GABRIEL ÁLVARES BORGES

EFFECTS OF CURCUMIN ON SIGNALING PATHWAYS AND CELL CYCLE IN HEAD AND NECK CARCINOMA CELLS

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Tese apresentada como requisito parcial para a obtenção do título de Doutor em Ciências da Saúde pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Brasília.

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Brasília
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„Die Wissenschaft fängt eigentlich erst da an, interessant zu werden, wo sie aufhört“

Justus Freiherr von **Liebig** (1803 - 1873)

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ABSTRACT

Due to antioxidant, anti-inflammatory, antibiotic, and antitumor effects, curcumin has been extensively studied for its potential as therapy for a variety of diseases, especially in the last decade. Curcumin interacts with several transcription factors, inflammatory mediators, and protein kinases to modulate diverse signaling pathways and biological events. The systematic review and the original research article that constitute this thesis indicate that, on head and neck carcinoma cells and animal models, curcumin was able to decrease cell viability, modify the organization of cytoskeleton, arrest cell cycle at G_2/M, induce apoptosis, and reduce the tumor burden. Curcumin also downregulated the expression of genes and proteins that are related to the PI3K-AKT-mTOR signaling pathway, which is known to be highly dysregulated and overactive in head and neck cancers. Such effects might be interrelated, and they highlight the promising therapeutic potential of curcumin to inhibit head and neck cancer growth and progression and to modulate the PI3K-AKT-mTOR signaling pathway.

Keywords: curcumin, head and neck neoplasms, systematic review, PI3K, AKT, mTOR.

RESUMO

Por conta dos efeitos antioxidante, anti-inflamatório, antibiótico e antitumoral, a curcumina e o seu potencial terapêutico contra várias doenças vêm sendo amplamente estudados, especialmente na última década. A curcumina pode interagir com diversos fatores de transcrição, mediadores inflamatórios e proteínas quinase para modular várias vias de sinalização e eventos biológicos. A revisão sistemática e o artigo de pesquisa original apresentados nessa tese indicam que, em linhagens celulares e modelos animais de carcinoma espinocelular de cabeça e pescoço, a curcumina foi capaz de reduzir a viabilidade celular, alterar a organização do citoesqueleto, interromper o ciclo celular em G_2/M, induzir a apoptose e reduzir o volume tumoral. A curcumina também controlou a expressão de genes e proteínas relacionadas à via de sinalização do PI3K-AKT-mTOR, sabidamente desregulada e hiperativa nos cânceres de cabeça e pescoço. Tais efeitos podem estar associados, e evidenciam o potencial terapêutico da curcumina para inibir o crescimento e a progressão do câncer de cabeça e pescoço e para modular a via de sinalização do PI3K-AKT-mTOR.

Palavras-chave: curcumina, neoplasias de cabeça e pescoço, revisão sistemática, PI3K, AKT, mTOR.

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LISTA DE ABREVIATURAS E SIGLAS

4E-BP1 - 4E-binding protein 1

4NQO - 4-Nitroquinoline 1-oxide

5-FU - 5-Fluorouracil

AIF - Apoptosis-inducing factor

AKT - Protein kinase B

Apaf-1 - apoptotic protease activating factor 1

ATCC - American Type Culture Collection

Bax - Bcl-2-associated X protein

Bcl-2 - B-cell lymphoma 2

Bik - Bcl-2-interacting killer

Bim - Bcl-2-like protein 11

BLI - Bioluminescence imaging

CDC25C - M-phase inducer phosphatase 3

CDC42 - Cell division control protein 42

CDK - Cyclin-dependent kinase

c-flip - Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein

cIAP2 - Cellular inhibitor of apoptosis 2

COX2 - Cyclooxygenase-2

DAPI - 4′,6-diamidino-2-phenylindole

DISC - Death-inducing signaling complex

DMBA - 7,12-Dimethylbenz[a]anthracene

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

E2F - E2 factor

EF-24 - a curcumin analog

EGCG - Epigallocatechin gallate

EGFR - Epidermal growth factor receptor

eIF4E - Eukaryotic translation initiation factor 4E

ELISA - Enzyme-Linked Immunosorbent Assay

Endo G - Endonuclease G

FADD - Fas-associated protein with death domain

FasL - Fas ligand

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

FLLL12 - a curcumin analog

GCO - Global Cancer Observatory

GLOBOCAN - Global Cancer Incidence, Mortality, and Prevalence

GPCR - G-protein coupled receptor

GRADE - Grading of Recommendations, Assessment, Development, and Evaluations

GTP - Guanosine triphosphate

HAT - Histone acetyltransferases

HDAC - Histone deacetylase

HNC - Head and neck cancer

HNSCC - Head and neck squamous cell carcinoma

HPV - Human papillomavirus

IARC - International Agency for Research on Cancer

IC_{50} - Half maximal inhibitory concentration

IL - Interleukin

ISR - Insulin receptor

mAb - Monoclonal antibody

MAPK - Mitogen-activated protein kinase

mTOR - Mechanistic target of rapamycin

mTORC - mTOR complex

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT - 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

NF-κB - Nuclear factor kappa B

p70 S6K - p70 ribosomal protein S6 kinase

p85 S6K - p85 ribosomal protein S6 kinase

pAb - Polyclonal antibody

PARP - Poly (ADP-ribose) polymerase

PCNA - Proliferating cell nuclear antigen

PCR - Polymerase chain reaction

PDK1 - Phosphatidylinositol-dependent kinase 1

PI - Propidium iodide

PI3K - Phosphoinositide-3-kinase

PICOS - Population, Intervention, Comparison, Outcome, and Study Design

PIP_2 - Phosphatidylinositol-4,5-biphosphate

PIP_3 - Phosphatidylinositol-3,-4,-5-triphosphate

PKC - Protein kinase C

PLD - Phospholipase D

pRB - Retinoblastoma protein

PRISMA - Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PTEN - Phosphatase and tensin homolog

PVDF - Polyvinylidene fluoride

RAC1 - Ras-related C3 botulinum toxin substrate 1

Rheb - Ras homolog enriched in brain

Rho GTPase - Ras homolog family of GTPases

RICTOR - Rapamycin-insensitive companion of mTOR

ROS - Reactive oxygen species

RTKs - Receptor tyrosine kinases

SGK - Glucocorticoid-induced kinase

SRB - Sulforhodamine B

SYRCLE's RoB – Systematic Review Centre for Laboratory animal Experimentation Risk of Bias tool

TNF - Tumor necrosis factor

TRADD - TNF receptor type 1-associated DEATH domain protein

TSC1 - Tuberous sclerosis complex 1

TSC2 - Tuberous sclerosis complex 2

TSI - Tumor Selectivity Index

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

WHO - World Health Organization

XIAP - X-linked inhibitor of apoptosis protein

XTT - 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

SUMÁRIO

1. INTRODUCTION

The Global Cancer Observatory (GCO) is an interactive web-based platform, maintained by the International Agency for Research on Cancer – World Health Organization (IARC-WHO), in which data on the epidemiological profile and world impact of cancer from different IARC's cancer surveillance projects are gathered [1]. Considering all cancer types, IARC estimated for 2018 approximately 18 million new cases worldwide, and more than 9.5 million deaths related to the disease. The estimated numbers for Brazil in 2018 were 559,371 new cases and 243,588 deaths. Specifically concerning head and neck cancers (HNC), the data are presented in Figure 1.

