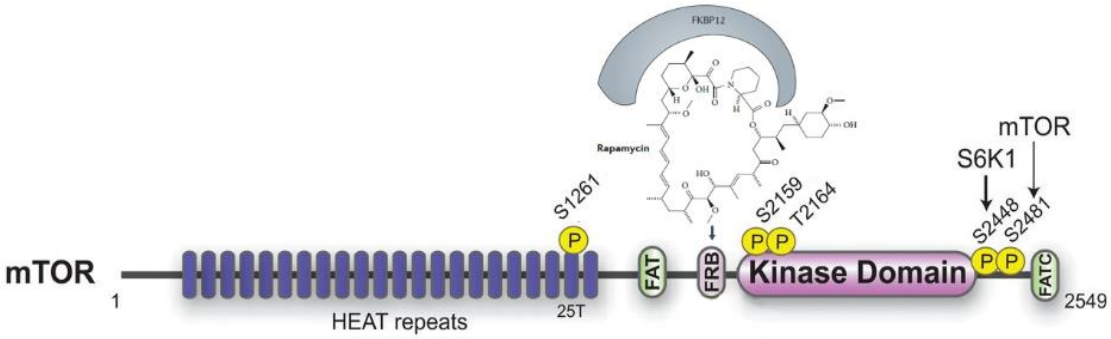

Figure 2. mTOR structure and rapamycin (Magnuson et al., 2012). mTOR contains tandem HEAT repeats, FAT domain, FATC domain and the kinase domain.

The rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2) are two structurally and functionally distinct complexes (Figure 3).

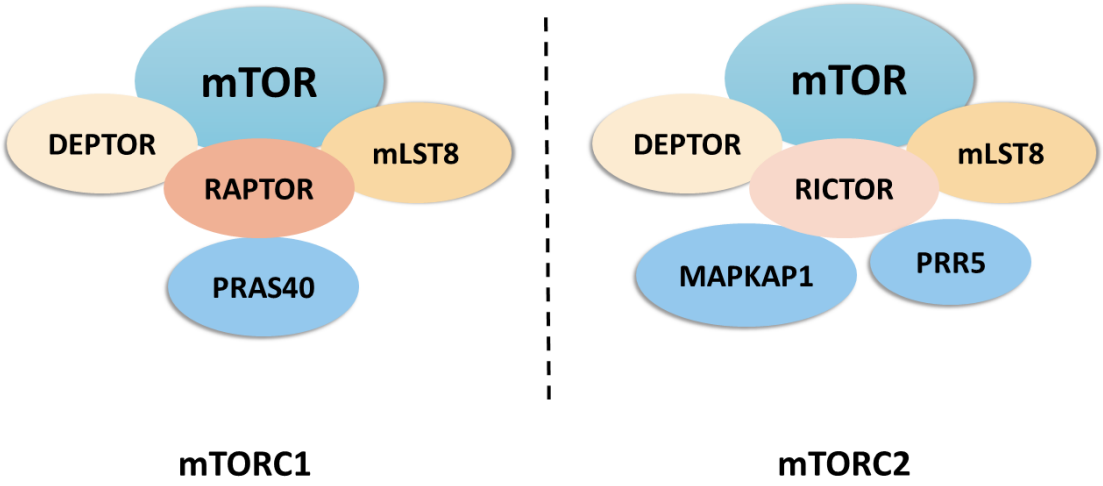

Figure 3. mTORC1 and mTORC2. Rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2) are two structurally and functionally distinct complexes

mTOR associates with mLST8 (mammalian lethal with SEC13 protein 8), DEPTOR (Dishevelled, Egl-10 and Pleckstrin (DEP) domain-containing mTOR-interacting protein), PRAS40 (proline-rich Akt substrate of 40 kDa, also known as AKT1S1) and RAPTOR (regulatory-associated protein of mTOR) to form mTORC1, while with mLST8, DEPTOR, PRR5 (proline-rich protein 5, also known as PROTOR1), MAPKAP1 (mitogen-activated protein kinase-associated protein 1, also known as mSIN1) and RICTOR (rapamycin-insensitive companion of mTOR) to generate mTORC2 (Benjamin et al., 2011).

2.2. mTOR SIGNALLING PATHWAY

2.2.1. TSC 1 and TSC 2 complex is a signal integration center

The TSC (tuberous sclerosis complex) 1/2 complex is a molecular signal integration center controlling mTOR signaling (Figure 4).

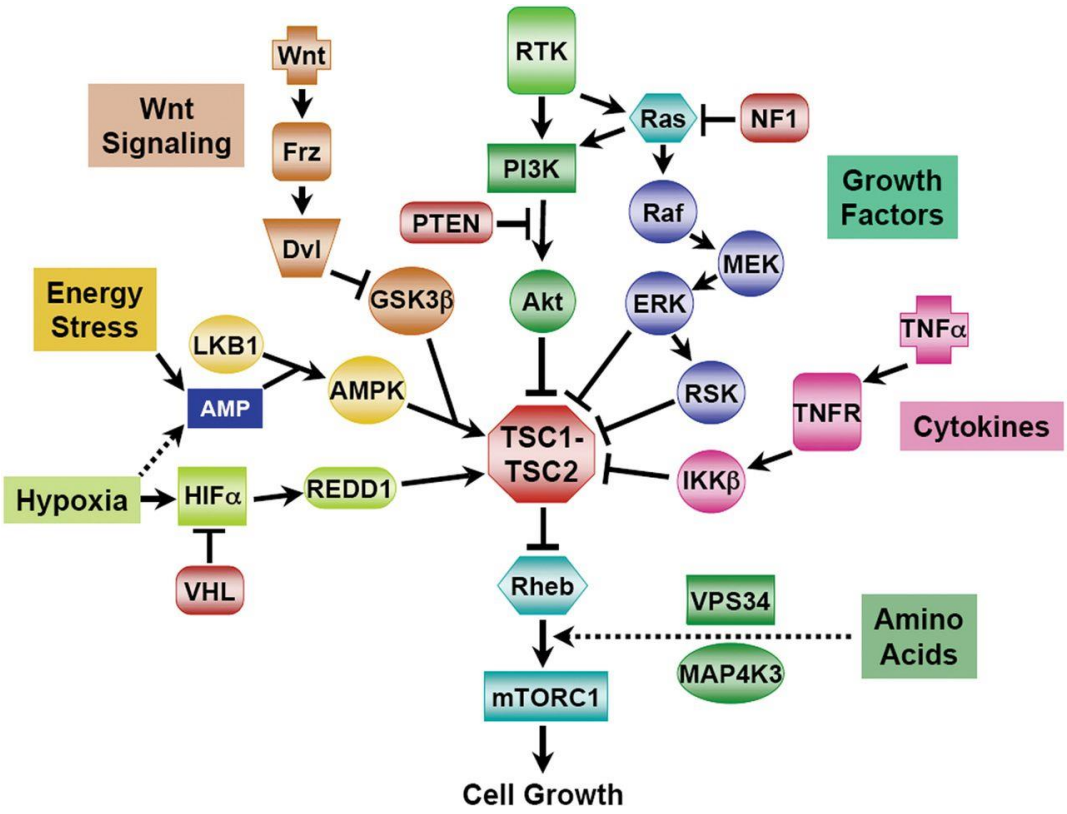

Figure 4. Signal integration by the TSC1/2 (Huang and Manning, 2008). TSC1/2 may sense plenty of distinct signaling pathways to either inhibit or activate the mTORC1

TSC1/2 may sense plenty of distinct signaling pathways to either inhibit or activate the mTORC1, suggesting that TSC1/2 is a signal integration center. The primary function of the TSC1/2 complex is as a critical negative regulator of

mTORC1 activation. Through inhibition of mTOR signaling, TSC1/2 complex inhibits cell growth. Mutation in TSC1 (hamartin) and/or TSC2 (tuberin) tumor suppressor genes could cause tuberous sclerosis complex, a multisystem disorder characterized by the development of numerous benign tumors (Young and Povey, 1998).

TSC2 is a guanosine triphosphatase (GTPase)-activating protein (GAP) toward the small GTPase, Rheb (rat sarcoma (ras) homologue enriched in brain) (Zhang et al., 2003). As a direct substrate of TSC2 GAP activity, this small GTPase has properties characteristic of Ras family and is expressed in a variety of tissues from yeast to mammals (Yamagata et al., 1994). As GTP-bound Rheb potently activates mTORC1, TSC1/2 can expedite the conversion of Rheb-GTP into Rheb-GDP, thereby inhibiting the mTORC1 signaling (Figure 5) (Huang and Manning, 2008).

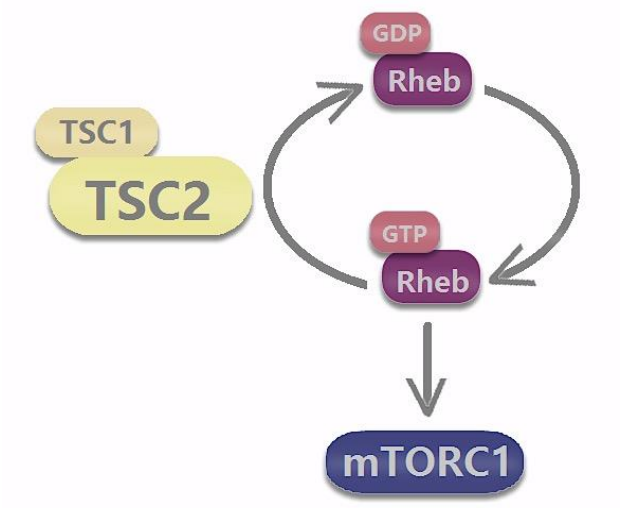

Figure 5. TSC1/2 negative regulates of mTORC1 by Rheb. TSC2 is a GTPase-activating protein (GAP) toward Rheb.

2.2.2. Upstream regulators of the TOR signaling

mTOR pathway responds to growth factors via the activation of the canonical insulin and Ras signaling pathways. The binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate (IRS), and subsequent recruitment of PI3K. PI3K bound to IRS converts phosphatidylinositol (4, 5)-bisphosphate (PIP2) in the cell membrane to Phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). PIP3 co-recruits PDK1 (phosphoinositide-dependent kinase-1) and Akt to the membrane, resulting in the phosphorylation and activation of Akt by PDK1. Increased phosphorylation of TSC2 by Akt leads to the inactivation of TSC1/2 and thus to the activation of mTORC1.

Protein synthesis requires sufficiently high levels of cellular energy. AMPK (Adenosine monophosphate (AMP)-activated protein kinase) is activated in response to energy depletion (low ATP (adenosine triphosphate): ADP (adenosine diphosphate) ratio) (Hardie, 2008). Activated AMPK direct phosphorylate TSC2 and results in the inhibition of mTORC1 signaling. As an upstream kinase for AMPK, the tumor suppressor LKB1 (liver kinase B1) is also linked to the TSC-mTORC1 signaling pathway (Figure 4) (Corradetti et al., 2004).

Amino acids have been proposed to represent a strong signal that positively regulates mTORC1 (Guertin and Sabatini, 2007) and the activation of mTORC1 by amino acids is known to be independent of TSC1/2 (Smith et al., 2005). The amino acid sensing pathways remains unknown, but recent studies have linked the class III

PI3K, VPS34 (vacuolar protein sorting 34) and the Ste20 (sterile20)-related kinase MAP4K3 (mitogen-activated protein 4 kinase 3) to TORC1 activation by amino acids (Figure 4) (Byfield et al., 2005; Findlay et al., 2007).

In addition to the signals described above, some other proteins have been demonstrated to interact with the TSC1/2 and to modulate the activity of mTOR1. P53 plays a critical role in sensing genotoxic and other stresses, and activated p53 could initiate a wave of starvation signals and trigger the activation of AMPK and subsequent activation of the TSC1/2 complex to inhibit the activity of mTOR (Feng et al., 2005). Furthermore, pro-inflammatory cytokines such as TNFα could activate mTOR pathway. Phosphorylation of TSC1 by IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta) interferes with the association of TSC1 with TSC2, which alters TSC2 membrane localization and activates mTOR pathway (Lee et al., 2007). Wnt signaling pathway also is able to stimulate mTOR via inhibiting GSK3 (glycogen synthase kinase 3) phosphorylation of TSC2. TSC2 is a physiological substrate of GSK3, and phosphorylation of TSC2 by GSK3 provide a clear mechanism to explain how different signaling pathways are integrated at a molecular level (Inoki et al., 2006).

2.2.3. Downstream targets of mTOR

mTOR signaling plays a role in various growth-related processes (Figure 6) and there are two well-characterized classes of downstream targets of mTORC1 related to protein synthesis: the translation regulators ribosomal S6 kinases S6K1 and eIF4E-BP

(eukaryotic initiation factor 4E (eIF4E) binding protein, also known as 4E-BP). Apart from the function of protein synthesis, mTOR also controls other important processes like lipid synthesis, autophagy and metabolism.

The S6 kinases are the directly targets of mTORC1 (Figure 6). There are two similar S6 kinases (S6K1 and S6K2) in mammalian cells. Although evidence suggested that S6K2 has greater activity, the effect of S6K1 on cell growth has been studied more extensively. S6K1 belongs to the protein kinase A, G, and C (AGC) family of protein kinases. The phosphorylation of Thr389 and the T loop is necessary to acquire the full function of S6K. mTORC1 is responsible for the phosphorylation on Thr389. Several S6KP substrates have been studied and 40S ribosomal protein S6 is one of them with significant meanings. The stimulation of S6K activity by mTORC1 leads to the phosphorylation of S6 and further increases in mRNA biogenesis. S6P can increase translational activation of 5′ tract of oligopyrimidine (TOP) mRNAs and through that S6K1 would upregulate general translation capacity. However, some recent findings suggested that S6 phosphorylation is not the only event that mediates the effect of S6Ks on cell growth and TOP mRNA translation. There should be other events that S6K1 regulates. (Hay and Sonenberg, 2004)

eIF4E-BPs are another relevant target of mTORC1 (Figure 6). Unphosphorylated 4E-BP1 suppresses mRNA translation. mTORC1 promotes mRNA translation initiation and progression by phosphorylating the eIF4E inhibitory proteins, 4E-BPs. Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E. eIF4E is then free to bind to eIF4G (Eukaryotic initiation factor 4 G), which completes the eIF4F

(Eukaryotic initiation factor 4F) complex on the 5' cap and eIF3 (eukaryotic initiation factor 3), 40S ribosomal subunits, and the ternary complex are also recruited to the cap, resulting in the assembly of the translation preinitiation complex (PIC) (Holz et al., 2005).

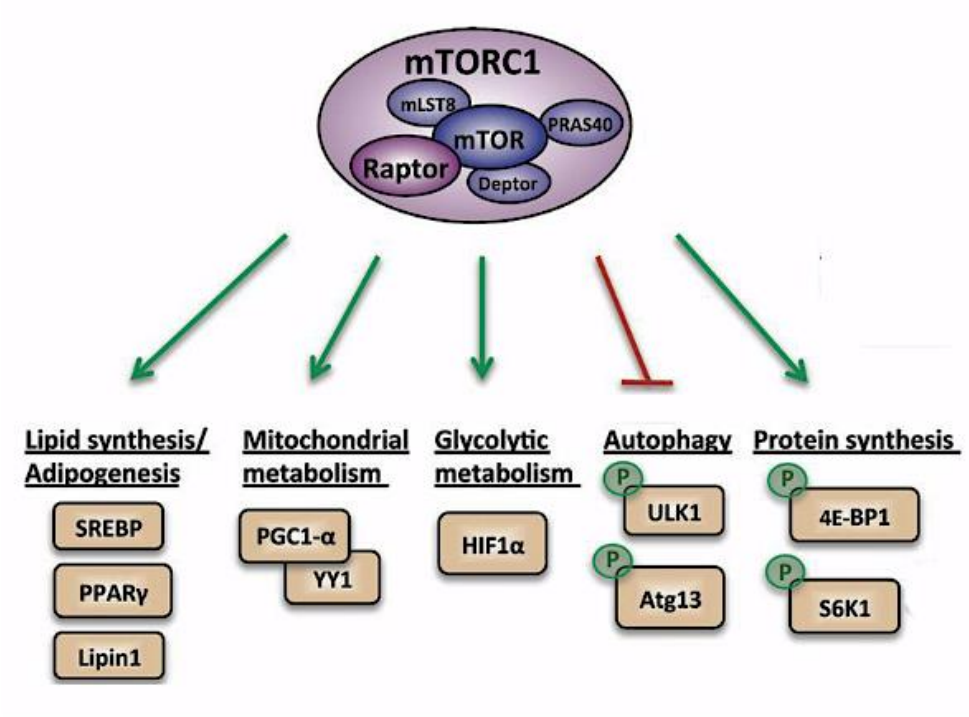

Figure 6 Effectors of mTORC1(Norrmén and Suter, 2013). mTOR signaling through multiple effector pathways, activates many different signal transduction pathways. Most of these pathways are involved in growth-related processes, autophagy, mitochondrial metabolism and glycolytic metabolism.

Some studies show that rapamycin could block the biosynthesis of ribosomes. That reflect that the activation of mTORC1 could also promote ribosome biogenesis (Mayer et al., 2004). mTORC1 promotes the translation of the mRNAs encoding ribosomal proteins and some translational machinery. mTOR regulates also RNA polymerase I (Pol I) transcription by modulating the activity of TIF-1A (transcription

initiation factor-1A), a regulatory factor that senses nutrient and growth-factor availability (Mayer et al., 2004). Rapamycin treatment leads to TIF1A inactivation and thus impairs formation of the transcription initiation complex. mTORC1 has been shown to closely involved in gene regulation at the promoters by regular Pol I and Pol III dependent gene (Tsang et al., 2010). Studies in yeast identified that forkhead-like transcription factor FHL1 (Forkhead-like 1) played a critical role in the regulation of polymerase II (Pol II)-dependent RP gene expression (Martin et al., 2004; Schawalder et al., 2004). FHL1 is constitutively bound to RP (ribosomal protein) gene promoters, and regulated by IFH1 (interacts with Forkhead 1) and by CRF1 (corepressor with FHL1 protein 1), coactivator and corepressor respectively. mTORC1 can maintain the corepressor CRF1 in the cytoplasm. Therefore, mTORC1 inactivation leads to the translocation of CRF1 into nucleus competing with IFH1 for binding to FHL1 and inhibiting RP gene transcription. mTOR can also control RP genes via zinc finger transcription factor SFP1 and histone modifying factors (Humphrey et al., 2004; Jorgensen et al., 2004).

By controlling the degradation of organelles and protein complexes, autophagy provides a source of biological substrate for anabolic processes and energy production during nutritional deficiencies, playing an impotent pro-survival role. It has been proved that mTOR can control autophagy (Figure 6). Activation of mTORC1 suppresses autophagy and inhibition of mTORC1 strongly induces autophagy. In yeast, Atg (autophagy-related protein) 13, which binds to and activates Atg1, is hyperphosphorylated in a Tor-dependent manner, reducing its affinity to Atg1,

disrupting the formation of the autophagosome and, conversely, starvation or rapamycin treatment can enhance protein kinase activity of Atg1 (Kamada et al., 2000). By phosphorylating ATG13 and UlK1 (Unc-51-Like Kinase 1), the mammalian homologues of yeast Atg13 and Atg1, mTORC1 blocks the formation of the ULK1–Atg13–FIP200 (family interacting protein of 200 kD) complex and autophagosome initiation. In contrast to yeast counterparts, nutrient conditions do not alter the complex formation (Hosokawa et al., 2009). Evidence also shows that pharmacological inhibition of mTOR kinase by rapamycin activates mitophagy and promotes the preferential clearance of mutant mtDNA (Dai et al., 2014).

mTORC1 also plays a role in regulating lipid synthesis (Figure 6), which is required for cell growth and proliferation. mTOR kinase activity is required for 3T3-L1 preadipocyte differentiation. CCAAT/enhancer-binding protein-α (C/EBP-α) and peroxisome proliferator–activated receptor-γ (PPAR-γ) are two key transcription factors, playing critical roles in adipogenesis and lipid accumulation. Rapamycin specifically disrupted the positive transcriptional feedback loop between C/EBP-α and PPAR-γ, by directly targeting the transactivation activity of PPAR-γ (Kim and Chen, 2004). mTORC1 positively regulates the activity of sterol regulatory element binding protein 1 (SREBP1). Silencing of SREBP blocks lipogenesis and causes a reduction in cell and organ size (Porstmann et al., 2008). Loss of TOR activity correlates with a decrease in fat accumulation. The *melted* mutant produces an animal with 40% less fat than normal, by reducing TOR activity and at the same time increasing FOXO (forkhead box O) activity (Teleman et al., 2005). TOR signaling network also controls

fat metabolism and may play a role in the development of metabolic disorders such as obesity and type 2 diabetes.

Some studies support the idea that mTOR plays a primary regulatory role on mitochondrial activity (Figure 6). Rapamycin treatment reduces mitochondrial membrane potential. mTOR activity and more precisely mTOR-raptor complex formation is tightly correlated with mitochondrial metabolism (Schieke et al., 2006). mTOR is necessary for the maintenance of mitochondrial oxidative function (Cunningham et al., 2007). Rapamycin can decrease gene expression of PPARγ coactivator 1α (PGC1α), estrogen-related receptor α and nuclear respiratory factors and decrease mitochondrial gene expression and oxygen consumption. The transcription factor yin-yang 1 (YY1) is the common target of mTOR and PGC-1α. However, some other studies do not observe the influence of mTOR on mitochondrial activity; e.g., Rapamycin treatment does not affect the mitochondrial potential (Desai et al., 2002). That indicates that the effect of mTOR on mitochondria is content-dependent. Besides, rapamycin was shown to have the ability to alleviate mitochondrial disease (Johnson et al., 2013). In a mouse model of Leigh syndrome, rapamycin administration improves the survival and attenuates disease progression. No precise mechanism was given to explain it, but the metabolic shift induced by rapamycin may take a credit.

Angiogenesis was recognized as a therapeutic target for inhibiting cancer growth four decades ago. Pathological angiogenesis is a hallmark of cancer. Rapid growth tumor needs enlarged and tortuous blood vessels to supply rapid consumed nutrition.

Pathological blood vessels are hyperpermeable to circulating macromolecules, resulting in extravasation of plasma fluid and proteins (Phung et al., 2006). Inhibition of angiogenesis could suppress tumor growth in humans (Folkman, 1971). Frost et al. (2013) reported that rapalogs could induce tumor cell apoptosis *in vivo* but not *in vitro*. After mTOR inhibitor administration in a B-cell lymphoma xenograft, they observed a downregulation of vascular endothelial growth factor (VEGF) protein expression, which is frequently highly expressed in cancers and plays a central role in angiogenesis. These results suggested anti-angiogenesis properties of mTOR inhibitors participated in its antitumor activity *in vivo*. The mTOR inhibitor rapamycin was reported to inhibit tumor growth in a liver metastatic model for colon cancer (Guba et al., 2002). Rapamycin administration not only diminished VEGF secretion but also markedly inhibited VEGF-dependent vascular endothelial cells proliferation and completely abrogated VEGF-induced tubular formation. Consistent with that, in breast cancer cells, the mTOR inhibitor temsirolimus exerted antiangiogenic effects by directly inhibiting VEGF production (Del Bufalo et al., 2006). Apart from that, temsirolimus can also down-regular VEGF production through the inhibition of hypoxia-stimulated hypoxia-inducible factor (HIF)-1α expression and transcriptional activation (Del Bufalo et al., 2006). mTOR signaling plays a key role in hypoxia-triggered smooth muscle and endothelial proliferation and angiogenesis *in vitro* (Kiefer et al., 2002). mTOR inhibition by rapamycin specifically abrogated hypoxia-mediated angiogenesis. Akt is important for VEGF-mediated angiogenesis. Sustained endothelial Akt activation is sufficient to increased blood volume and leaky

blood vessels in a xenograft model by decreasing Akt S473 phosphorylation in the endothelial cells surrounding the tumor (Phung et al., 2006). This Akt signaling in the tumor vascular stroma is sensitive to rapamycin. Rapamycin as an antiangiogenic agent can block pathological blood vessel formation and reduces tumor growth. Considered the demonstrated role of mTORC2 in Akt activity, rapamycin may have more unidentified connection with mTORC2 and through that exerts its antiangiogenic properties.

2.3. NEGATIVE FEEDBACK REGULATION OF mTOR SIGNALING

There is a negative feedback loop from the TSC/mTOR/S6K pathway to the upstream, insulin-responsive IRS/PI3K/PDK1/Akt pathway (Figure 7). Inappropriate activation of the TSC/mTOR/S6K pathway attenuates IRS-dependent processes. mTORC1 hyperactivation triggers an S6K1-dependent negative feedback loop. S6K1 regulates IRS1 through direct phosphorylation, thereby impairing IRS1 adaptor function. Downregulation of IRS1 and IRS2 limits the signal transmission from the insulin receptor to PI3K. Furthermore, this results in dampened Akt activation. Um et al. (2004) reported that S6K1-deficient mice displayed insulin sensitive because of the loss of a negative feedback loop from S6K1 to IRS. Accordingly, there should be an increased Akt activity when mTOR is inhibited. Rapamycin-based therapeutic approaches may have encountered a stumbling block in the S6K1-mediated feedback loop, the inhibition of which leads to a severe upregulation of PI3K signalling and provides important pro-survival and proliferative signals through Akt and other AGC kinases (O'Reilly et al., 2006; Zoncu et al., 2011). Under conditions of chronic

activation of mTORC1, such as TSC1/TSC2 complex deficiency or excess of nutrients, the negative feedback loop constitutively dampens Akt signaling and the downstream substrates involved in cell survival and proliferation will also be inhibited. Tumors in TSC patients are less aggressive because the feedback loop squelches PI3K/Akt signaling (Guertin and Sabatini, 2007). Taking the FOXO family for example, the FOXO family are important transcription factors in promoting apoptosis and mediating cell cycle arrest, which can be inhibited by Akt. Signaling through the feedback loop results in dampened Akt activation, which in turn, activates the FOXO family function (Sykes et al., 2011).

It is worth mentioning at this point that mTORC2 also performs a positive role in the activation of Akt (Figure 7). S6K1 directly phosphorylates the Rictor subunit of mTORC2 on Thr^{1135} and stimulates 14-3-3 binding to Rictor (Dibble et al., 2009). S6K1-mediated phosphorylation of Rictor negatively regulates the ability of mTORC2 to phosphorylate Akt-Ser^{473} in cells (Dibble et al., 2009). Full activation of Akt requires phosphorylation at 2 sites, Thr^{308} and Ser^{473}. Sarbassov et al. (2005) found that mTORC2 directly phosphorylated Akt on Ser473 *in vitro* and facilitated Thr^{308} phosphorylation by phosphoinositide-dependent kinase 1 (PDK1). Thus, mTORC2 plays an important role in the regulation of cell survival and proliferation through phosphorylation of its substrate Akt. Therefore, mTORC1 is indirectly regulated by mTORC2. That reminds us that mTORC2 could be served as an anti-tumor target and inhibiting both mTOR complexes has great clinical importance.

mTORC2 was originally identified as rapamycin insensitive. But there are study

showing that prolonged rapamycin treatment inhibits the assembly of mTORC2 and reduces the levels of mTORC2 (Sarbassov et al., 2006). In AML cells, rapamycin was shown to block Akt activation via inhibition of mTORC2 formation and presented its therapeutic potential in leukemia (Zeng et al., 2007)

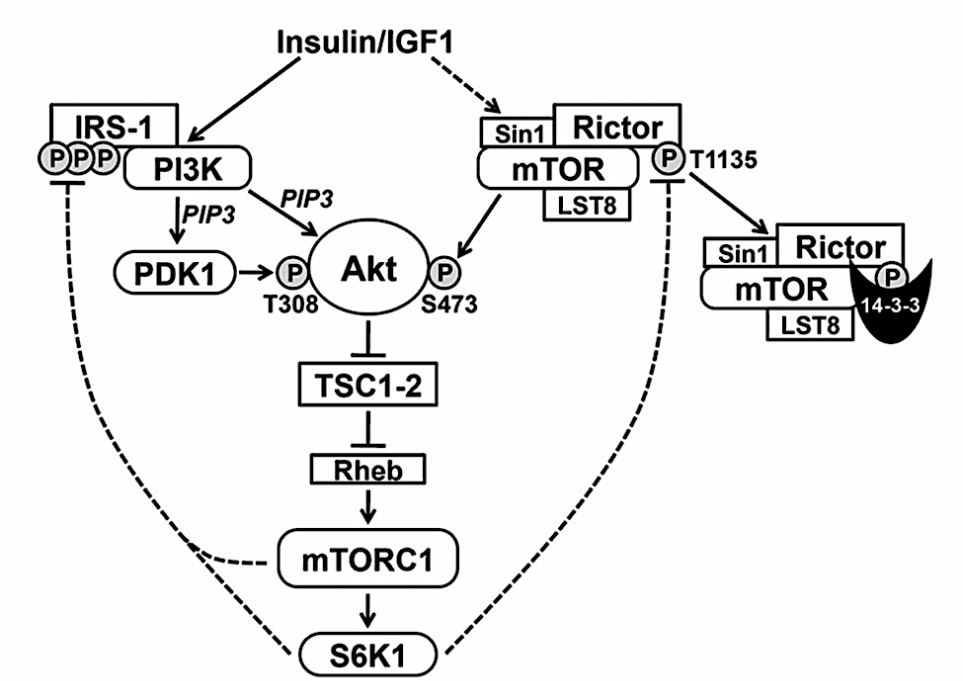

Figure 7. The feedback loop affects insulin-stimulated activation of Akt (Dibble et al., 2009). S6K1 regulates IRS1 through direct phosphorylation and limits the signal transmission from the insulin receptor to PI3K. S6K1-mediated phosphorylation of Rictor also negatively regulates the ability of mTORC2 to phosphorylate Akt-Ser^{473} in cells

The existence of the negative feedback loop strongly obstructs the application of rapamycin and its derivatives as universal cancer therapeutics. Losing feedback inhibition of Akt could be a potential therapeutic problem and promote resistance to

chemotherapy because the Akt regulars various pro-survival signaling (Gines et al., 2003; Thakkar et al., 2001). However, the inhibition of mTORC2 of rapamycin is cell-type-dependent, we need to distinguish the cancer type that is respond favorably to rapamycin and its derivatives from that are not. In addition, combined with other anticancer agents that inhibit PI3K or Akt, rapamycin and its derivatives could overcome the shortcomings from the negative feedback loop. Some studies show that combined inhibition of IGF-1R and mTOR is highly effective in multiple myeloma and might represent a potential new treatment strategy (Baumann et al., 2009). The combination of resveratrol and rapamycin maintains inhibition of mTORC1 signaling, while preventing upregulation of Akt activation in estrogen receptor positive and negative breast cancer cells (Alayev et al., 2015).

It is also necessary to develop next generation of mTOR inhibitors: ATP-competitive inhibitors of mTOR. The new generation of mTOR inhibitors competes with ATP for binding to the kinase domain in mTOR. The ATP analogues block the phosphorylation of all known downstream targets of both mTORC1 and mTORC2 (Matter et al., 2014).

Dual PI3K/mTOR inhibitors are one of ATP-competitive inhibitors. PI-103 is the first agent of this class. To define targets critical for cancers driven by activation of PI3K, PI-103 was developed. Although this drug was originally regarded as PI3K inhibitors, it also shown to effectively inhibit mTORC1 and mTORC2 (Fan et al., 2006). Dual PI3K/mTOR inhibitors can inhibit the both upstream and downstream of Akt, thus avoiding the negative feedback loops.

Dual PI3K/mTOR inhibitors may related with high toxicity (Chiarini et al., 2015). To reduce the toxicity, mTORC1/mTORC2-selective inhibitors are drawing more attentions. These kinds of inhibitors are ATP-competitive mTORC1/mTORC2 inhibitors that block only the mTOR catalytic domain.

Several of these drugs have been or are being investigated in clinical trials, but some of these inhibitor have a biphasic effect on Akt (Rodrik-Outmezguine et al., 2011). Inhibition of mTOR kinase cause marked activation of receptor tyrosine kinases (RTK), leading to subsequent PI3K activation and re-phosphorylation of Akt T308 which is sufficient to reactivate Akt activity and signaling. The addition of RTK inhibitors can prevent this reactivation of Akt signaling and cause profound cell death and tumor regression *in vivo* (Rodrik-Outmezguine et al., 2011). The successful application of the RTK inhibitors highlights the need for combinatorial approaches to block the negative feedback loop pathways.

2.4. mTOR INHIBITORS ASSOCIATED SIDE EFFECTS

mTOR inhibitors have been evaluated in clinical trials for the treatment of a variety of cancers (Martins et al., 2013). An intravenous formulation of temsirolimus (CCI-779, Wyeth Pharmaceuticals Inc., Philadelphia, PA) was approved by US FDA (Food and Drug Administration) in 2007 for the treatment of advanced renal cell carcinoma (RCC). In 2009, an orally administered drug for use in patients with advanced metastatic RCC, everolimus (RAD001, Novartis Pharmaceuticals Corporation, East Hanover, NJ) gained FDA approval. Ridaforolimus (AP23573,

Ariad Pharmaceuticals Inc., Cambridge and Merck & Co., Inc., Whitehouse Station, NJ), a drug for the treatment of metastatic soft tissue and bony sarcomas, was approved by FDA in 2011 (Hudes et al., 2007; Martins et al., 2013; Motzer et al., 2008). The studies on these agents are extensive and numerous. All of these agents, alone or in combination with other chemotherapy agents, are also under investigation to text the effective on various forms of cancers (Martins et al., 2013).

During the clinical application, cancer patients with mTOR inhibitors treatment often reported suffering a number of unique toxicities. A Phase II study of temsirolimus in heavily pretreated patients with breast cancer revealed the most common temsirolimus-related adverse events of all grades were mucositis (70%), maculopapular rash (51%), and nausea (43%) and the most common, clinically important grade 3 or 4 adverse events were mucositis (9%), leukopenia (7%), hyperglycemia (7%), somnolence (6%), thrombocytopenia (5%), and depression (5%) (Chan et al., 2005). In a Phase I study of a potent dual PI3K/mTOR inhibitor, BGT226, the most common side effects were on the gastrointestinal system (nausea (68%), diarrhea (61%), vomiting (49%), and fatigue (19%)) (Markman et al., 2012).

The most prevalent adverse events (AEs) reported are stomatitis, skin rash, hyperglycemia, hyperlipidemia, thrombocytopenia, fatigue, and anemia (de Oliveira et al., 2011; Martins et al., 2013). Among them, stomatitis has been identified repeatedly as one of the most common dose-limiting toxicities, whatever the mode of administration (per os, intravenous). These side effects may cause severe social or personal impairments and may result in cessation of mTOR therapy. There are no

explanations about how these mTOR inhibitors associated AEs occurred.

Until now, it is not clear whether rapamycin is cytostatic which is to attenuate cell cycle progression and cell growth or can also eliminate tumor cells by inducing cell death. Therefore, the most promising approach for the use of rapalogs in cancer patients seems to be their combination with traditional chemotherapeutic agents or other targeted agents. This strategy could also lead to a lower drug dose that may lessen the systemic side effects of the drugs.

3. MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine, aMT) is a highly conserved molecule found widely throughout nature. Melatonin displays high lipid solubility which facilitates passage across cell membranes (Pardridge and Mietus, 1980). The origin of this indoleamine molecule can be traced back an estimated 2.5 billion years.

Melatonin was first isolated and structurally identified chemically in bovine pineal tissue by Lerner et al. in 1958 (Lerner et al., 1958). Melatonin is a small molecule, with a molecular weight of 232 Daltons, derivative of the amino acid tryptophan (Axelrod and Weissbach, 1960). The circadian production of melatonin by the pineal gland explains its chronobiotic influence on organismal activity, including the endocrine and non-endocrine rhythms. Other functions of melatonin, including its antioxidant and anti-inflammatory properties, its genomic effects, and its capacity to modulate mitochondrial homeostasis, are linked to the redox status of cells and

tissues.

Because the discovery of melatonin was in pineal tissue, for many years the pineal was considered the exclusive source of melatonin in vertebrates. However, the presence of melatonin has been detected in multiple extrapineal tissues including the brain, retina, lens, cochlea, Harderian gland, airway epithelium, skin, gastrointestinal tract, liver, kidney, thyroid, pancreas, thymus, spleen, immune system cells, carotid body, reproductive tract, and endothelial cells. In most of these tissues, the melatonin-synthesizing enzymes have been identified. Melatonin is present in essentially all biological fluids including cerebrospinal fluid, saliva, bile, synovial fluid, amniotic fluid, and breast milk. In several of these fluids, melatonin concentrations exceed those in the blood. The importance of the continual availability of melatonin at the cellular level is important for its physiological regulation of cell homeostasis, and may be relevant to its therapeutic applications (Acuña-Castroviejo et al., 2014).

Melatonin is also produced in invertebrates, unicells, and plants. That is to say that melatonin is indispensable. An hypothesis proposed by Tan et al. (2013a) may explain that. They presumed that melatonin was evolved in purple nonsulfur bacteria, likely in *Rhodospirillum rubrum* to protect them from an oxidizing environment. These melatonin-producing bacteria were subsequently phagocytized by eukaryotes, where they eventually evolved into mitochondria. From that point of view, all eukaryotic cells may have retained the ability to produce melatonin because of mitochondrial existence (Tan et al., 2013a).

3.1. MELATONIN SYNTHESIS

3.1.1. Classic melatonin synthetic pathway in animals

Axelrod's group (Axelrod and Weissbach, 1960; Weissbach et al., 1960) reported biosynthesis of melatonin for the first time. The essential amino acid, tryptophan, is the initial precursor for melatonin synthesis. Melatonin formation needs four consecutive enzymatic steps. Briefly, Tryptophan is first hydroxylated to form 5-hydroxy-tryptophan (5HTP) by tryptophan-5-hydroxylase (TPOH). This product is subsequently decarboxylated to 5-hydroxy-L-tryptamine (serotonin or 5-HT) under the catalytic action of aromatic amino acid decarboxylase (AADC). After that, serotonin was first acetylated to form N-acetylserotonin by arylalkylamine N-acetyltransferase (AANAT) and, finally, N-acetylserotonin was methylated to melatonin by hydroxyindole-O-methyl transferase (HIOMT) that currently known as N-acetyl-Serotonin methyltransferase (ASMT) (Figure 8).

The concentrations of melatonin in the serum, which mainly from the pineal gland, shows a circadian pattern. ASMT can be inhibited by light and was considered as rate limiting enzyme in melatonin synthesis (Wurtman et al., 1963). However, AANAT, the enzyme that acetylates serotonin to form N-acetylserotonin, is regulated by circadian clock and generally regarded as determinant of maximal melatonin production peaked until today. The pineal gland receives input from postganglionic fibers, leading to the release of noradrenaline and increased production of cyclic AMP, thus activating the enzyme AANAT (Luboshizsky and Lavie, 1998).

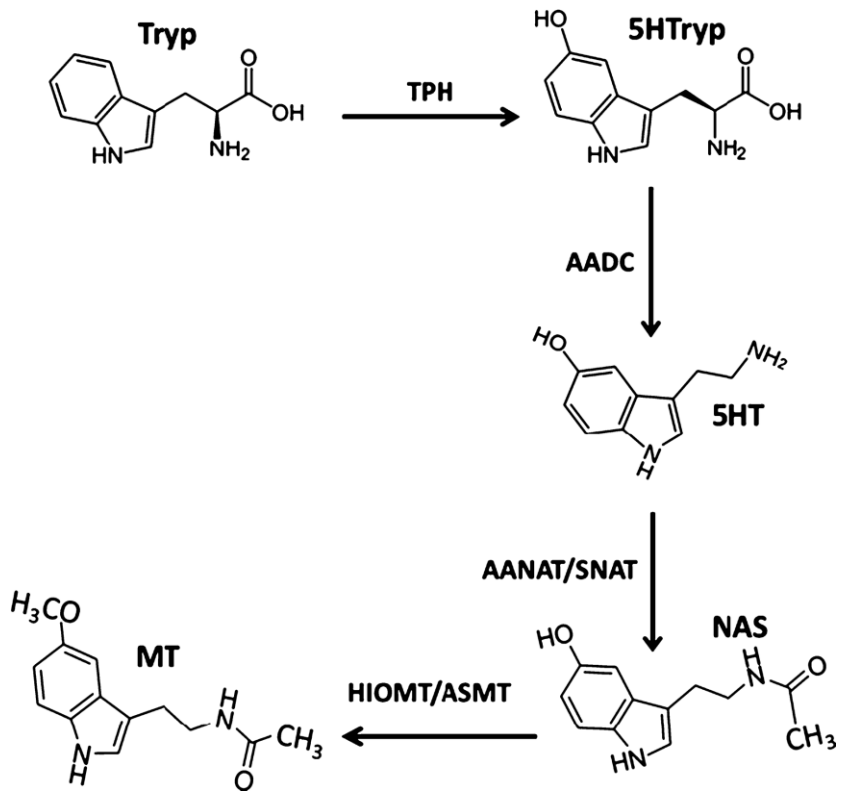

Figure 8. Classic melatonin synthetic pathway

3.1.2. Alternate melatonin synthetic pathway in animals

Melatonin synthesis may be entirely different at other extrapineal sites in animals. Tan et al (2016a) think that the classic melatonin pathway is not generally the prevailing route of melatonin production. An alternate pathway is known to exist, in which serotonin is first O-methylated to 5-methoxytryptamine (5-MT) and, thereafter, 5-MT is N-acetylated to melatonin (Tan et al., 2016a). They hypothesized that 5-MT may be the immediate precursor of melatonin (Tan et al., 2016a). Some studies have proven that 5-MT administration significantly increases melatonin levels in animals

(Ho et al., 1985; Lang et al., 1985). However, the alternatives have never been studied systematically. More sophisticated studies related to the enzyme kinetics of purified ASMT and AANAT, including their subforms, are required in different species to confirm their hypothesis.

3.2. MELATONIN FUNCTION AND MECHANISMS

3.2.1. Receptor-mediated actions

Two membrane receptors of melatonin, identified as the melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2), were characterized and cloned. They bind melatonin with high affinity (Venegas et al., 2013). MT1 receptor is 350 amino acids in length, whereas MT2 consists of 363 amino acids (Dubocovich and Markowska, 2005). They share a high degree of sequence homology and are both members of the superfamily of transmembrane, G-protein-coupled receptors.

MT1 and MT2 melatonin receptors signal by coupling to heterotrimeric Gi proteins formed by α, β and γ subunits. Activation of these receptors promotes dissociation of G proteins into α and βγ dimmers, which interact with various effector molecules involved in the transmission of cell signaling (Dubocovich and Markowska, 2005).

Melatonin may also interact with a group of nuclear receptors to carry out some activities (Acuña-Castroviejo et al., 1993; Acuña‐Castroviejo et al., 1994; García et al., 2015). The superfamily members of nuclear receptors that reportedly bind melatonin include the RORα, RZRα, RORα2, and RZRβ, which are referred as

retinoid orphan receptors (ROR) or retinoid Z receptors (RZR). These nuclear receptors contain an NH2-terminal domain, a DNA binding domain, a ligand binding domain (in the COOH terminal), a zinc double finger, and a hinge region. However, in most cases, their specific functions are under debate (Reiter et al., 2014b).

There is also what is referred to as an MT3 (melatonin receptor type 3) receptor located in the cytosol of a few cells. It is not coupled to a G protein and exhibits low affinity for iodo-melatonin (Reiter et al., 2014b). However, solid data support the idea that the MT3 melatonin binding site is the enzyme quinone reductase II (QR2), rather than a membrane melatonin receptor (Tan et al., 2007). Tan et al. (2007) hypothesized that melatonin is a co-substrate of QR2 and probably influences the activity of QR2.

3.2.2. Antioxidant properties of melatonin

The direct free radical scavenging actions of melatonin and its metabolites are one of the main functions of melatonin (Figure 9). The antioxidant activity of melatonin has been known for almost 25 years (Acuña-Castroviejo et al., 2007; Escames et al., 2006b; Escames et al., 2003; Tan et al., 1993). Melatonin is uncommonly effective in reducing oxidative stress because of the number of means it has as a direct free radical scavenger and indirect antioxidant. Thus, melatonin functions in this capacity in both the aqueous and lipid portions of the cell (J Reiter et al., 2013) and as a result it protects lipids (Deng et al., 2016; García et al., 2014), proteins (Mollaoglu et al., 2007; Waseem et al., 2016), and DNA (Chua et al., 2016; Reiter et al., 1998) from oxidative damage. It is more highly concentrated in the

regions of the cells where many of the free radicals are formed, e.g., mitochondria (Venegas et al., 2012). It may be synthesized in the mitochondria (He et al., 2016) and at this site its synthesis may be inducible (Tan et al., 2015). However, not only melatonin but a number of its metabolites also function as radical scavengers (Tan et al., 2015). Melatonin binds transition metals which reduces the formation of the most aggressive ROS, i.e., ·OH (Galano et al., 2015).

The detoxification of ROS/RNS is achieved by melatonin and a number of its metabolic kin in what is later referred to as the antioxidant cascade (Tan et al., 1993). Hence, the derivatives of melatonin that are formed when it directly neutralizes a free radical, often by electron donation (Galano et al., 2011), are equally as effective, and sometimes more so, than melatonin itself in reducing oxidative stress (Reiter et al., 2014a). Cyclic-3-hydroxymelatonin (c3OHM) is the primary metabolite and it is formed when melatonin scavenges two free radicals. c3OHM as well as the secondary and tertiary downstream metabolites, N-acetyl-N-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) all can function as free radical scavengers. So, a single melatonin molecule along with its metabolites can scavenge numerous reactive oxygen species (ROS).

Furthermore, melatonin stimulates the activity of a number of antioxidative enzymes (Reiter et al., 2000) and it promotes the synthesis of another important antioxidant such as glutathione (Urata et al., 1999). Melatonin was also found to stimulate anti-oxidative enzymes including glutathione peroxidase (GPx) and glutathione reductase (GRd) (Escames et al., 2006b; Ortiz et al., 2014; Pablos et al.,

1995). The enhancement of superoxide dismutase (SOD) activity was also reported (Liu and Ng, 2000; Öztürk et al., 2000). Melatonin increased mRNA levels of both CuZnSOD and MnSOD (Antolín et al., 1996; Kotler et al., 1998). Moreover, melatonin maintain also glutathione (GSH) homeostasis decreasing the ratio glutathione disulfide (GSSG)/GSH (Martín et al., 2000).

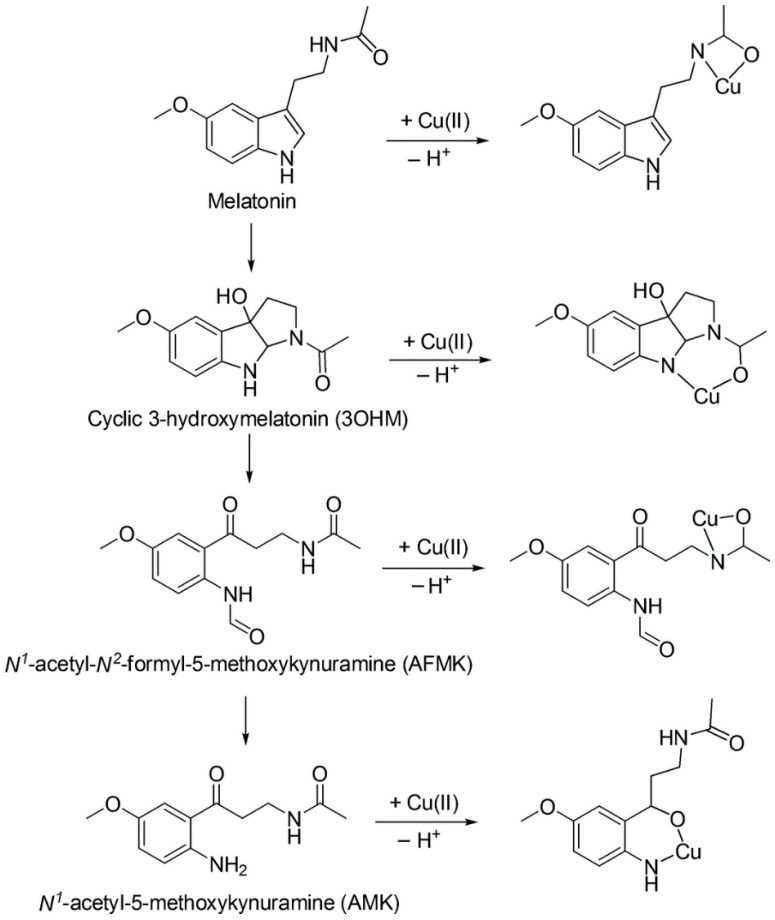

Figure 9. Free radical scavenging cascade (vertical) and metal-chelating cascade (horizontal) of melatonin and its metabolites.

3.2.3. Melatonin and mitochondria interaction

Based on the observations that the cytoplasm of pinealocytes is rich in mitochondria and the morphology of these mitochondria changes dynamically with the light/dark cycle as well as with the activity of the pinealocytes in different species, Tan el al. (2016b) presume that mitochondria may be the major sites for melatonin synthesis. Melatonin synthesis may be the additional function of mitochondria and melatonin can be used locally for defense against oxidative stress and inflammation (Tan et al., 2016b). The authors did not exclude the possibility that melatonin is also synthesized in the cytosol. But they believed that cytosolic melatonin synthesis is far less efficient than that in mitochondria (Tan et al., 2016b).

Melatonin is a highly lipophilic molecule that can cross cell membranes and easily reach subcellular compartments like mitochondria (Menendez-Pelaez and Reiter, 1993). Melatonin can accumulate in mitochondria against a concentration gradient (Acuña-Castroviejo et al., 2007; Acuña‐Castroviejo et al., 2001). It was reported that glucose transporters have a central role in melatonin uptake into cells (Hevia et al., 2015a). A recent study indicated that peptide transporters 1 and 2 (PEPT1/2), which are localized in the mitochondrial membrane, are responsible for melatonin transport into mitochondria (Tan et al., 2016b). This active transport may be the reason of mitochondrial melatonin accumulation.

Therefore, melatonin is taken up by mitochondria (Lopez et al., 2009), providing an in situ protection against oxidative damage (Acuña-Castroviejo et al., 2002;

Acuña-Castroviejo et al., 2007; Acuña-Castroviejo et al., 2011; Jou et al., 2010; Lopez et al., 2009; Paradies et al., 2010) (Acuna et al. 2002, 2011; Acuna-Castroviejo et al. 2007; Jou et al. 2010; Lopez et al. 2009; Paradies et al. 2010). Under physiological and pathological conditions, the ability of melatonin to maintain mitochondrial homeostasis has been reportedly showed (Acuña-Castroviejo et al., 2011; Acuña‐Castroviejo et al., 2001; Escames et al., 2007; Lopez et al., 2006a; Lopez et al., 2006b) (Acuna et al. 2011; Acuna-Castroviejo et al. 2001; Escames et al. 2007; Lopez et al. 2006a, b). Melatonin, but not other antioxidants including vitamins C and E, and N-acetylcysteine, was highly efficient to maintain mitochondrial GSH homeostasis in extremely oxidative conditions, closing the mitochondrial permeability transition pore (MPTP) (Paradies et al., 2010) and promoting mitochondrial survival (Martín et al., 2000; Martín et al., 2002). In normal isolated mitochondria, melatonin increased the activity of the respiratory chain complexes, reduced oxygen consumption and decreased slightly the mitochondrial inner membrane potential, decreasing ROS (Lopez et al., 2009).

In vivo, melatonin also restored the normal mitochondrial function impaired in different inflammatory status, including neurodegenerative diseases (Acuña-Castroviejo et al., 2011; Cardinali et al., 2010; Khaldy et al., 2003; Olcese et al., 2009; Tapias et al., 2009), aging (Carretero et al., 2009; Escames et al., 2010), neuromuscular disorders (Chahbouni et al., 2010), metabolic dysfunction (Cardinali et al., 2011; Koziróg et al., 2011), and sepsis (Escames et al., 2006a). In these conditions, melatonin reduces mitochondrial ROS production to normal levels, recovers the

normal respiratory chain activity and ATP production, and maintains the GSH homeostasis, closing the MPTP. Melatonin also blunted inducible nitric oxide synthase (iNOS)/(mitochondrial iNOS) i-mtNOS expression during sepsis, recovering full mitochondrial function (Crespo et al., 1999; Escames et al., 2007; Escames et al., 2006b; Escames et al., 2003; Lopez et al., 2006a; Lopez et al., 2006b). Although the initial mechanism proposed for these effects of melatonin therapy was related to the inhibition of the NF-κB pathway activation, the capability of mitochondria to concentrate melatonin, and the ability of the latter to reduce mitochondrial ROS production, suggest that the indoleamine may also inhibits the mitochondrial ROS-dependent NLRP3 (NOD-like receptor (NLR) family pyrin domain containing 3) inflammasome activation. The relationship between melatonin and autophagy, which seems to involve a reduction in the oxidative stress by the indoleamine, further support this hypothesis (Coto-Montes and Tomás-Zapico, 2006; Garcia et al., 2011; Yoo and Jeung, 2010).

3.3. MELATONIN-INDUCED ONCOSTASIS AND CLINICAL RELEVANCE

Melatonin is effective in suppressing neoplastic growth in a variety of tumors (Cardinali et al., 2016). Melatonin is one of the natural molecules that are effective in treating neoplastic malignancies and has the ability to reduce severe DNA damage, limiting the frequency of cancer initiation. Melatonin can also regulate the expression of growth and differentiation-related genes to contribute to a variety of stages of malignant transformation. Different mechanisms of the antitumor effect of melatonin also have been suggested (Mills et al., 2005).

3.3.1. Mechanism of melatonin antitumor action

As shown in Figure 10, studies have suggested several mechanisms for the effects of melatonin. Present knowledge on the possible mechanisms of melatonin oncostasis induce direct and indirect antioxidant effects, regulation of metabolism, anti-angiogenic and antimetastatic effects, the capacity to decrease telomerase activity and the ability to regulate the immune system (Figure 10).

3.3.1.1. Anti-oxidative actions

ROS can be involved in cancer initiation as well as in malignant transformation (Tafani et al., 2015). Melatonin has a marked anti oxidative effect as we discussed above. So melatonin could provide protection against damage from carcinogenic substances and act as a free radical scavenger to inhibit tumor growth.

3.3.1.2. Regulation of metabolism

Tumor growth can be stimulated by uptake linoleic acid (LA). Its metabolite 13-hydroxyoctadecadienoic acid (13-HODE) has the ability to activate EGFR (epidermal growth factor receptor) / MAPK (mitogen-activated protein kinase) mitogenic signaling. It has been shown that melatonin treatment induce a rapidly and reversibly inhibition of LA uptake and therefore, a decrease of 13-HODE (Blask et al., 1999).

Warburg observed that cancer cells have high rates of aerobic glycolysis to satisfy its increased requirements for ATP production (Warburg, 1956). As the rate of ATP

production by glycolysis is low, malignant cells have an increased need for glucose uptake (Macheda et al., 2005). The glucose transporters (GLUT) are responsible for glucose uptake and the upregulation of GLUT1 was observed in a variety of tumors (Macheda et al., 2005). The consistent overexpression of GLUT1 is clearly associated with tumor progression. Hevia et al. (Hevia et al., 2015b) found that melatonin interacts with GLUT1 at the same site where glucose does and in that way, melatonin decreased glucose uptake, resulting in the reduction of tumor cells growth.

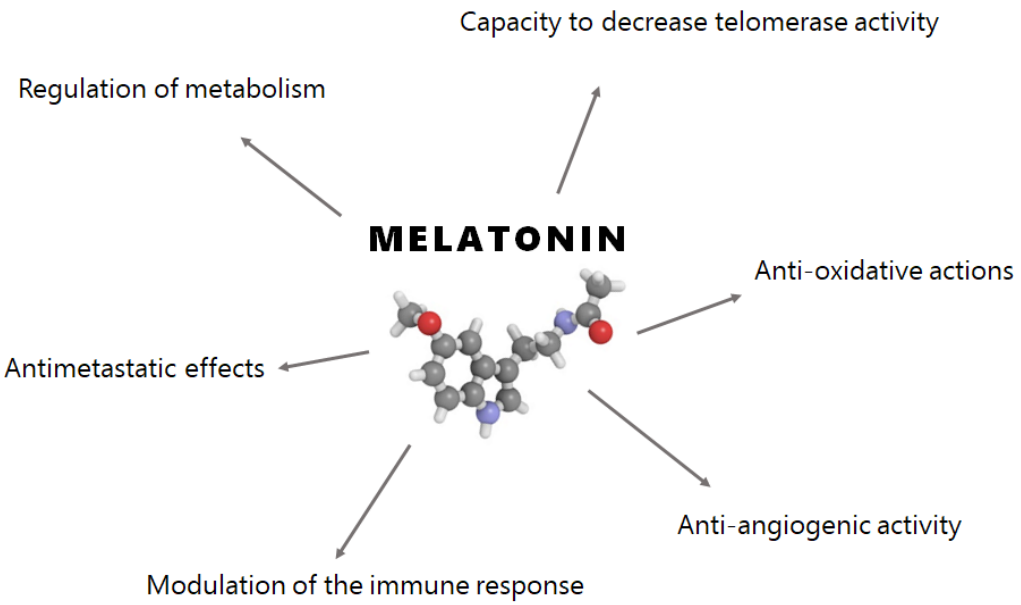

Figure 10. Several mechanisms through which melatonin can exert oncostatic effects.

3.3.1.3. Anti-angiogenic activity

Angiogenesis is an essential event involved in tumor's progression and development. Angiogenesis can be induced surprisingly early during the multistage

development of tumor (Hanahan and Weinberg, 2011). VEGF is the most active endogenous pro-angiogenic factor, and hypoxia or oncogene signaling can upregulate VEGF gene expression to promote new blood vessel formation.

The anti-angiogenic properties of melatonin have been reported in numerous studies. Melatonin reduces VEGF production and VEGF mRNA expression in diferent cancer cells (Alvarez-García et al., 2013). This effect was associated with a reduction in HIF-1α protein expression, nuclear localization, and transcriptional activity (Carbajo-Pescador et al., 2013). Under hypoxia, melatonin suppressed HIF-1 transcriptional activity, leading to a decrease in VEGF expression (Park et al., 2010). These observations suggested melatonin could play a pivotal role in tumor suppression via inhibition of HIF-1-mediated angiogenesis.

3.3.1.4. Capacity to decrease telomerase activity

Some oncogenic changes may active or upregulate telomerase and in these cases, cells with short telomeres may escape senescence and become immortal (Shay, 2016). Telomerase is a highly attractive target for cancer therapeutics. Melatonin can increase telomerase activity in normal cells. However, melatonin has the ability to attenuate telomerase activity in cancer cells and cause a significant reduction of tumors size and metastases (Leon-Blanco et al., 2003). In related *in vitro* studies, melatonin decreased telomerase reverse transcriptase activity and the RNA subunit in a dose-dependent manner (Leon-Blanco et al., 2003).

3.3.1.5. Antimetastatic effects

Although the vast majority of studies of oncostatic role of melatonin put emphasis on tumor development and progression at the primary site, mounting evidence indicates that melatonin can also counteract tumor metastases.

Downregulation of E-cadherin expression is a decisive disruptor of epithelial homeostasis, increased tumor formation, and augmenting tissue invasiveness (Craene and Berx, 2013). Melatonin can increase E-cadherin expression in cancer cells to a lower invasive status (Cos et al., 1998; Wu et al., 2016).

Tight junction is a kind of cell-to-cell adhesion complexes which has a crucial role in maintaining cell integrity. Alterations in the expression and/or distribution of TJ proteins can make cancer cells to become invasive and then ultimately result in metastasis (Su et al., 2017). Melatonin up-regulates the expression of occludin, a transmembrane protein found in the TJ, and enhances occludin to locate on the cell surface in different cancer cells such as lung adenocarcinoma cell line (Zhou et al., 2014).

Melatonin exhibits versatile regulatory actions on matrix metalloproteinases gene expression, which could degrade extracellular matrix to liberate growth factors and promote metastatic spread. Melatonin induce a significant reduction in pro- and active MMP-9 activity, which is associated with cancer invasion and metastasis (Rudra et al., 2013). Furthermore, Akt-mediated JNK (Jun N-terminal kinases) 1/2 and ERK (extracellular signal–regulated kinases) 1/2 signaling pathways were also involved in

melatonin-regulated MMP-9 transactivation and cell motility (Lin et al., 2016). Melatonin can also inhibit cancer cell migration via attenuation of MMP-9 expression and activity mediated by decreasing histone acetylation (Yeh et al., 2016).

Epithelial–mesenchymal transition (EMT) is a morphogenesic process in which epithelial cells shift toward the mesenchymal state and become migratory. EMT is a mechanism to generate cancer stem cells endowed with an invasive and metastatic phenotype (Han et al., 2013). Melatonin impedes the EMT process and cancer cell dissemination through interference with NF-κB signaling (Su et al., 2017; Wu et al., 2016). Mao et al. (2016) found that both endogenous and exogenous melatonin can significantly repress invasive/metastatic phenotype in breast cancer cells by inhibiting Rsk (ribosomal s6 kinase) 2. Rsk2 is a key signaling node induced and activated by the Her (human epidermal growth factor receptor) 2/Mapk/Erk pathway, which promotes numerous downstream signaling pathways associated with the development of EMT

3.3.1.6. Modulation of the immune response

Melatonin has been reported to stimulate the production of interleukin (IL)-2, interferon (IFN)-γ and IL-6 by human peripheral blood mononuclear cells as well as activate human monocytes to produce IL-1 and IL-12.

Melatonin administration also results quantitative and functional enhancement of natural killer (NK) cells (Genovese et al., 2005). NK cells are well-established killers of virus-infected cells and a wide variety of tumor cells. In addition to direct

cytotoxicity, NK cells can also regulate the anti-tumor adaptive immune response. The action of melatonin on NK cells may be through increased IL-2 production via melatonin receptors in T-helper cells and this IL-2 serves as an exquisite stimulant of NK cells (Miller et al., 2006). Considered of the ability of melatonin to modulate immune cell production and function, this might be exploited to boost the efficacy of cancer vaccines in the future.

3.3.2. Melatonin as an adjuvant in the treatment of cancer

Melatonin has a number of properties which are not limited to antitumor effect (Acuña-Castroviejo et al., 2014; Ortiz et al., 2015b; Reiter et al., 2015). Melatonin has anxiolytic and antidepressant ability which underline its possible application in depression and anxiety of cancer patients. Melatonin can be served as an effective adjuvant of chemotherapy and radiotherapy to increase the efficacy of treatment and decrease side effects. Taken melatonin as an adjuvant could effectively lower the threshold for chemotherapy or radiotherapy and protect normal tissues from being sensitized to the cytotoxicity of the therapies (Abdel Moneim et al., 2017; Fernández-Gil et al., 2017a; Ortiz et al., 2015b). In most of the combination trials where melatonin was used in conjunction with therapeutic drugs, the presence of melatonin was found to prolong disease progression-free time and overall survival as well as improve patient suffering (Jung and Ahmad, 2006; Tahamtan et al., 2015; Yun et al., 2014). The concomitant administration of melatonin significantly reduces the frequency of thrombocytopenia, neurotoxicity, cardiotoxicity, stomatitis and asthenia (Lissoni et al., 1999). Moreover, chemotherapy is better tolerated in patients treated

with melatonin ans melatonin can enhance the immunological activities of patients (Lissoni et al., 2003).

Melatonin alone is able to induce a significant increase of disease stabilization and survival time in patients with untreatable metastatic solid tumors (Lissoni et al., 2008).

II. HYPOTHESIS AND OBJECTIVES

Aberrant Akt activation is a widespread oncogenic phenomenon. PI3K/Akt/mTOR pathway is also frequently activated in cancer. The anticancer activity of mTOR inhibitor rapamycin is demonstrated by innumerable scientific and clinical studies. However，when rapamycin analogues are investigated for HNSCC treatment, these drugs show poor tolerability and treatment leads to therapy resistance. These counterintuitive observations are mainly caused by the negative feedback loop from S6K1 to Akt signaling. Losing the feedback inhibition of Akt because of rapamycin treatment may actually increase the survival of cancer cells and even result in more severe tumors. Besides, in clinical trials of mTOR inhibitors, a number of adverse events have been identified and among them, mucositis is regarded as the most frequent one, leading to dose reduction or interruption of therapy.

Melatonin is effective in suppressing neoplastic growth in a variety of tumors. Melatonin exerts direct anticancer actions by inhibiting the proliferation and growth of tumor cells. Melatonin can also induce both intrinsic and extrinsic apoptotic pathway in cancer cells. Notably, melatonin is shown to downregulate the phosphorylation of mTOR and Akt in various cancers. Besides, melatonin has a number of properties which are not limited to antitumor effect. Melatonin has anxiolytic and antidepressant ability which underline its possible application in depression and anxiety of cancer patients. The free radical scavenging/antioxidant properties of melatonin can also reduce side effects of different therapies. Our previous work presented a melatonin gel that can prevent mucosal disruption and ulcer formation.

In light of these properties, we hypothesize that melatonin could be potentially served as a combination anti-cancer agent to enhance the therapeutic effect of rapamycin and to minimize the toxic side effects of this drug in HNSCC.

The main objective of this project was to evaluate the interaction between melatonin and rapamycin in HNSCC. The effects of combination between rapamycin and different concentrations of melatonin were analyzed in head and neck cancer cell lines Cal-27 and SCC-9 *in vitro*, and xenografts mice *in vivo*.

The specifics objectives were:

Objective 1: To study the effects of melatonin on rapamycin-induced HNSCC cell death, and to identify the potential cross-talk pathways. We analyzed the dose-dependent effects of melatonin in rapamycin-treated HNSCC cell lines (Cal-27 and SCC-9). To elucidate this objective, we examined the relationships between inhibition of the mTOR pathway, ROS, and apoptosis and mitophagy

Objective 2: To study the potential synergistic effects of melatonin with rapamycin in Cal-27 xenograft mice. We investigated whether the combined therapy is more effective at inhibiting tumour growth compared to rapamycin treatment alone.

Objective 3: To measure the toxicity of rapamycin in liver, heart, kidneys and lung and examine the protective effects of melatonin on these heathy organs.

III. MATERIALS AND METHODS

1. CHEMICALS, CELL CULTURE AND TREATMENTS

Human squamous cell carcinoma lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cal 27 cells were maintained in Dulbecco's modified Eagle medium (DMEM), high glucose, glutamax supplemented with 10% fetal bovine serum (FBS), 2% antibiotic/antimycotic solution. SCC-9 cells were grown in a 1:1 mixture of Ham's F-12 and DMEM containing 0.5 mM sodium pyruvate, 0.4 μg/mL hydrocortisone (Sigma-Aldrich), 10% FBS, 1% L-glutamine, 2% antibiotic/antimycotic solution. All cell culture reagents were purchased from Gibco®-Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) unless otherwise indicated. The cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

1.1. PREPARATION OF MELATONIN AND RAPAMYCIN SOLUTION

Melatonin (Fagron Ibérica S.A.U., Terrasa, Spain) stock solution was prepared in 15% propilen glycol (PG) and filter-sterilized through a 0.2-μm pore filter (Sartorius Biotech GmbH, Gottingen, Germany). Melatonin was diluted to a desired concentration directly in the culture medium prior the experiments.

Rapamycin (Alfa Aesar, Ward Hill, MA, USA) was dissolved in ethanol (5μg/500μL) and stored at -20ºC. Rapamycin solution was freshly prepared by diluting the rapamycin stock in an aqueous solution of 5% Tween 80 and 5% polyethylene glycol and filter-sterilized through a 0.2-μm pore filter before use.

2. *IN VITRO* TREATMENT

Cells were grown to 60% to 70% confluence, serum-starved for 24 h, and treated with or without melatonin (0.1, 0.5, 1mM) every 48h. Cells were treated with or without rapamycin (20 nM) 1h after the second treatment of melatonin. After another 48 h, cells were harvested for further analysis (Figure 11).

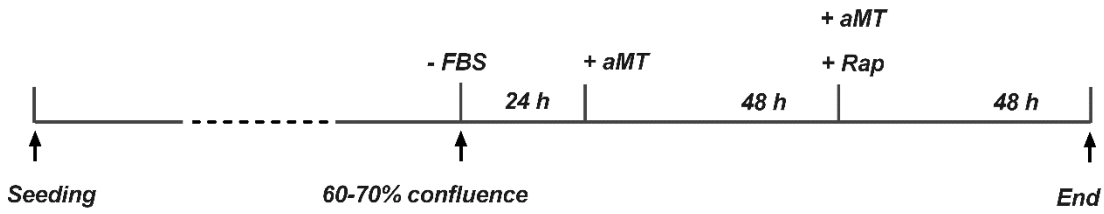

Figure 11. *In vitro* experimental model diagram.

3. ANIMAL STUDIES

Athymic (nu/nu) nude mice (Harlan Sprague-Dawley), 5 to 6 weeks old, housed in appropriate sterile filter-capped cages, fed and given water *ad libitum*, were used in the study. All experiments were conducted in accordance with the University of Granada's Ethical Committee; the Spanish Protection Guide for Animal Experimentation (R.D. 53/2013; and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123).

3.1. ESTABLISHMENT OF HNSCC TUMOR XENOGRAFTS

1) Cells were grown in complete medium and any contamination was excluded.

2) When cells were 70-80% confluent, we removed medium and wash cells with PBS. We detached the cells with trypsin-EDTA and we harvest them.

3) We immediately centrifuged at 1000 *g* for 5 min at 4℃.We counted cells in a Neubauer chamber using trypan blue staining to exclude dead cells.

4) Cells were resuspended in no-phenol red DMEM.

5) We cleaned and sterilized the inoculation area of the mice with ethanol.

6) We mixed cells and drawed the cells into a syringe without a needle. Needle causes a strong and negative pressure which can cause cell damage.

7) We injected 4×10^6 cells in 200 µL subcutaneously (s.c.) into the left flank of each mouse.

8) Animals were monitored twice a week for tumor development. Drug treatments were initiated when tumor volume reached 100 to 200 mm^3. Tumor diameters were measured with a digital caliper, and the tumor volume in mm^3 was calculated by the formula:

$$\text{Tumor Volume} = (\text{width})^2 \times \text{length}/2$$

3.2. *IN VIVO* TREATMENT

Rapamycin (1 mg/kg) was intraperitoneally (i.p.) injected every two days for ten days. To investigate the influence of melatonin, some mice also received s.c. injection of melatonin (300 mg/kg) one day before each rapamycin administration (Figure 12). We used a high dose of melatonin to reach mitochondria of cancer cells.

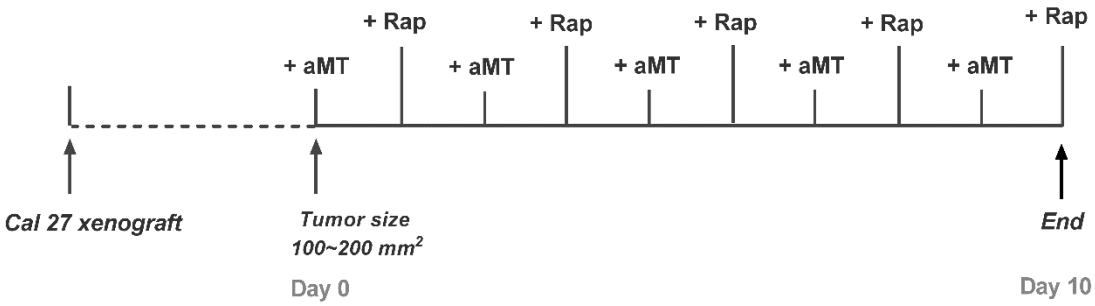

Figure 12. *In vivo* experimental model diagram.

Melatonin was dissolved in PG, and then diluted 1:2 in saline and filted through a 0.2-μm pore. Rapamycin was dissolved in DMSO, and then diluted in an aqueous solution of 40% PEG, 20% PG, and 5% Tween 80 in PBS and filter-sterilized through a 0.2-μm pore filter.

4. CELLULAR MORPHOLOGY, VIABILITY, AND COLONY FORMATION ASSAY

4.1. CELLULAR MORPHOLOGY

Cell morphology was observed under an inverted phase-contrast microscope, and

pictures were acquired using a camera (OlympusBX61, Tokyo, Japan).

4.2. VIABILITY

Trypan blue staining was performed to assess cell viability. Trypan Blue is a stain that is actively extruded from viable cells, but which readily enters and stains dead cells. Therefore, blue-stained cells were counted as dead cells and viable cells will be unstained. Trypan Blue was sterile filtered before using.

Procedure

1) Cells were trypsinized and resuspended in the complete medium.

2) We took a small sample (10 µL) of suspension cells and added 0.4% 2X trypan blue buffer.

3) We setted up a Neubauer chamber and cover slip. Immediately, we placed 10 µL of cell mixture on the Neubauer chamber.

4) Cells were counted under the microscope (Nikon Eclipse Ti, Tokyo, Japan) in four 1 x 1 mm squares of one chamber and the average number of cells per square was determined.

5) Calculations:

Cell viability (%) = Viable cells (unstained) / Total cells (stained and unstained)

4.3. COLONY FORMATION ASSAY

Colony formation assay or clonogenic assay is an *in vitro* cell survival assay

based on the ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Colony formation assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents.

Procedure

1) We harvested cells and plated an appropriate number of cells (800 and 1000 cells/well for Cal-27 and SCC-9 respectively) per well on a 6-well plate, at least in duplicate.

2) Cells were treated with melatonin alone at different concentrations. 48 hours after, the medium was changed and then treated again with melatonin and treated with rapamycin.

3) Colonies were allowed to grow for 2 weeks to form colonies of at least 50 cells each one, following the melatonin treatment each 48 hours.

4) The colonies were fixed with absolute ethanol and stained with crystal violet.

5) Pictures of each well were taken and then clones were counted with Image J software.

5. MEASUREMENT OF GSH AND GSSG LEVELS

Glutathione is a tripeptide that contains L-cysteine, L-glutamic acid and glycine. It can prevent cell damage caused by ROS such as free radicals and peroxides. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. An increased ratio of GSSG/GSH is an indication of oxidative stress.

GSH and GSSG were measured using an established fluorometric method (Hissin and Hilf, 1976) in a microplate reader microplate reader (Power-Wave$_x$ Microplate Scanning Spectrophotometer, BioTek Instruments, Inc., Winooski, VT). Both GSSG and GSH were measured using o-phthalaldehyde (OPA) as a fluorescent reagent. The reaction of GSH with OPA is at pH 8 and of GSSG with OPA is at pH 12. GSH is complexed to N-ethylmaleimide (NEM) to prevent interference of GSH with measurement of GSSG.

Procedure

1) Reagent preparation

a. We prepared GSH and GSSG solution stock (0.4 mg / mL) using 0.1 M phosphate buffer (PB)-0.005 M EDTA buffer (pH 8.0) and kept at -80ºC until use.

b. OPA solution was prepared daily just prior to use. We dissolved 1 mg OPA in 1 ml methanol.

c. NEM solution was prepared daily just prior to use. We dissolved 5 mg NEM in 1 ml MilliQ water.

2) Sample preparation

a. We harvested cells by centrifugation and resuspended in 50 μL of ice cold PB-EDTA Buffer.

b. We sonicated 3 times for ~10 sec to complete cell lysis. We took an aliquot for Bradford.

c. We added 1 volume ice-cold 10% (w/v) trichloroacetic acid (TCA) into 1 volume of sample and vortexed briefly to mix well.

d. We incubated on ice for 10 minutes.

e. We centrifuged samples at 20,000 × *g* for 15 minutes at 4°C and transferred supernatant to a fresh tube. We measured volume of supernatant.

3) We prepared GSSG standard by diluting the GSSG stock solution in 0.1 N NaOH and mixing well by vortex.

4) We prepared GSH standard by diluting the GSH stock solution in PB-EDTA buffer and mixing well by vortex.

5) *GSSG Assay*

a. We took 30 μL of supernatant, mixing with 12 μL of NEM. We incubated at room temperature for 40 min protected from light.

b. After the incubation, we added 58 μL of 0.1 N NaOH.

c. We added 45 μL mixture from step 5b and GSSG standard dilutions into a 96

well plate.

d. We added 145 µL of NaOH and 10 µL OPA to each well.

e. We incubated at room temperature for 15 minutes protected from light.

f. We measured it on a fluorescence microplate reader at Ex/Em= 350/420 nm.

6) GSH assay

a. We added 10 µL of supernatant from step 2 and GSH standard dilutions into a 96 well plate.

b. We added 180 µL PB-EDTA and 10 µL OPA to each well.

c. We incubated at room temperature for 15 minutes protected from light.

d. We measured it on a fluorescence microplate reader at Ex/Em= 350/420 nm.

6. MEASUREMENT OF GPX ACTIVITY

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxides (H_2O_2).

The measurement of activity of GPx is indirectly by a coupled reaction with glutathione reductase (GRd) which recycles of GSSG back to GSH and NADPH (b-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled

reactions.

$$R\text{-}OOH + 2\,GSH \xrightarrow{GPx} R\text{-}OH + GSSG$$

$$GSSG + NADPH + H^{+} \xrightarrow{GRd} NADP^{+} + 2GSH$$

Procedure

1) We collected the cells by centrifugation and homogenized cell pellet in cold buffer (50 mM Tris-HCl, Ph 7.5, 5 mM EDTA and 1 mM DTT).

2) We centrifuged at 10000 × *g* for 15 minutes at 4ºC.

3) We collected the supernatant for assay and store on ice. The samples were frozen at – 80ºC for at least one month.

4) We prepared the solution for reaction as following:

Solution A: 0.2 mM NADPH in buffer A (100 mM PB-1 mM EDTA pH 7.5).

Solution B: 4 mM NaN_3, 4 mM GSH 0.2 mM NADPH and 0.5 U/mL GRd in buffer A (100 mM PB-1 mM EDTA pH 7.5).

5) We prepared the 96 well plate as shown in the Table 1. Each sample had two replicates at least.

Table 1.

Non-enzymatic well	Sample well
10 µl of sample	10 µl of sample
240 µl Solution A	240 µl Solution B

Table 1. Reaction system used to measure GPx activity.

6) We incubated the plate for 5 minutes at 37º C).

7) We initiated the reaction by adding 10 µl cumene hydroperoxide to all the wells. We added the cumene hydroperoxide as quickly as possible.

8) We read the absorbance at 340 nm for three minutes on a Bio-Tek Power-Wave Microplate Scanning Spectrophotometer.

9) We calculated the GPx activity using the following formula：

GPx activity = (ΔA340/min × 26)/0.00622 × 1000 = ΔAbs × 83.6 µmol/min

Where： ΔA_{340}/min. = $\Delta A_{\text{sample well}}$/min. - $\Delta A_{\text{Non-enzymatic well}}$/min.

$\Delta A_{\text{sample well}}$/min. =A (time 2) - A (time 1)/Time 2 (min.) - Time 1 (min.)

0.00622 is the extinction coefficient for NADPH and 26 is the dilution factor.

7. MEASUREMENT OF LIPID PEROXIDATION (LPO) LEVELS

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) were assayed as a marker of lipid peroxidation (Esterbauer and Cheeseman, 1990).

Procedure

1) Reagent preparation

a. R1 solution: we prepared 0.26635% N-methyl-2-phenylindole in acetonitrile. We diluted it 3:1 in methanol immediately before use. We protected R1 solution from light.

b. R2 solution: methanesulfonic acid (MSA)

c. 100 mM MDA + 4-HNE standard stock was purchased from Bioxytech LPO-568 assay kit (OxisResearch, Portland, OR, USA).

2) Sample preparation

Tissue homogenate

a) We removed blood *in situ* by rinsing with ice-cold isotonic saline (i.e., 0.9% NaCl).

b) We weigh tissue and prepared tissue homogenate in Tris buffer (20 mM, pH 7.4).

c) After homogenization, we centrifuged the homogenate 3000 *g* at 4ºC for 10 minutes.

d) The supernatant was used for the assay. Aliquots were taken for sample replicates, as well as for protein determination. 200 µL from these samples were required to use in this assay. The supernatant were kept on ice prior to analysis, or, if not analyzed immediately after preparation, frozen at –80ºC for longer storage.

Samples of cells

a. We harvested and resuspended cells ice-cold Tris buffer (20 mM, pH 7.4).

b. We sonicated 3 times for ~10 *sec* to complete cell lysis.

c. We followed the steps c and d of the tissue procedure.

3) We prepared standard curve as Table 2.

4) We added 200 µL of sample to polypropylene microcentrifuge tube and each group were run in triplicate.

5) We added 650 µL of R1 reagent and mixed gently.

6) We added 150 µL R2 reagent, mixed well and stoppered the tube.

7) We incubated at 45°C for 40 minutes.

8) We centrifuged turbid samples (e.g., 15,000 × *g* for 10 minutes) to obtain a clear supernatant.

9) We transferred the clear supernatant to a 96-well plate (Table 2).

10) We measured absorbance at 586 nm.

Table 2.

Well	R1(µL)	H_2O (µL)	Standard Stock (µL)	Supernatant (µL)	R2 (µL)	Standard (µM/mL)
White	650	200	0	-	150	0
S1	650	200	0	-	150	0
S2	650	194	6.5	-	150	0.65
S3	650	187.5	12.5	-	150	1.25
S4	650	175	25	-	150	2.5
S5	650	150	50	-	150	5
S6	650	100	100	-	150	10
S7	650	50	150	-	150	20
Sample	650	-	-	200	150	-

Table 2. Amounts of reagent, standard stock and supernatant used for the preparation of the standard graph and measurement. Standard (µM/mL) is the final concentration for the standard graph. Each group should be run in triplicate.

8. MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

Free radical production by cultured cells can be measured using permeant reagent 2', 7' –dichlorofluorescin diacetate (DCF-DA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell. After diffusion into the cell,

DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively.

Procedure

1) The day before the experiment, we harvested treated cells and seeded them in a dark bottom 96-well microplate with 10000 cells per well. We allowed cells to adhere overnight.

2) We removed the media and washed the cells with PBS.

3) We added culture medium without phenol red (DMEM, high glucose, HEPES, no phenol red, 21063-029, Life Technologies).

4) We stained cells by adding 100 μM/well of DCF-DA solution.

5) We incubated cells with the DCF-DA solution for 30 minutes at 37°C in the dark.

6) We removed DCF-DA solution and then wash the cells with PBS.

7) We filled each well with Krebs-Ringer bicarbonate buffer and measured fluorescence immediately.

8) We measured the fluorescence on a fluorescence plate reader at Ex/Em= 485/535 nm for 45 minutes each 5 minutes.

9. MEASUREMENT OF MITOCHONDRIAL MASS

10-n-Nonyl-Acridine Orange (NAO) is an acridine orange derivative and is generally used as a fluorescent marker of the inner mitochondrial membrane in whole cells. NAO accumulation in the cell seems to be related to specific interactions with mitochondrial membrane proteins and/or lipids, such as cardiolipin, and is largely independent of mitochondrial membrane potential. The structure and integrity of mitochondria can be assessed using 10-N-nonyl-acridine orange.

Procedure

1) The day before the experiment, cells were trypsinized and re-plated at 40000 cells/well in a 96-well plate.

2) After cells were attached (overnight), we removed the medium carefully.

3) We washed cells with PBS and removed all the liquid in each well.

4) Cells were stained with 2.5 μM NAO (Invitrogen-Life Technologies, Madrid, Spain) for 30 min at 37°C in darkness.

5) After incubation, cells were washed with PBS and re-suspended in no-phenol red medium.

6) NAO fluorescence was measured at 485/538 nm (ex/em) in triplicate using the spectrofluorometer (BioTek Instruments, Inc., Winooski, VT).

10. MITOCHONDRIAL DNA QUANTIFICATION

Human mitochondrial DNA (mtDNA) was quantified by real-time PCR using a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Inc., CA, USA). We used primers and probes for the human 12S gene (mtDNA) and 18S (nuclear DNA, nDNA). The mtDNA values were normalized to nDNA values (mtDNA/nDNA ratio).

Procedure

1) We resuspended the cell pellet in 300 μL lysis buffer with Proteinase K. After vortex to completely resuspend cell pellets, we incubate them overnight at 37 °C.

2) We added an equal volume of phenol/chloroform and mixed well by inverting the tube until the phases are completely mixed.

3) We spun at max speed for 5 min at RT. We got a white layer (protein layer) in the aqueous- phenol/chloroform interface.

4) We carefully transferred the upper aqueous phase to a new tube.

5) To precipitate DNA, we added 2.5 or 3 volume of cold isopropanol.

6) We spun at max speed for 5 min.

7) We discarded the supernatant and rinse the DNA pellet with 1 ml 70% ethanol.

8) We spin at max speed for 5 min and carefully discarded the supernatant and air-dry the DNA pellet. We resuspended DNA in water.

9) To quantify the total cellular DNA content we used the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). PCR reactions were performed in a Stratagene Mx3005P qPCR System (Agilent Technologies) using primers and probe complementary to sequences of the nuclear gene 18 S (TaqMan Gene Expression Assays Hs99999901_s1, Applied Biosystems).

10) For the amplification of mtDNA content the following primers and probe, which are complementary to sequences of the 12 S gene: forward (5′CCACGGGAAACAGCAGTGATT3') and reverse (5'CTATTGACTTGGGTTAATCGTGTG A3') primers, and mtDNA probe (12S RNA Oligo 5'TGCCAGCCACCGCG3'), all of them purchased from Eurofins FisherScientific were used. For DNA amplification, reactions were performed as follows: 5 min denaturalization at 95°C, followed by 40 cycles of 1 *sec* at 95°C, 5 *sec* at 65°C and 6 *sec* at 72°C. Results shown are a summary of 3 independent experiments (in duplicate) and are expressed as a ratio of mtDNA/nDNA copy.

11. MITOCHONDRIAL RESPIRATION

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) as described in the figure 13 (Wu et al., 2007). The day before the experiment, treated cells were seeded in XF 24-well cell culture microplate (Seahorse Bioscience) at 8×10^4 cells/well and incubated overnight at 37°C in 5% CO_2

atmosphere. After that, assays were initiated by replacing the media with assay medium (Seahorse Bioscience) pre-warmed to 37°C and incubated for 60 *min* at 37° C allow the temperature and pH to reach equilibrium. The microplate was then placed into the XF24 instrument to measure OCR. Under typical *in vitro* cell culture conditions, the rate of OCR is an indicator of mitochondrial respiration (Shah et al., 2013). The final concentration and order of injected substances was as follows: 1 µM oligomycin, 1 µM FCCP, 1 µM rotenone, and 1 µM antimycin. We recorded an average of four readings for each cell line, which was normalized according to the number of cells.

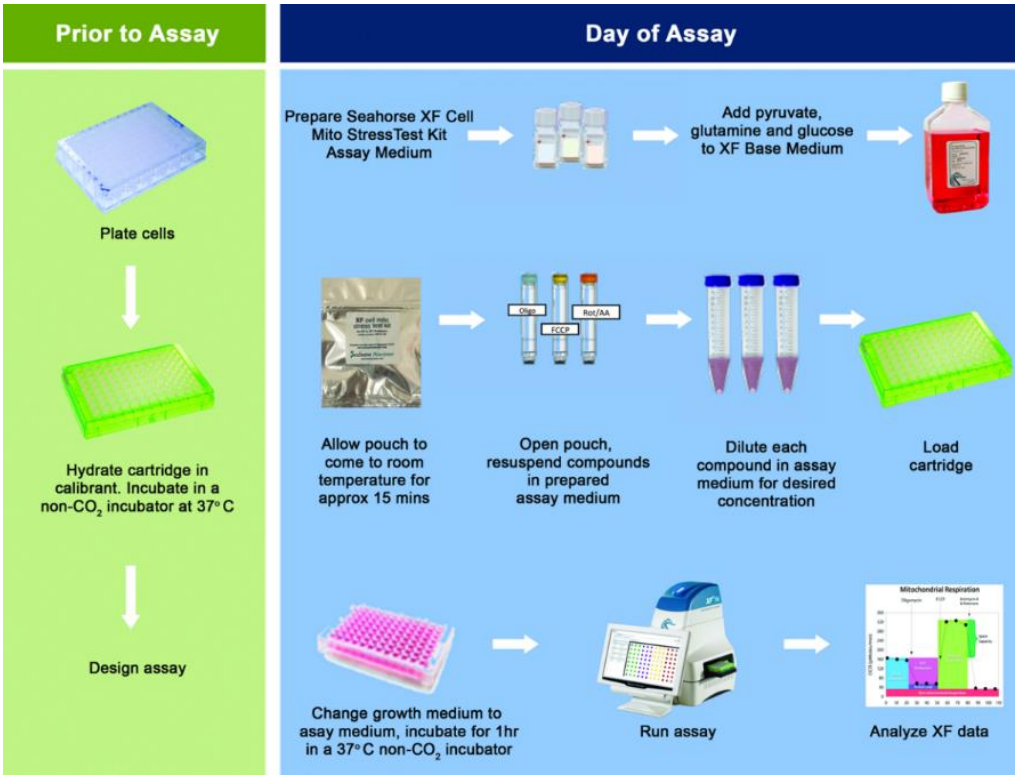

Figure 13. Agilent Seahorse XF Cell Mito Stress Test assay workflow.

12. WESTERN BLOT ANALYSIS

Adequate amounts of proteins were separated on 12.5% or 7% PhastGel homogeneous gels (GE healthcare life, Barcelona, Spain) and transferred onto Hybond™-ECL™ nitrocellulose membranes (GE healthcare life) using the PhastSystem (GE healthcare life). The membranes were incubated in blocking buffer (5% BSA in PBS plus 0.1% Tween 20) and then with the primary antibody diluted in blocking buffer overnight at 4 °C. The primary antibodies used in this study included GAPDH (1:200; Cat# sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA), Bax (1:200; Cat# sc-526, Santa Cruz Biotechnology), Bcl2 (1:200; Cat# sc-492, Santa Cruz Biotechnology), Total mitochondrial oxidative phosphorylation (OXPHOS) Rodent WB Antibody Cocktail (1:1000; Cat# ab110413, Abcam Inc., Cambridge, MA), Akt (1:1000; Cat# 4685, Cell Signaling Technology, Barcelona, Spain), Phospho-Akt (Thr308) (1:1000; Cat# 2965, Cell Signaling Technology), Phospho-Akt (Thr473) (1:1000; Cat# 4058, Cell Signaling Technology), P62 (1:1000; Cat# 18420-1-AP, Proteintech, Manchester, UK), NIX (1:1000, Cat# N0399, Sigma-Aldrich, Madrid, Spain), LC 3 (1:1000; Cat# NB100-2220, Novus, Abingdon, UK), Phospho-S6 (Ser235/236) (1:1000; Cat# 4856, Cell Signaling Technology). Anti-mouse (1:1000, BD Biosciences Pharmigen, San Jose, CA, USA), anti-rabbit (1:5000, Thermo Scientific, Madrid, Spain), and anti-goat (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) HRP-conjugated secondary antibodies were used according to manufacturer's instructions. The immunoreaction was detected using the ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life

Sciences). Plots were digitized on a Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA) and quantified using 1D Image Analysis software 3.6.

Procedure

1) Sample preparation

a. We prepared the homogenization buffer for cell as Table 3.

Table 3.

Buffer	Concentration	Volume
Tris-HCl pH7.6	50nM	4.8mL
DTT	7.7mg/mL	100µL
Coctail inhibitors	100×	50µL
EDTA	100×	50µL
Total	-	5mL

Table 3. Amounts of reagent and buffer used for the preparation of the homogenization buffer.

b. We added ~60 µL of homogenization buffer to each cell pellet.

c. We sonicated 3 times for ~10 *sec* to complete cell lysis.

d. We centrifuged at 3000 *rmp* for 20 min at 4°C.

e. We transferred the supernatant to a fresh tube, also kept on ice, and we discarded the pellet. We aliquoted the protein samples for long term storage at

–80ºC.

f. We prepared a small volume to determine the protein concentration.

g. We prepared 4× loading buffer (Laemmli buffer) as shown in Table 4.

h. We prepared 20 μg of each sample with 5 μL 4× loading buffer.

i. Samples were thermomixered 10 min at 99℃。

j. We performed the gel electrophoresis or storaged at -80ºC.

Table 4.

Buffer	Concentration
SDS	10%
2-mercaptoethanol	20%
Glycerol	20%
Bromophenol blue	0.004%
Tris-HCl	10 mM

Table 4. Amounts of reagent and buffer used for the preparation of the 4× loading buffer.

2) We prepared the gel compartment and placed the PhastGels on the separation bed.

3) We placed the PhastGel buffer strip holder over the gels and inserted the buffer strips into it.

4) Samples were applied to gels with PhastGel sample applicators.

5) We pressed ’SEP start/stop” and enter the programmed methods to start the run.

6) When the alarm sounded, we stopped it.

7) We transferred to HybondTM-ECLTM nitrocellulose membranes using

PhastTransfer Kit. Before transfering, we incubated the membranes and PhastGels in transfer buffer at least 10 *min*.

8) After the transferring, we stained the membrane with Ponceau Red solution to visualize protein bands. We marked the position of the molecular weight markers.

9) We blocked the membrane in fresh blocking buffer for 60 minutes at room temperature with constant agitation.

10) We diluted the primary antibody to the recommended concentration/dilution in fresh blocking solution.

11) We incubated the membrane in the primary antibody solution overnight at 4°C with agitation.

12) After the incubation, we washed the membrane three times for 5 minutes each time with PBS containing 0.05% Tween-20.

13) We incubated the membrane in the secondary antibody reagent of choice for 1 hour at room temperature with agitation.

14) We washed the membrane three times for 15 minutes each time with PBS containing 0.05% Tween-20.

15) We performed the detection of proteins using the ECLTM Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) on a Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY).

13. HISTOLOGY AND HISTOPATHOLOGY

Procedure

1) Animals were cut open below the diaphragm and the ribcage was cut to expose the heart.

2) Animals were transcardially perfused with a saline solution wash assisted by a peristaltic pump injecting it in the left ventricule and cutting off the right atrium to allow the blood flow out. We washed until liver began to blanch.

3) Then, we perfused with 4% formaldehyde. We visualized the animal's extremities for evidence of tremors resulting from the aldehyde-crosslinking of nerves and muscle. This is an indication that fixation is taking place.

4) We removed tumors and other organs carefully and stored them in 20 volumes of 4% formaldehyde for 24 hours at room temperature.

5) The fixed samples was then dehydrated, cleared, and embedded in paraffin following conventional protocols.

6) For morphological evaluation, we stained histological sections with hematoxylin-eosin. We used picrosirius (PS) stain to evaluate collagen fibre reorganization and periodic acid-Schiff histochemical stain (PAS) to confirm the mucin synthesis associated with the glandular-like zones of tumors.

14. MELATONIN DETERMINATION BY HPLC

Melatonin was extracted with trichloromethane, and the organic phase was evaporated to dryness (SPD 2010 Speed Vac System; Fisher Scientific, Madrid, Spain). The samples were analyzed by HPLC (Shimadzu Europe GmbH, Duisburg, Germany) with a Waters Sunfire C18 column (150 x 4.5 mm, 5 μm). After stabilizing the column with the mobile phase, samples (20 L) were injected onto the HPLC system at a 1-mL/min flow rate, and the fluorescence of melatonin was measured in a fluorescence detector (Shimadzu RF-10A XL fluorescence detector) with an excitation and emission wavelength of 285 and 345 nm, respectively. Retention time was 8.9 *min.* A standard curve for melatonin was used for calculates the amount of melatonin in samples according to the peak area.

Procedure

1) We dissected the tumor tissue and it was frozen in liquid nitrogen as soon as possible.

2) We placed the tissue in round bottom microfuge tubes

3) We added ~1 mL MiliQ water to the tube and homogenize the tissue with an electric homogenizer. We centruguged the homogenate at 3000 x *g* for 10 *min* at 4 ^{o} C . We took the supernatant and made aliquot for Bradford.

4) To perform the extraction we used dry tubes washed with 1 ml of chloroform and shook in a thermomixer at 1400 *rpm* for 10 *min*.

5) We added 1 ml of chloroform, 450 µl 0.1 M acetate / acetic buffer and 450 µL of sample to each clean tube.

6) We incubated the tube at 15ºC in Thermomixer at 1400 *rpm*, and then we centrifuged at 11000 x *g* for 10 *min* at 4 ° C.

7) We removed aqueous phase and membrane (use curved clamps), and we then added 500 µl 0.1 N NaOH.

8) We incubated the tube at 15ºC in Thermomixer at 1400 rpm, and then we centrifuged at 11000 *g* for 10 *min* at 4 ° C.

9) We removed aqueous phase and transfer the organic phase to a new tube and not its volum.

10) We removed all the solvent and got the dry melatonin using Speedvac for 45 *min*.

11) We measured content of melatonin by HPLC with a 150 × 4.5 mm Waters Sunfire C18 5m column.

15. STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 6 Scientific software (GraphPad Software, Inc., La Jolla, CA), using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post test. The data were expressed as the mean ± SEM of a minimum of three independent experiments. A P

value of < .05 was considered to indicate statistical significance.

IV. RESULTS

1. MELATONIN INHIBITS THE RAPAMYCIN-INDUCED FEEDBACK ACTIVATION OF AKT IN HNSCC

We first investigated how melatonin might influence the effects of rapamycin. To this end, we analysed mTOR pathway activation following melatonin treatment, with or without rapamycin, in the HNSCC cell line Cal-27. The cells were treated for 48 h with 0, 0.1, 0.5, or 1 mM melatonin and/or 20 nM rapamycin. Then we performed western blotting to evaluate the downstream target of mTORC1, ribosomal protein S6 (phospho-S6, p-S6). Rapamycin alone significantly decreased S6 phosphorylation in Cal-27 cells, whereas such phosphorylation was not affected by melatonin alone (Figures 14A, E). However, p-S6 was significantly reduced in all samples co-treated with rapamycin and any tested concentration of melatonin, indicating that melatonin did not interfere with rapamycin-induced mTOR activation.

We next measured Akt expression, which can be elevated via a feedback loop (Tennant et al., 2010). Rapamycin decreased the level of Akt phosphorylation at the Thr site (p^{T308}Akt; the mTORC1 phosphorylation site on Akt), in correlation with the reduction of p-S6 (Figure 14B, E). On the other hand, rapamycin increased Akt phosphorylation at the Ser site (p^{S473}Akt; the mTORC2 phosphorylation site on Akt) (Figure 14C, E). These results are consistent with previous findings that rapamycin increases pAkt (S473) levels in several human cancer cell lines (O'Reilly et al., 2006; Sun et al., 2005). Feedback activation of mTORC1 via phosphorylation of Akt at serine residue 473 by mTORC2 is considered a substantial limitation of mTORC1-specific inhibitors (Tennant et al., 2010).

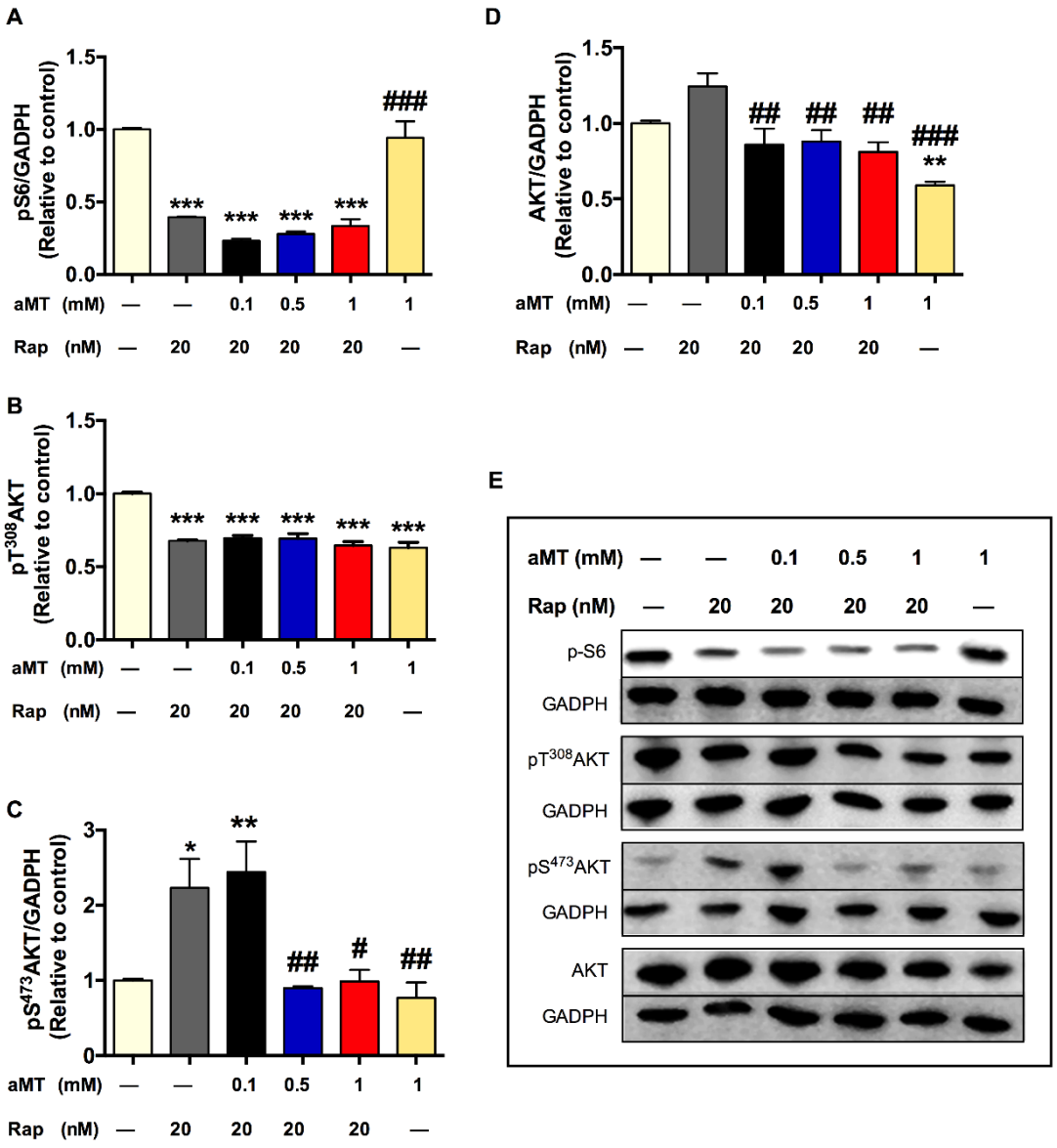

Figure 14. Combined treatment with melatonin and rapamycin modulated the mTOR pathway. Western blot analysis and densitometric quantification of pS6 (A, E), pT308Akt (B, E), pS473Akt (C, E), and Akt (D, E) in the HNSCC cells line Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

Therefore, the induction Akt phosphorylation is an unexpected and potentially undesirable consequence of mTOR inhibition. However, treatment with melatonin

alone reduced pAkt levels (both S473 and T308), and thereby attenuated the rapamycin-induced increase of p^{S473}Akt (Figure 14B–E). Consistent with the above results, treatment with a combination of the two agents significantly decreased total expression of Akt proteins compared to treatment with rapamycin alone (Figure 14D, E).

2. MELATONIN ENHANCES EFFECTS OF RAPAMYCIN, SENSITIZING CELLS TO TREATMENT

We next investigated the combined effects of melatonin and rapamycin on HNSCC cell viability using both Cal-27 and SCC-9 cells. Observation of morphological changes after the treatments revealed that melatonin administration resulted in cell detachment and cell shrinkage (Figure 15A, B).

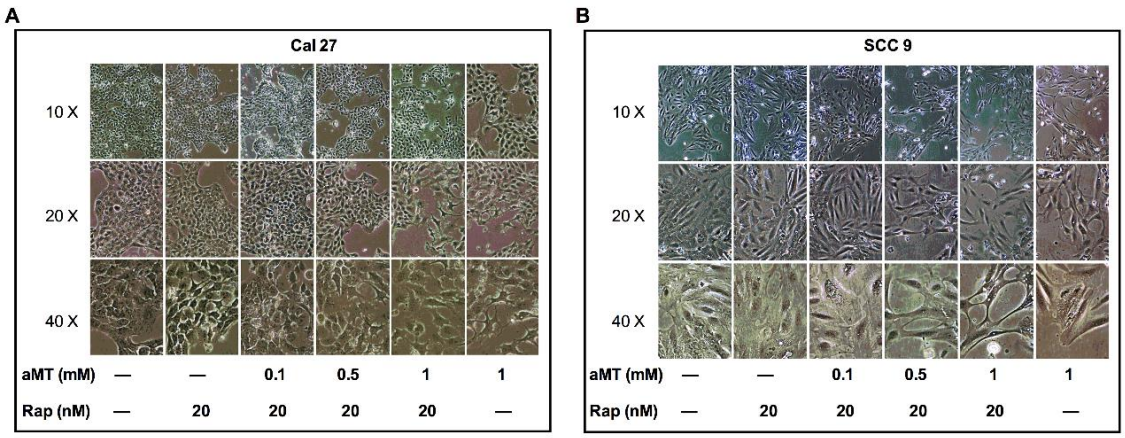

Figure 15. Morphological alterations in HNSCC cell lines Cal-27 and SCC-9. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. *n* = 6 per group.

Surprisingly, trypan blue staining revealed that rapamycin had no significant effects on the viability of either cell line (Figure 16A, B). However, melatonin decreased cell viability in a dose-dependent manner, with the maximal effect achieved at a concentration of 1 mM.

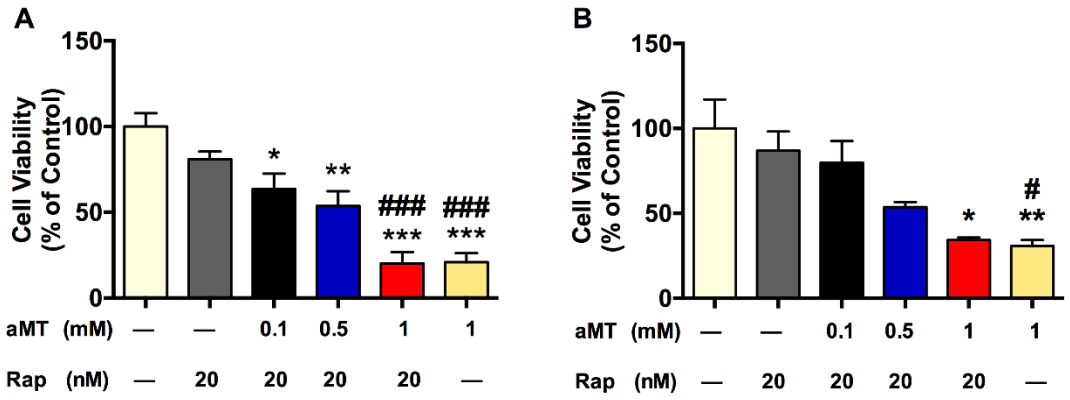

Figure 16. Cell viability in HNSCC cell lines Cal-27 and SCC-9. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and #P<.05, ###P<.001 vs. rapamycin-treated group.

Consistent with these results, both Cal-27 and SCC-9 cells lost their clonogenic capacity after treatment with higher concentrations of melatonin (Figure 17A–C). After three weeks, the surviving fraction of rapamycin-treated cells showed only a slight decline compared with controls. However, colony numbers were remarkably lower following melatonin treatment at all concentrations in Cal-27 cells, and particularly with treatment with 1 mM melatonin in SCC-9 cells. The clonogenic assay results confirmed that melatonin caused cell reproductive death. Together, our findings demonstrated that rapamycin had no detectable anti-clonogenic capacity in

HNSCC Cal-27 and SCC-9 cells. In contrast, the combination of melatonin and rapamycin exerted potent dose-dependent anti-proliferative effects in both HNSCC cell lines, although SCC-9 cells were more resistant than Cal-27 cells.

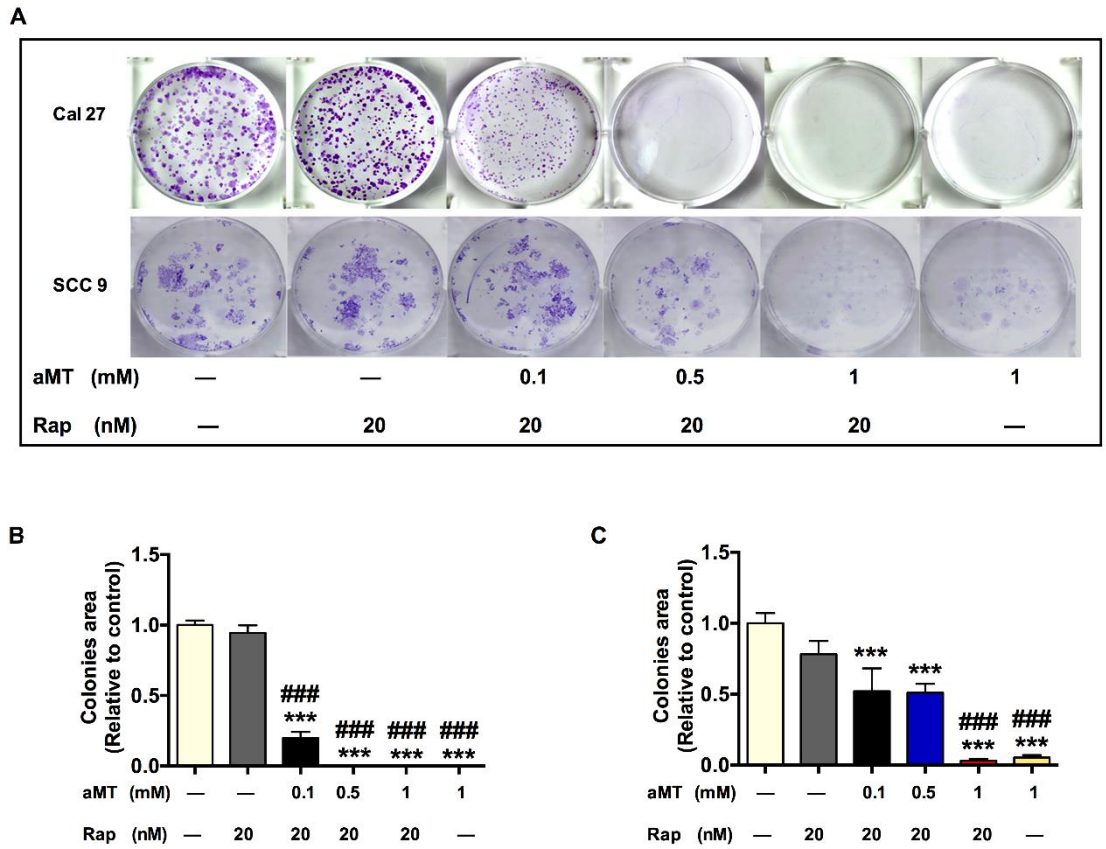

Figure 17. Clonogenic assay results in HNSCC cell lines Cal-27 and SCC-9. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. *n* = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and #P<.05, ###P<.001 vs. rapamycin-treated group.

Apoptosis pathway analysis generated similar results. Compared to control, combined treatment with rapamycin and melatonin at concentrations of 0.5 and 1 mM increased expression of the pro-apoptotic protein Bax (Figure 18A, D), and decreased

expression of the anti-apoptotic protein Bcl2 (Figure 18B, D), increasing the Bax/Bcl-2 ratio (Figure 18C). The Bax/Bcl-2 ratio did not indicate an increase in apoptosis pathway activation upon treatment with rapamycin alone or combined with a low concentration of melatonin (0.1 mM). Thus, only high melatonin concentrations (0.5 and 1 mM) enhanced the cytotoxic effects of rapamycin, leading to apoptosis in HNSCC cell lines.

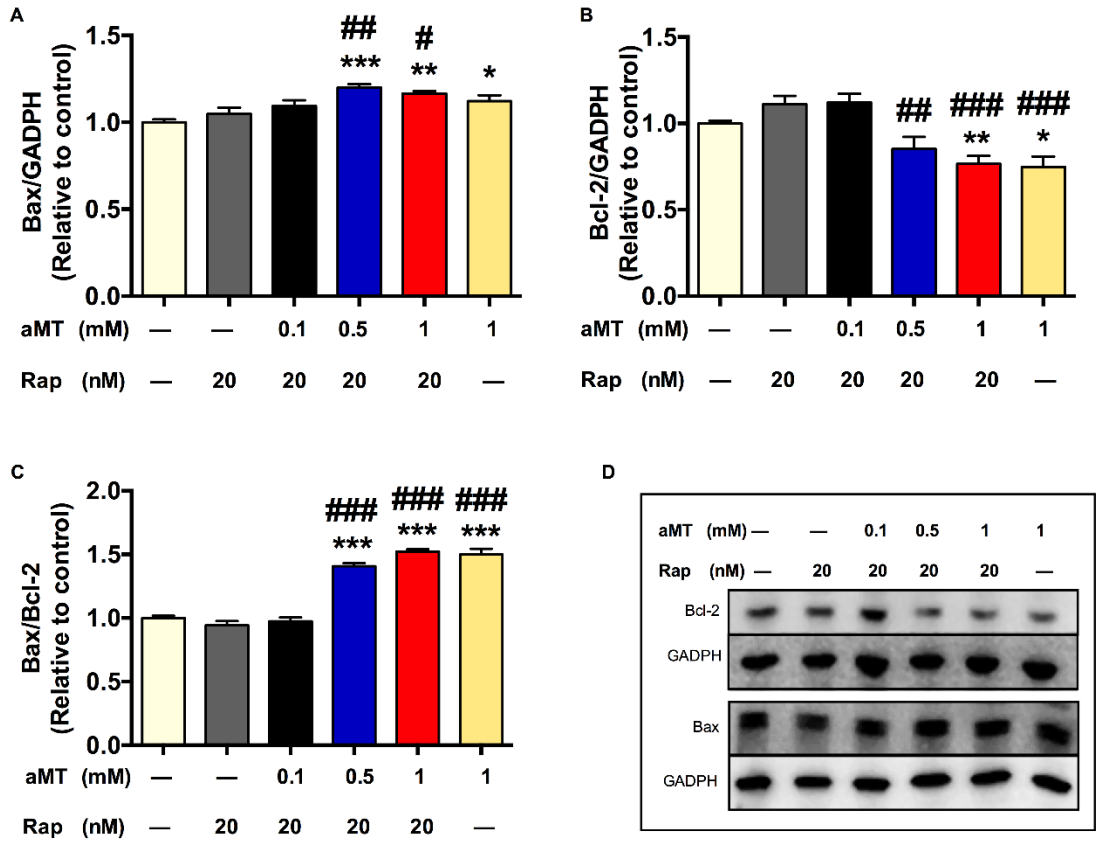

Figure 18. Combined treatment with melatonin and rapamycin synergistically induces apoptotic cell death. (A–C) Western blot analysis and densitometric quantification of Bax (A,D) and Bcl2 (B,D), and the Bax/Bcl2 ratio (C) in the HNSCC cell line Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

These results suggested that the tested cell lines showed *in vitro* resistance to rapamycin. Moreover, combining rapamycin with melatonin significantly decreased cell viability and colony number, and increased apoptosis in a dose-dependent manner. Thus, we demonstrated that rapamycin induced Akt activity, and that melatonin inhibited this induction. Notably, mTOR blockade in HNSCC cells reduces the expressions of key molecules involved in cellular metabolism (Cheng et al., 2008), and mitochondria are the main target of melatonin (Acuña-Castroviejo et al., 2003). Therefore, we further explored the possibility that melatonin could enhance the oncostatic effects of rapamycin via other mechanisms impacting cellular metabolism.

3. MELATONIN DIRECTLY CONTROLS MITOCHONDRIAL FUNCTION, ENHANCING THE BIOLOGICAL EFFECTS OF RAPAMYCIN

The ability of melatonin to enhance the cytotoxic effects of rapamycin in HNSCC cells may be related to melatonin-induced changes in mitochondrial function. Previous studies show that rapamycin-induced inhibition of mTOR decreases mitochondrial capacity; however, the mechanism by which mTOR is targeted to the mitochondria remains unclear (Ramanathan and Schreiber, 2009). Thus, we next measured mitochondrial respiration capacity in Cal-27 and SCC-9 cells, and found that treatment with rapamycin alone resulted in reduced basal OCR in both Cal-27 (Figure 19A, C) and SCC-9 cells (Figure 20A, C).

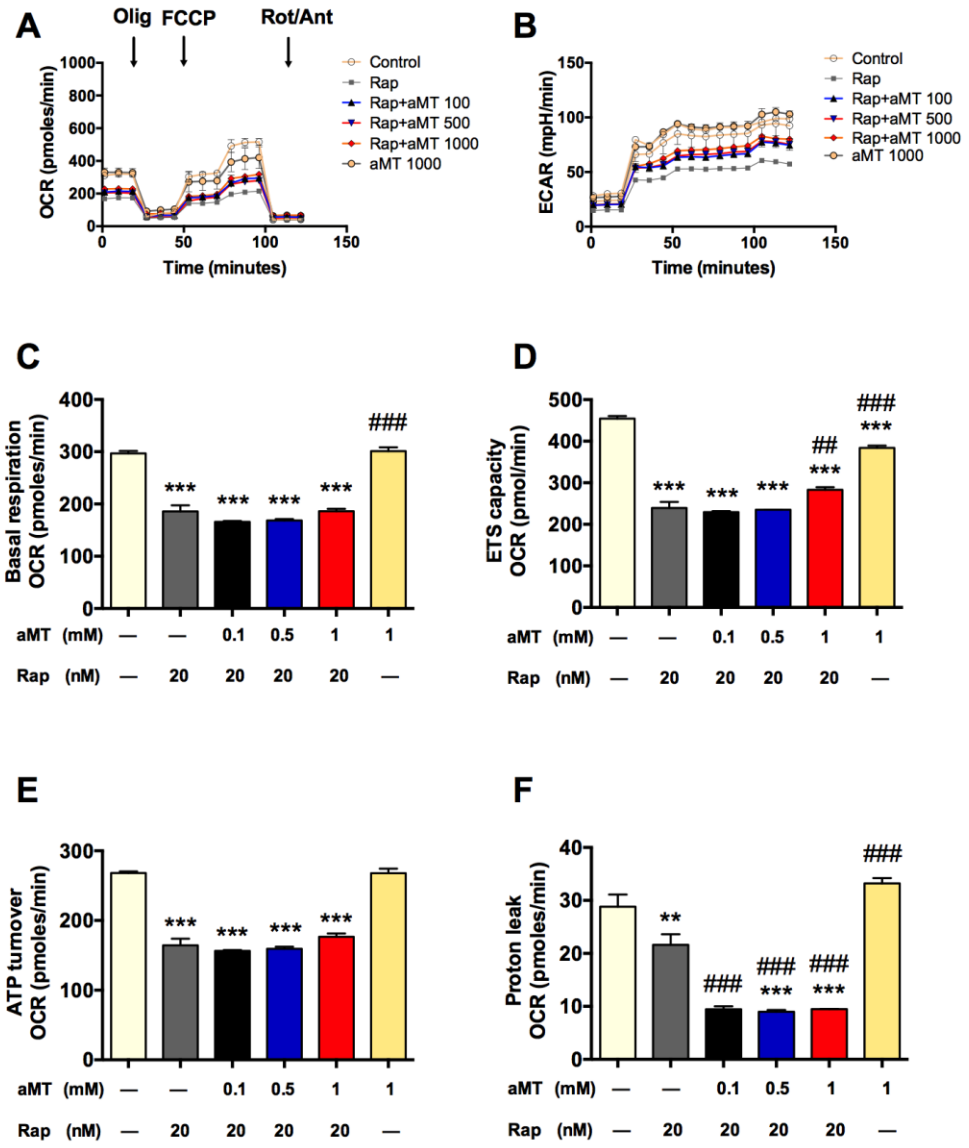

Figure 19. Effects of the combination of melatonin and rapamycin on mitochondrial respiration in Cal 27. Oxygen consumption rate (OCR) (A), extracellular acidification rate (ECAR) (B), basal respiration (C), maximal respiratory capacity (ETC) (D), ATP turnover (E) and proton leak (F) in the HNSCC cell lines Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

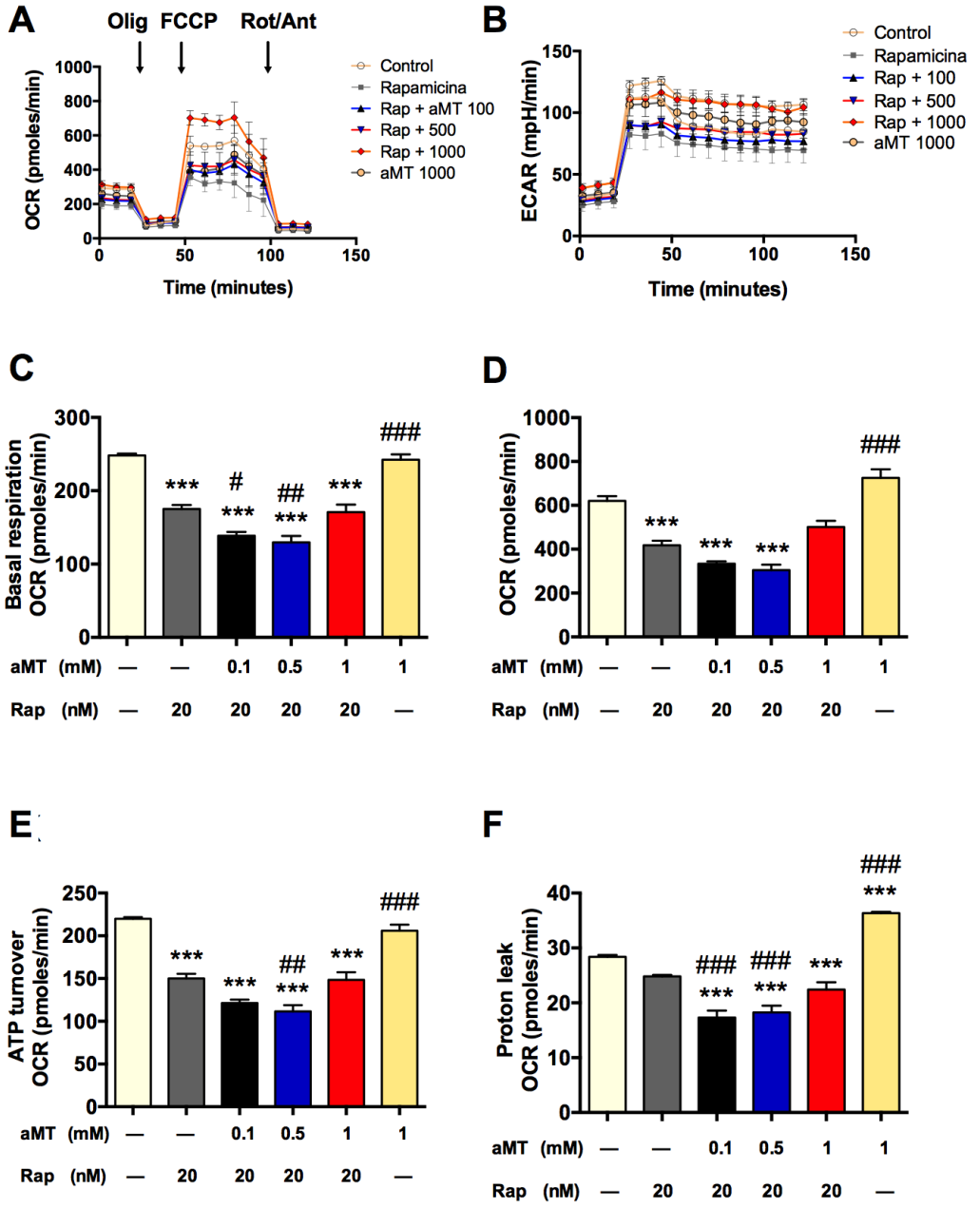

Figure 20. Effects of the combination of melatonin and rapamycin on mitochondrial respiration in SCC-9. Oxygen consumption rate (OCR) (A), extracellular acidification rate (ECAR) (B), basal respiration (C), maximal respiratory capacity (ETC) (D), ATP turnover (E) and proton leak (F) in the HNSCC cell lines SCC-9. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

We further conducted experiments to assess functionality of the electron transport chain in the presence of rapamycin. While measuring oxygen consumption rates, we sequentially added oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and a combination of rotenone and antimycin to the cells to assess the electron transport chain integrity. Upon addition of the ATP synthase inhibitor oligomycin, both cell lines showed decreased oxygen consumption while maintaining detectable respiration. This suggested the occurrence of ATP synthesis in conjunction with oxygen consumption, and that rapamycin did not uncouple energy production from the electron transport. The cells were then treated with FCCP to uncouple respiration, followed by treatment with a combination of rotenone and antimycin to inhibit complex I and III activity, respectively. As expected, FCCP addition resulted in elevated oxygen consumption to above basal levels, i.e., to maximal respiratory capacity (ETS). Addition of rotenone and antimycin completely inhibited mitochondrial respiration.

Our data showed that rapamycin decreased basal respiration, the electron transport chain (ETC), ATP turnover, and proton leak (Figure 19, 20). Together, these findings suggested that rapamycin-treated cells exhibited reduced capacity for oxidative phosphorylation, despite possessing an intact and functional mitochondrial electron transport chain. Moreover, rapamycin decreased ECAR compared to the basal control in both Cal-27 (Figure 19B) and SCC-9 cells (Figure 20B). These results clearly showed that rapamycin could decrease metabolic rate in HNSCC. Importantly, combined treatment with both rapamycin and melatonin induced a sustained reduction

in oxygen consumption (Figure 19, 20), and a marked decrease of proton leak. Melatonin also dose-dependently increased the ECAR values in both Cal-27 (Figure 19B) and SCC-9 cells (Figure 20B). Both cell lines showed greater proton leak with the combination of melatonin and rapamycin versus rapamycin alone. Interestingly, compared to in SCC-9 cells, Cal-27 cells showed a greater difference in proton leak between the groups treated with and without melatonin. These results were consistent with the greater resistance of SCC-9 to the combined treatment in terms of clonogenic capacity. Since rapamycin-induced mTOR inhibition decreased oxygen consumption and mitochondrial capacity in both Cal-27 and SCC-9 cells, and the results were more significant in Cal-27 cells, we used this cell line in subsequent experiments.

The results regarding respiration were in line with the observation that melatonin enhances the effects of rapamycin, in terms of decreasing the number of mitochondria or the number of functional mitochondria. To confirm this hypothesis, we next examined OXPHOS, mitochondrial mass, and mtDNA. Analysis of OXPHOS by western blot revealed that treatment with melatonin alone led to significant rises in the expressions of respiratory complexes I, III, and IV (Figure 21A, B). In contrast, treatment with rapamycin alone did not significantly alter the mitochondrial complexes. However, combined treatment increased the levels of complexes I and III compared to the control or treatment with rapamycin alone (Figure 21A, B), with the greatest effects observed at the highest melatonin concentrations (0.5 and 1 mM).

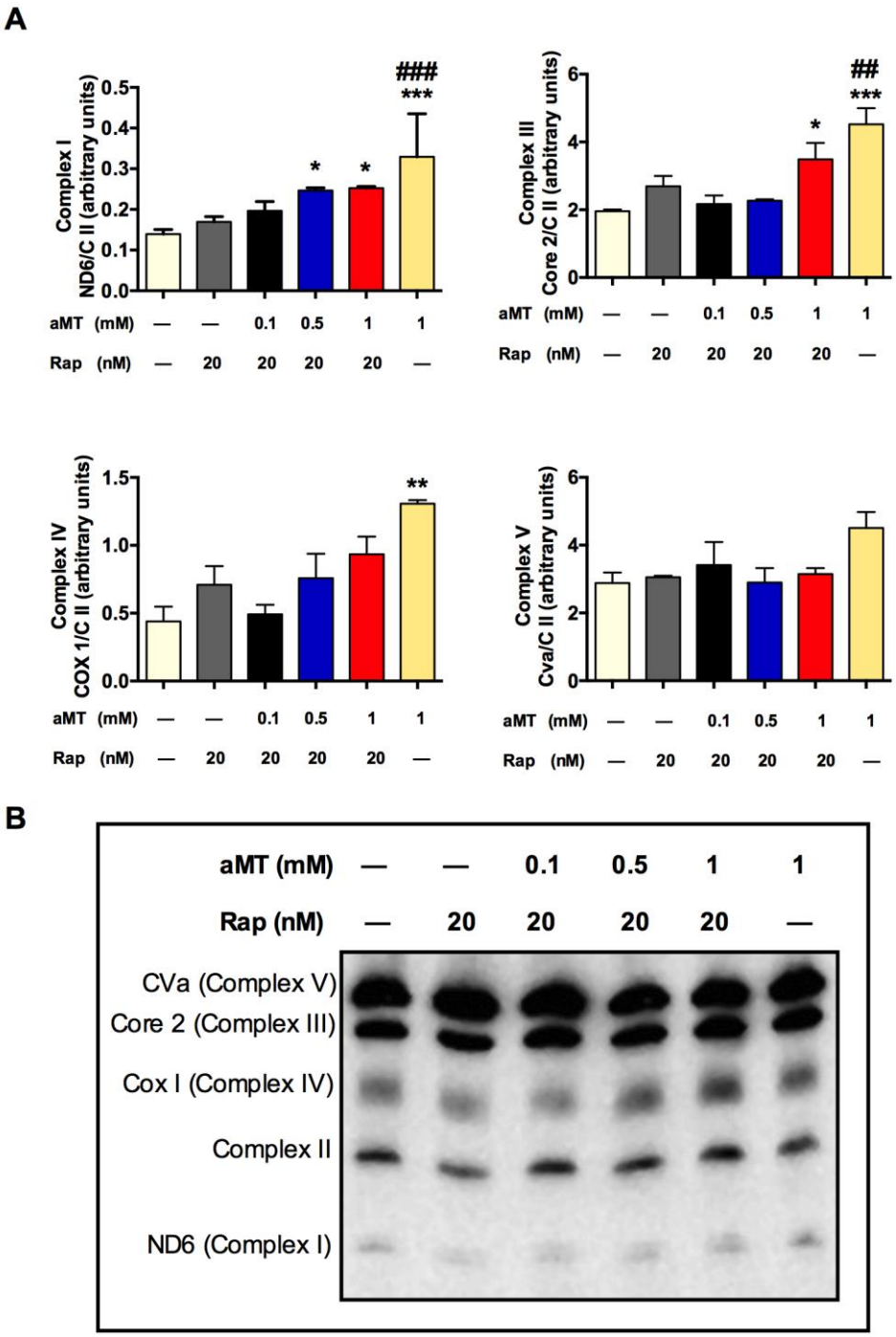

Figure 21. Western blot analysis and densitometric quantification of OXPHOS. (A): The intensities of the signals in (B) were analysed by densitometry. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM.. n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

To examine mitochondrial mass, we used acridine orange. Fluorescence data revealed that the combination of rapamycin and melatonin significantly increased mitochondrial mass compared to treatment with only rapamycin (Figure 22A). We also evaluated the synergism between melatonin and rapamycin by analysing mtDNA. The mtDNA/nDNA ratio indicated that combined treatment with rapamycin and 1 mM melatonin significantly increased mtDNA (Figure 22B). Overall, our results suggested that melatonin enhanced the cytotoxic effects of rapamycin by augmenting the number of dysfunctional mitochondria.

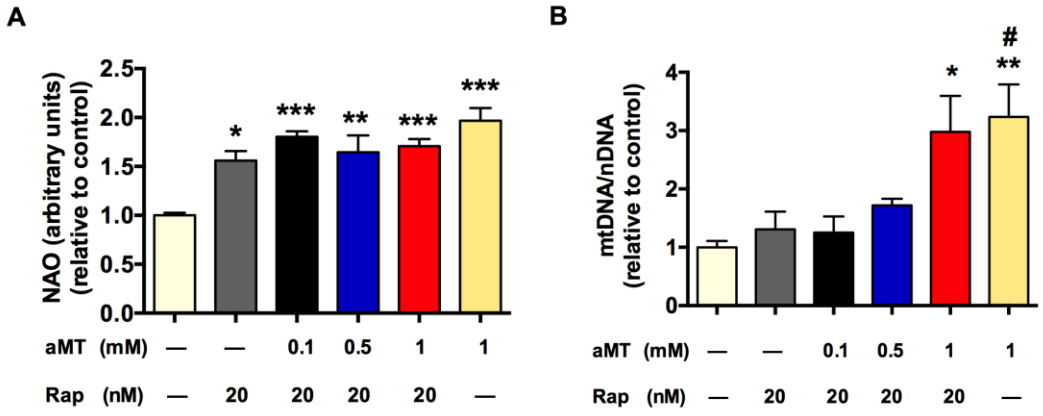

Figure 22. Melatonin promotes mitochondrial function in a dose-dependent manner. Mitochondrial mass (A), and mtDNA (B) in the HNSCC cell line Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM.. n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

4. MELATONIN AFFECTS REDOX HOMEOSTASIS IN HNSCC CELLS

Since melatonin reportedly produces intracellular ROS in tumour cells (Bejarano et al., 2011; Yun et al., 2016), we next analysed the redox homeostasis. Mitochondria or, more accurately, complex I and complex III of the ETC, are major sites of ROS production, and our results demonstrated that combined treatment with melatonin plus rapamycin significantly increased the expressions of complex I and complex III. As expected, combined treatment with rapamycin and melatonin significantly and dose-dependently elevated ROS generation compared with rapamycin alone. However, the enhancement of ROS generation by the melatonin/rapamycin combination was abrogated by the use of 1 mM melatonin (Figure 23A, C). Importantly, these results were consistent with analyses of LPO levels (Figure 23B, D), which also showed that oxidative stress was increased by treatment with rapamycin along with 0.1 or 0.5 mM melatonin, but decreased when using 1 mM melatonin.

Glutathione plays a central role in maintaining redox-homeostasis. The cycling of GSH participates in the process of converts H_2O_2 to H_2O and the GSSG/GSH ratio is regarded as a marker of oxidant stress. The GSSG/GSH ratio also confirmed the redox state that we observed from ROS and LPO generation analysis (Figure 24A). In agreement with these results, melatonin also dose-dependently suppressed the activity of antioxidant enzyme activity, such as GPx, with the most significant effect at 1 mM (Figure 24B).

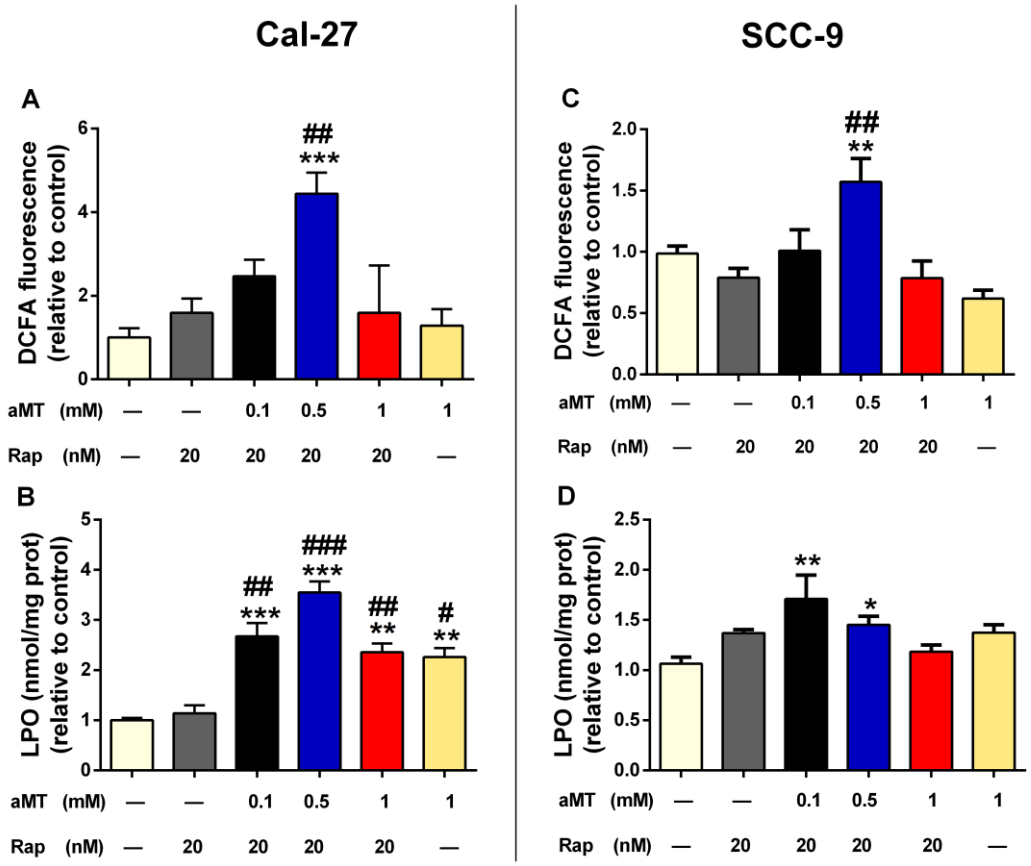

Figure 23. Melatonin induces oxidative stress in rapamycin-treated HNSCC. ROS generation in Cal-27 (A) and SCC-9 (C), LPO levels in Cal-27 (B) and SCC-9 (D). Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM.. n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

These experimental findings supported the possibility that melatonin enhances the effects of rapamycin via inhibition of Akt pathways, and induction of oxidative stress. However, treatment with 1 mM melatonin increased apoptosis and induced the loss of mitochondrial function. To more clearly define the involvement of mitochondrial injury in melatonin/rapamycin-induced cell death, we next analysed mitophagy.

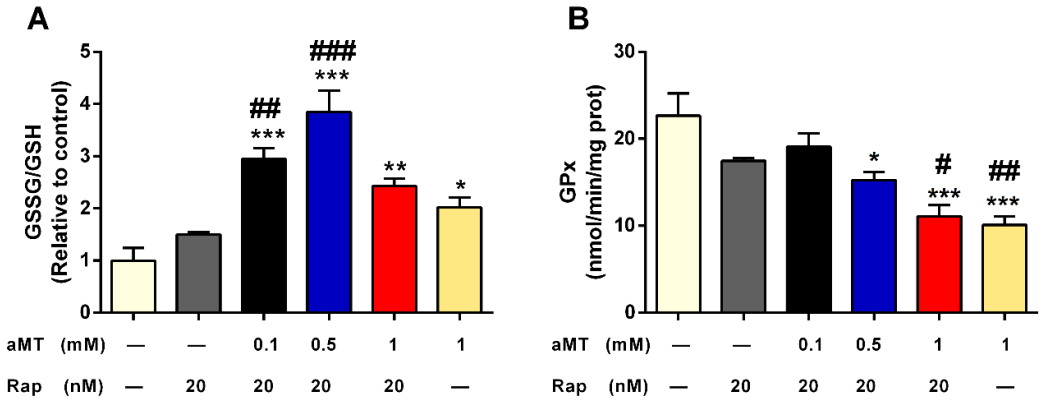

Figure 24. Melatonin regulates GSSG/GSH ratio (A), and GPx activity (B) in the HNSCC cell line Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. n = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and #P<.05, ##P<.01, ###P<.001 vs. rapamycin-treated group.

5. MELATONIN INDUCES MITOPHAGY

Mitophagy can facilitate the death of tumour cells due to excessive removal of mitochondria (Sentelle et al., 2012). We hypothesized that melatonin may induce apoptosis as well as autophagy in HNSCC cells (Sentelle et al., 2012).

To test this hypothesis, we determined the levels of the mitophagy-related proteins LC3-II and NIX (Figure 25). Sequestosome 1 (SQSTM1, p62) is an ubiquitin-binding protein that interacts with the autophagosomal membrane protein LC3 during mitophagy. Lysosomal degradation of autophagosomes leads to a drop in p62 levels. After treatment with higher concentrations of melatonin, we observed a significant

decrease of p62 (Figure 25B, D) and the formation of LC3 II (Figure 25A, D). Melatonin also induced an accumulation of NIX (Figure 25C, D). Together, these data indicated that combined treatment resulted in mitophagy, especially with a 1 mM concentration of melatonin.

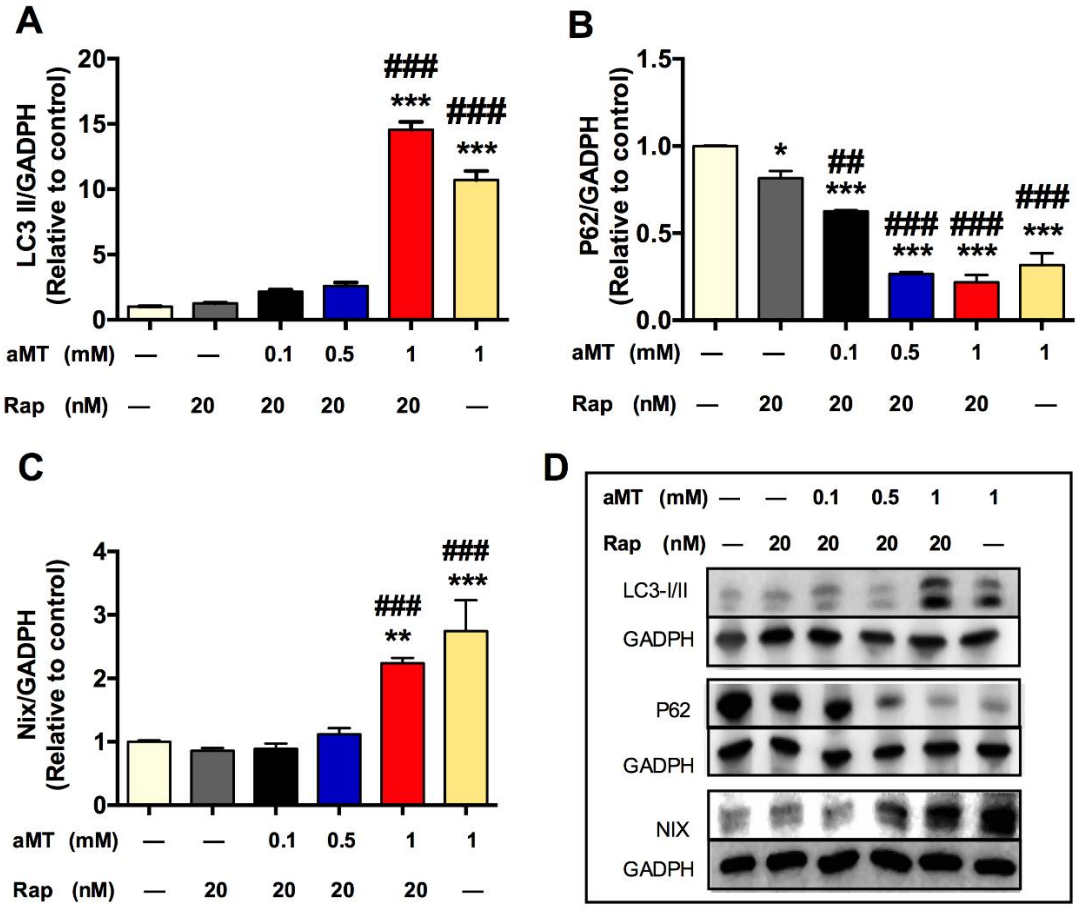

Figure 25. High melatonin concentration induces mitophagy in rapamycin-treated HNSCC. Western blot analysis and densitometric quantification of LC3 (A, D), P62 (B, D), and NIX (C, D) in the HNSCC cell line Cal-27. Treatment groups include control (vehicle), rapamycin 20 nM, melatonin (aMT) 1 mM, and rapamycin plus aMT 0.1, 0.5, or 1 mM. Western blots were quantified by densitometry. n = 6 per group. Data are presented as mean ± SEM. *P<.05, **P<.01, ***P<.001 vs. control, and ##P<.01, ###P<.001 vs. rapamycin-treated group.

6. EFFECT OF COMBINED TREATMENT WITH MELATONIN AND RAPAMYCIN IN HNSCC XENOGRAFTS

Based on the effects of combined rapamycin and melatonin treatment *in vitro*, we investigated whether this combination therapy was more effective at inhibiting tumour growth compared to rapamycin treatment alone. For this purpose, athymic nude mice were injected with Cal-27 cells. Tumour-bearing mice were randomly divided into three groups: untreated control group (injections of solvent alone), rapamycin treatment, and treatment with both rapamycin and melatonin (300 mg/kg). Both rapamycin treatment alone and combined treatment with melatonin and rapamycin resulted in similar significant reductions of tumour volume compared to control mice (Figure 26A, B).

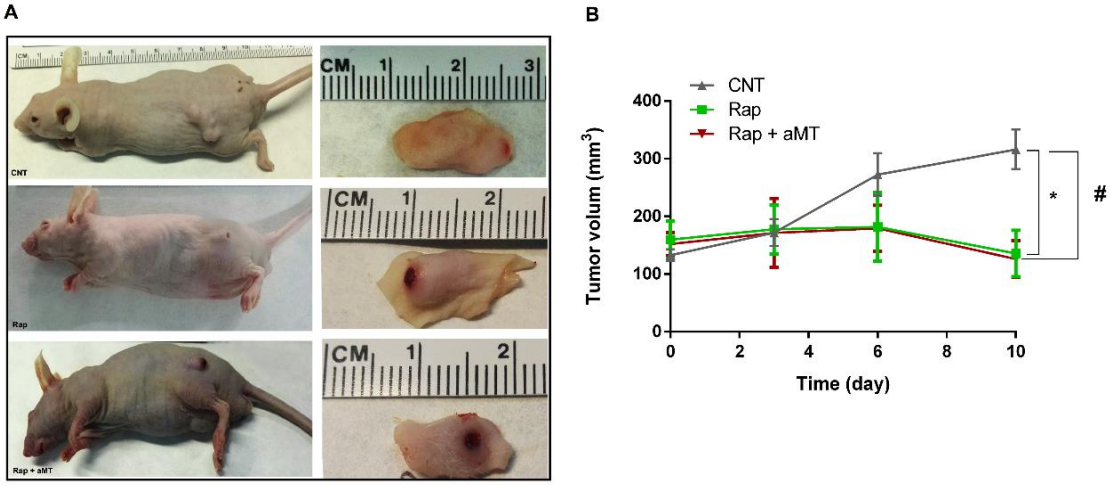

Figure 26. The combination of melatonin and rapamycin affected tumour growth in a xenograft model of HNSCC cancer. Representative pictures show tumours from xenograft mice (A), tumour growth (B). Treatment groups include control (mice with tumours treated with vehicle), mice treated with rapamycin (Rap), and mice treated with rapamycin plus melatonin at 300 mg/kg (Rap + aMT). n = 6 per group. Data are presented as mean ± SEM. *P<.05 vs. control, and #P<.05 vs. rapamycin-treated group.

This was in contrast to the expectation that the combined treatment would be more therapeutically effective than treatment with rapamycin alone, based on our above-described *in vitro* results. Thus, our *in vivo* results suggested that melatonin was not reaching the mitochondria at a sufficient concentration, even though the tumours showed a melatonin concentration of 87 ng/mg protein (Figure 27).

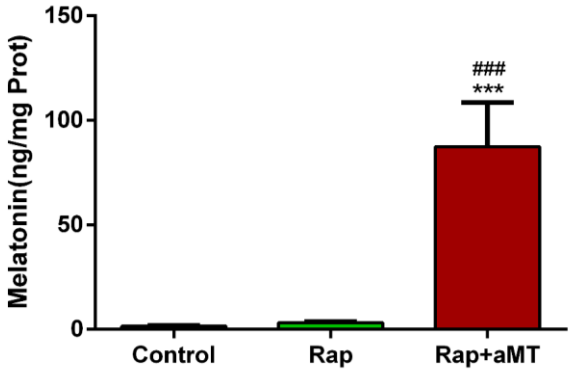

Figure 27. Tumour melatonin levels after treatment. Melatonin was accumulated in tumor cells after 10 days subcutaneous injection. Treatment groups include control (mice with tumours treated with vehicle), mice treated with rapamycin (Rap), and mice treated with rapamycin plus melatonin at 300 mg/kg (Rap + aMT). n = 6 per group. Data are presented as mean ± SEM. ***P<.001 vs. control, and ###P<.001 vs. rapamycin-treated group.

To further investigate the apparent discrepancy between melatonin's enhancement of rapamycin's effect *in vitro* and the effects of the combined treatment in HNSCC xenografts, we performed morphological evaluation with standard H&E staining and immunostaining. H&E staining revealed a high number of cells in mitosis in the controls (Figure 28A), while both treated groups had tumour cells that exhibited signs of apoptosis, characterized by cellular volume reduction (pyknosis), nuclear fragmentation (apoptotic bodies), and chromatin condensation (Figure 28B, C). However, the group treated with both melatonin and rapamycin exhibited significantly

greater accumulation of apoptotic bodies and more tumour cell necrosis (Figure 28C).

In all conditions, histological evaluation revealed a squamous cell carcinoma component mixed with a variable amount of PAS-positive pseudoglandular component, compatible with an adenosquamous carcinoma (Figure 28D–L). The three treatment conditions showed differences related to tumour differentiation, degree of inflammation, and encapsulation. The control was characterized by large and complex cystic cavities surrounded by solid and highly cellular tumour walls. Control tumour cells showed moderate-to-well differentiated squamous zones with horn pearl formation, and irregular pseudoglandular structures containing mucin (PAS-positive reaction) (Figure 28D–F). Additionally, the tumour interior exhibited tumour necrosis, which was surrounded by a discontinued collagen-based capsule (Figure 28D–F).

In the group treated with rapamycin alone, histological examination revealed large cystic areas, a well-differentiated squamous component, and PAS-positive areas consisting of pseudoglandular structures. Surrounding the tumour mass, we observed a delicate capsule and variable amounts of infiltrating lymphocytes (Figure 28G–I). Finally, in the group treated with rapamycin plus melatonin, histological examination revealed a well-differentiated squamous cell carcinoma with a pseudoglandular component (PAS positive) (Figure 28J–L). The cystic cavity was reduced relative to the other groups. Curiously, the tumour was mainly surrounded by a collagen-rich capsule (Figure 28J, K). Table 5 summarizes additional information related to other histopathological findings

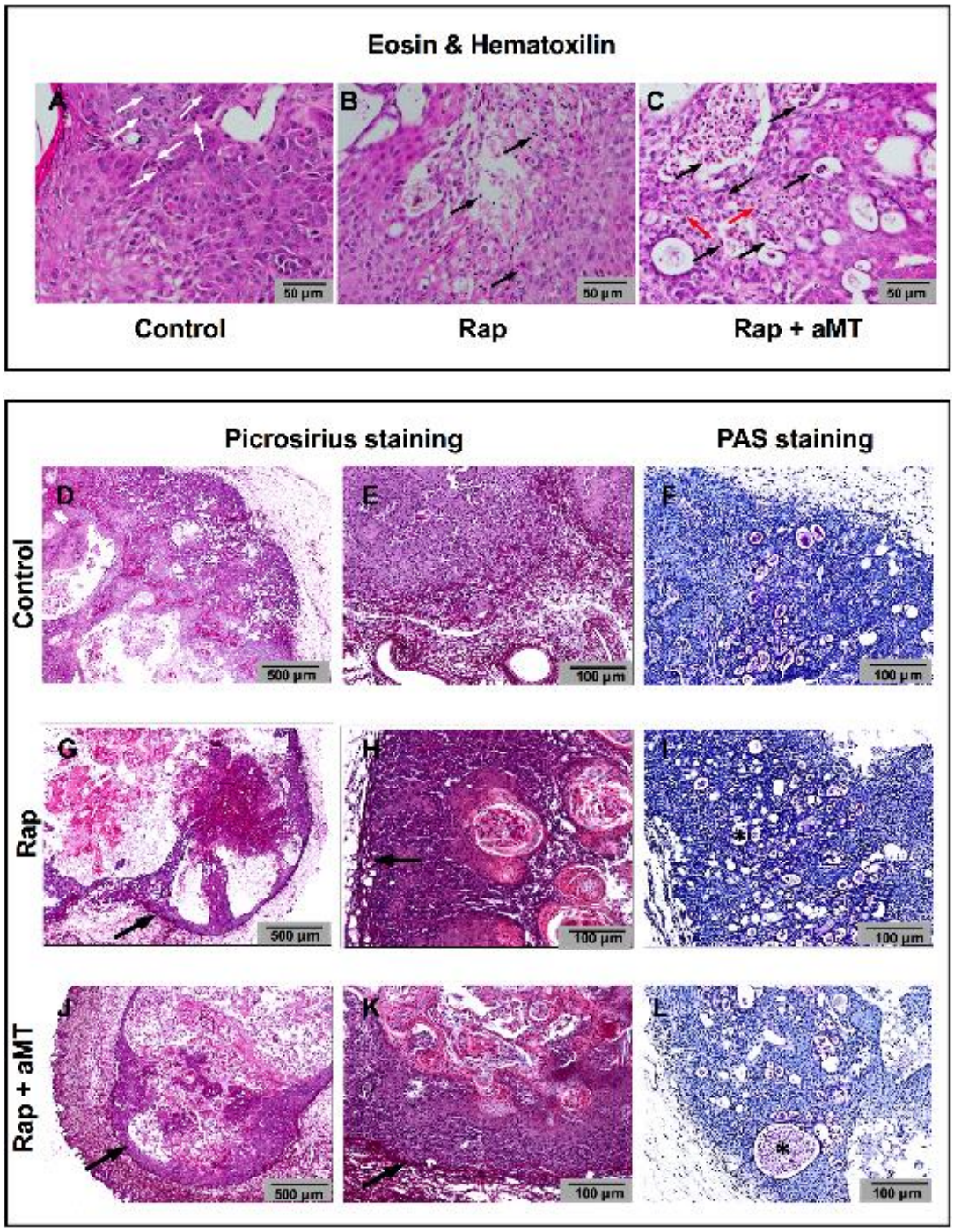

Figure 28. Histological and histochemical results. H&E stained tumour from the control group (mice with tumours treated with vehicle) (A), mice treated with rapamycin (Rap) (B), or mice treated with rapamycin plus melatonin at 300 mg/kg (Rap + aMT) (C). Mitosis is indicated by white arrows, apoptotic bodies by black arrows, and necrosis by red arrows. (D–L) Representative pictures of tumours treated with vehicle (Control) (D–F), rapamycin (Rap) (G–I), and rapamycin plus melatonin (Rap + aMT) (J–L). Black arrows indicate the capsule stained red by the picrosirius histochemical method, which is most evident in the Rap + aMT group (J,K). Asterisks indicate the pseudoglandular structures present in all groups.

Table 5

Histological features	Control	Rap	Rap+aMT
Squamous differentiation (0-+++)	++	+++	+++
Horn pearl formation	Yes	Yes	Yes
Glandular differentiation (0-+++)	+	+	+
Cyst formation	Yes	Yes	Yes
Capsule formation	Diffuse	Yes	Yes, Well-Formed
Stromal invasion	Yes	Yes	Yes
Perivascular invasion	Yes	Not Observed	Yes
Perineural invasion	Not Observed	Not Observed	Not Observed
Inflammation	Yes, moderate	Yes, abundant Peritumoral Infiltration of Lymphocytes	Yes, moderate
Necrosis	Yes (Tumoral)	Yes (Tumoral)	Yes (Tumoral)

Table 5. Summary of the histological elements present in tumours following treatment with vehicle (control), rapamycin alone (Rap), or rapamycin and melatonin (Rap + aMT).

7. MELATONIN PREVENTS OXIDATIVE STRESS AGAINST RAPAMYCIN-INDUCED TOXICITY IN NON-CANCER TISSUES, LUNGS, KIDNEYS, AND LIVER

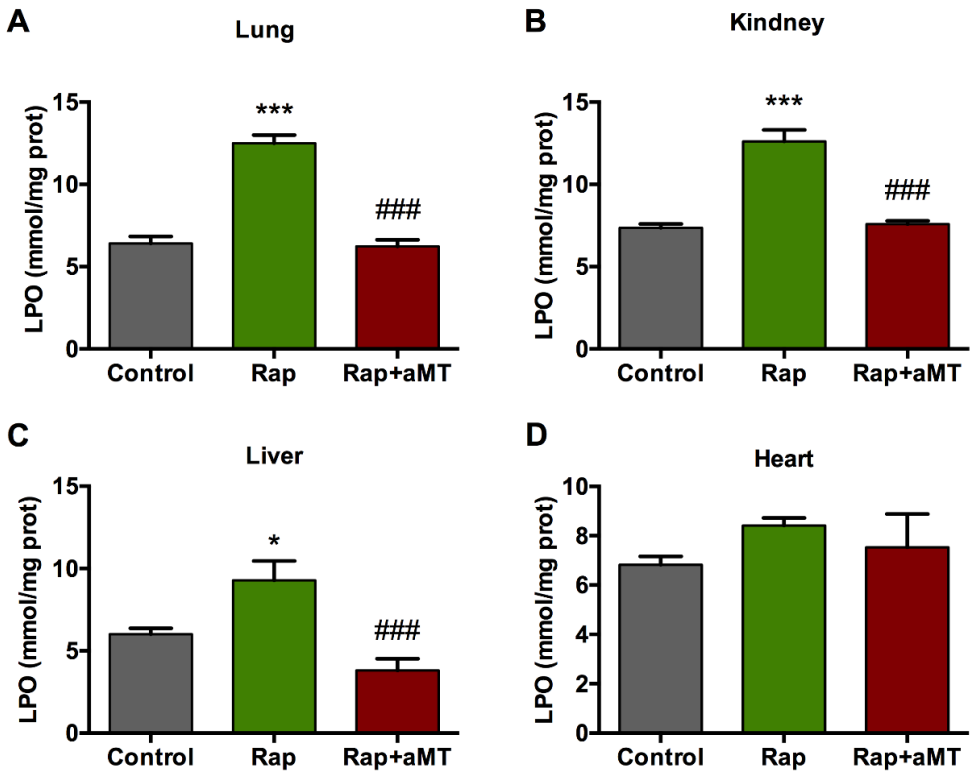

Figure 29. Melatonin prevented LPO in various organs from mice treated with rapamycin. LPO in lung (A), kidney (B), liver (C), and heart (D). Treatment groups include control (mice with tumours treated with vehicle), mice treated with rapamycin (Rap), and mice treated with rapamycin plus melatonin at 300 mg/kg (Rap + aMT). n = 6 per group. Data are presented as mean ± SEM. *P<.05, ***P<.001 vs. control, ###P<.001 vs. rapamycin-treated group.

Melatonin reportedly protects various organs from chemotherapy- and radiotherapy-induced toxicity (Abdel Moneim et al., 2017; Fernández-Gil et al., 2017b; FIC et al., 2007; Hassan et al., 1999; Ortiz et al., 2015a). Thus, the combination of melatonin with rapamycin may both achieve an enhanced oncostatic

effect and overcome a major limitation of anticancer treatment. Supporting this possibility, we found that melatonin decreased rapamycin-induced LPO in the liver, kidneys, and lungs (Figure 29). This indicated that melatonin treatment induced significant protection against rapamycin toxicity in normal cells.

V. DISCUSSION

The results of our present study demonstrated a synergistic effect of melatonin and rapamycin with regards to anti-cancer activity against HNSCC cells. Rapamycin increased p^{S473}Akt levels, while melatonin abrogated this induction, thus enhancing rapamycin's anti-tumour effects. Moreover, our findings provide the first evidence that melatonin triggers mitophagic and apoptotic processes via modulation of mitochondrial function in HNSCC.

Rapamycin is an inhibitor of the mTOR pathway, and shows a strong anti-cancer phenotype in both preclinical and clinical trials. However, many cancer types are not sensitive to rapamycin (Benjamin et al., 2011; Inoki et al., 2006), partially because rapamycin can elevate Akt activity through a feedback loop (O'Reilly et al., 2006). Akt can phosphorylate and negatively regulate some substrates such as FOXO transcription factors, GSK3β, Bad, which can block several apoptotic processes (Sasaki et al., 2004). Akt is located at a bifurcation point where differential phosphorylation of distinct residues may lead to activation of mTOR-dependent pathways and/or to the inhibition of apoptotic processes. Therefore, rapamycin-induced Akt activation in tumour cells is likely to reduce its antitumor actions by activating pathways that attenuate its effects on proliferation and apoptosis. In our present study, rapamycin effectively inhibited mTORC1, but also prompted the phosphorylation of Akt (p^{S473}Akt) in HNSCC. Rapamycin is an allosteric inhibitor of mTORC1, without a direct inhibitory mTORC2 effect (Bhagwat et al., 2011). This selective effect on mTOR inhibition is likely to limit the effectiveness of rapamycin on several cancer lines. In the field of cancer research, there is much interest in the

use of multiple targeting or dual-targeting agents against mTORC1 and mTORC2 to overcome feedback activation and resistance (Coppock et al., 2016). However, increased toxicity renders many treatments and drug combinations intolerable, limiting the usefulness of many such approaches.

Importantly, our present results showed that melatonin neutralized the rapamycin-induced increase of phosphorylated Akt. Notably, this effect was only observed with a high concentration of melatonin, while low-dose treatment did not seem to impact Akt phosphorylation. Reinforcing our findings, other authors report that pharmacological concentrations of melatonin can lead to inhibition of PI3K/Akt phosphorylation in breast and ovarian carcinoma (Ferreira et al., 2014; Wang et al., 2012). AKT substrates specificity is likely dependent on the ratio between the phosphorylation status of Ser473 and Thr308 (Yung et al., 2011). Selective phosphorylation of the Ser473 residue is thought to provide protection from stress/drug-induced apoptosis, while melatonin specifically down-regulates Ser473 phosphorylation in breast cancer cells (Proietti et al., 2014). Moreover we showed that the combination of melatonin and rapamycin overcame the rapamycin-induced apoptosis in the HNSCC cells lines Cal-27 and SCC-9, although rapamycin alone did not increase apoptosis pathway activation. This was observed in association with an up-regulation of pro-apoptotic proteins, such as Bax, and down-regulation of the anti-apoptotic protein Bcl-2. It has been previously described that melatonin can enhance cytotoxic and pro-apoptotic actions of various chemotherapeutic agents through the participation of melatonin receptors (Pariente et al., 2017; Pariente et al.,

2016). However, in the present study, the Bax/Bcl2 ratio was strongly regulated by high-dose compared with low-dose melatonin treatment.

Melatonin-induced apoptosis in cancer cells is associated with ROS production (Bejarano et al., 2009; Casado-Zapico et al., 2011; Leja-Szpak et al., 2010), and ROS seems to be involved in pathways critically associated with life-span and cell cycle, including the mTOR pathway (Gharibi et al., 2014). Since the mitochondrial electron transport chain and OXPHOS are the main sources of cellular ROS, we next addressed the possibility that melatonin acted directly in cancer cell mitochondria to enhance the cytotoxic effects of rapamycin. Our results indicated that the combination of melatonin and rapamycin affected mitochondrial homeostasis in the HNSCC cells lines. Melatonin decreased mitochondrial respiration capacity, causing a dose-dependent increase of ECAR, and increasing OXPHOS. These results suggest that even though HNSCC cells lines exhibited depolarized mitochondria and decreased oxygen consumption, they still had active mitochondrial respiration. The cells possessed functional OXPHOS machinery, but most likely executed respiration decoupled from ATP production (Vega-Naredo et al., 2014). The mitochondrial electron transport chain and OXPHOS are the most efficient means for ATP production and the free radicals are by-products of this energy metabolism in cells (Tan et al., 2013b). However, well documented studies have been testifying Warburg's hypothesis that cancer cells exhibit increased glycolysis and give rise to enhanced lactate production even in the presence of O_2 (Dell'Antone, 2012; Warburg, 1926). This kind of aerobic glycolysis constitutes an advantage for tumor growth. Cancer

cells can use intermediates of the glycolytic for anabolic reactions, and the end products of glycolysis provide an acids environment that can favor tumor invasion and suppress anti-cancer immune effectors (Fischer et al., 2007; Gatenby and Gillies, 2004; Koukourakis et al., 2006; Swietach et al., 2007). Mitochondrial dysfunction is one of reason that the metabolic reprogramming in cancer cells. Tumor mitochondria are reported to have relatively small cristae and mutations in some Krebs cycle enzymes (López-Ríos et al., 2007; Ward et al., 2010). Cancer cells benefit greatly from suppression of mitochondrial function: decreased mitochondria-driven apoptosis, increased proliferation and angiogenesis. On the contrary, to rescue mitochondrial function of cancer cells can be considered as a potential strategy to reduce cells proliferation.

We also found that melatonin increased mitochondrial mass and mtDNA. However, considering that mitochondrial ATP production is positively related to mtDNA copy numbers (Lee and Wei, 2005), our results suggested an increase in non-functional mitochondria. We propose that melatonin directly affects the electron transport chain, causing increased ROS production. Our present results showed a significant and substantial increase of intracellular ROS following combined treatment with melatonin and rapamycin compared to treatment with rapamycin alone. High ROS levels inhibit cell growth and cause apoptosis and necrosis in many tumour cells (Burdon, 1996). Prior studies demonstrate that melatonin increases intracellular ROS in various cancer cells (Radogna et al., 2009; Uguz et al., 2012).

ROS production was unexpectedly inhibited with melatonin treatment at a

concentration of 1 mM. This discrepancy cannot readily be explained, considering that 1 mM melatonin also decreased activity of the antioxidant enzyme GPx. We inferred that at high melatonin concentration, ROS accumulation under respiratory conditions may have resulted in mitochondrial protein degradation and even mitophagy. Mitophagy is the main cellular mechanism for removing damaged mitochondria and proteins (Wang and Klionsky, 2011). Although, cytosolic ubiquitin–proteasome system (UPS) and mitochondrial proteases can be accomplished by the cell to remove misfolded, denatured or oxidized mitochondrial proteins, extensive mitochondrial damage need selective mitophagy for mitochondria clearance. LC3 proteins are core autophagy apparatus proteins which act as scaffolds to recruit various proteins. NIX functions as a receptor targeting mitochondria and takes mitochondria aggregates to the autophagosome via its binding with LC3. p62 protein binds to ubiquitinated proteins on the mitochondrion and through LC3, the complex can be tethered to the autophagosome. In accordance with this hypothesis, we detected increased expressions of LC3-II and NIX, and degradation of p62 following treatment with high melatonin concentrations. These results indicate that high concentrations of melatonin contribute to initiating mitophagy and eliminating dysfunctional mitochondria. That is likely because ROS accumulation causes severe damage to mitochondrial proteins, triggering mitophagy to limit the mitochondrial damage. That also explained the fact that the mitochondrial oxygen consumption and ROS production were decreased after high concentration melatonin administration. Cells can be benefit from elimination of dysfunction mitochondria and maintaining a

healthy population of mitochondria. However, the continuous and excessive mitophagy actually may promote cell death (Kim et al., 2007).

The role of mitophagy in cancers is controversial. Mitophagy can facilitate survival by allowing adaptation to stress, or can promote cell death due to excessive removal of mitochondria.(Kulikov et al., 2017) Sentelle and coworkers(Sentelle et al., 2012) showed that mitophagy induction can lead to tumour cell death via an mechanism involving autophagy. Studies also demonstrate that some anti-tumour drugs activate autophagy while inducing apoptosis.(Eom et al., 2010; Sasaki et al., 2010) These results are consistent with our present findings showing that 1 mM melatonin enhanced the melatonin-induced apoptosis and mitophagy. Our present study is the first to show a link between melatonin-induced mitophagy and apoptosis in HNSCC, which could lead to the development of new strategies for cancer treatment.

We next addressed the possibility that melatonin could enhance rapamycin's cytotoxicity in tumour cells *in vivo*. Surprisingly, we observed similar growth-inhibitory effects following treatment with rapamycin alone and in combination with melatonin. These results suggested that the melatonin concentration achieved in the tumour was insufficient to enhance rapamycin's cytotoxic effect *in vivo*. However, histological analysis revealed damaged tumour cells following *in vivo* treatment with both rapamycin alone and in combination with melatonin, with increased apoptosis and greater cell differentiation in the group that received combined treatment compared to rapamycin alone.

Numerous recent reports show that complexes I and III play pivotal roles in the production of ROS, particularly H_2O_2, and could play important roles as cellular messengers and be involved in differentiation and apoptosis (Pacini and Borziani, 2016). Consistently, our present study revealed that combined treatment with rapamycin and melatonin increased apoptosis and differentiation of tumour cells *in vivo*. We previously reported that pharmacological doses of melatonin increase neural stem cell differentiation into mature neurons, in a manner that involves an increase of mitochondrial function (Mendivil-Perez et al., 2017). The current findings indicate that the observed melatonin-dependent anti-proliferative effects require an adequate concentration of melatonin to reach the mitochondria. If melatonin acts in a mitochondria-mediated manner, then efficient reduction of tumour growth might require higher melatonin concentrations or increase treatment duration. Overall, our findings highlight the importance of mitochondria and cell differentiation therapies when using melatonin against cancer cells.

On the other hand, numerous adverse events associated with mTOR inhibitors were reported in clinical practice (Kaplan et al., 2014; Pallet and Legendre, 2013). We found that rapamycin administration induced LPO in different organs of mice and melatonin counteracted these deleterious effects. The protective effect of melatonin is supposed to be related with its anti-oxidant effects. However, although it is well known that melatonin acts difference towards cancer and healthy cells, the precise mechanism remains unknown (Zhang and Zhang, 2014). Fhurthermore, from the evidences of clinical trials, melatonin administration did not demonstrate any

significant adverse effects except drowsiness (Chen et al., 2014; Panzer and Viljoen, 1997). Therefore, our results together with data from other authors suggest that melatonin can be used as adjuvant with rapamycin and minimize the side effects in tumor therapy.

In conclusion, our present results demonstrated that combined treatment with rapamycin and melatonin enhanced the cytotoxic effects of rapamycin in HNSCC cells. Moreover, we found that this effect involved relationships between mTOR signalling pathways, impaired oxygen consumption, mitochondrial hyperpolarization, and oxidative stress. Moreover, our findings suggest that the administration of a high dose of melatonin as an adjuvant in cancer therapy with rapamycin may ameliorate the adverse effects of rapamycin. These observations have important implications for HNSCC treatment, particularly considering the presently low overall 5-year survival (50%), which has not improved much over the past three decades.

VI. CONCLUSIONS

1. Rapamycin has not significant effect on the viability of NHSCC cell lines. Melatonin, however, decreases the cell viability in a dose-dependent manner. The combination of melatonin and rapamycin exerts potent dose-dependent anti-proliferative effects in HNSCC cell lines.

2. Melatonin does not interfere with rapamycin-induced mTOR inhibition. High concentrations of melatonin attenuate the rapamycin-induced increase of phosphorylation of Akt. Melatonin enhances the effects of rapamycin via inhibition of Akt pathways.

3. Rapamycin-treated cells exhibit reduced capacity for oxidative phosphorylation. Rapamycin can decrease metabolic rate in HNSCC. Rapamycin does not significantly alter the mitochondrial complexes. Melatonin leads to significant rise in the expressions of respiratory complexes. Melatonin enhances the cytotoxic effects of rapamycin by augmenting the number of dysfunctional mitochondria.

4. Melatonin affects the ROS generation in a dose dependent manner. Melatonin enhances the effects of rapamycin via induction of oxidative stress. ROS accumulation resultes in severe oxidative damage of mitochondria leading to mitophagy or even apoptosis.

5. The combination of melatonin plus rapamycin significantly increases apoptotic bodies and cell necrosis in HNSCC xenografts compared with rapamycin treatment alone. Melatonin enhances the rapamycin's effect *in vitro*.

6. Melatonin administration protects normal cells against rapamycin-associated toxicity.

VI. CONCLUSIONES

1. La rapamicina no tiene efectos significativos sobre la viabilidad de las líneas celulares NHSCC. Sin embargo, la melatonina disminuye la viabilidad celular de una manera dependiente de la dosis. La combinación de melatonina y rapamicina ejerce potentes efectos antiproliferativos dependientes de la dosis en las líneas celulares HNSCC.

2. La melatonina no interfiere con la inhibición de mTOR inducida por rapamicina. Altas concentraciones de melatonina atenúan el aumento de la fosforilación de Akt inducida por rapamicina. La melatonina aumenta los efectos de la rapamicina a través de la inhibición de las vías de Akt.

3. Las células tratadas con rapamicina muestran una disminución de la capacidad de la fosforilación oxidativa. La rapamicina puede disminuir la tasa metabólica de las HNSCC. La rapamicina no altera significativamente los complejos mitocondriales. La melatonina conduce a un aumento significativo en la expresión de los complejos respiratorios. La melatonina aumenta los efectos citotóxicos de la rapamicina al aumentar el número de mitocondrias disfuncionales.

4. La melatonina afecta a la generación de ROS de una manera dosis dependiente. La melatonina aumenta los efectos de la rapamicina a través de la inducción del estrés oxidativo. La acumulación de ROS produce un daño oxidativo severo en las

mitocondrias conduciendo a un proceso de mitofagia e incluso apoptosis.

5. La combinación de melatonina más rapamicina incrementa de forma significativa la aparición de cuerpos apoptóticos y de necrosis en las células tumorales de xenoinjertos HNSCC en comparación con el tratamiento de rapamicina sola. La melatonina aumenta el efecto de la rapamicina *in vitro*.

6. La administración de melatonina protege a las células normales de la toxicidad asociada a la rapamicina.

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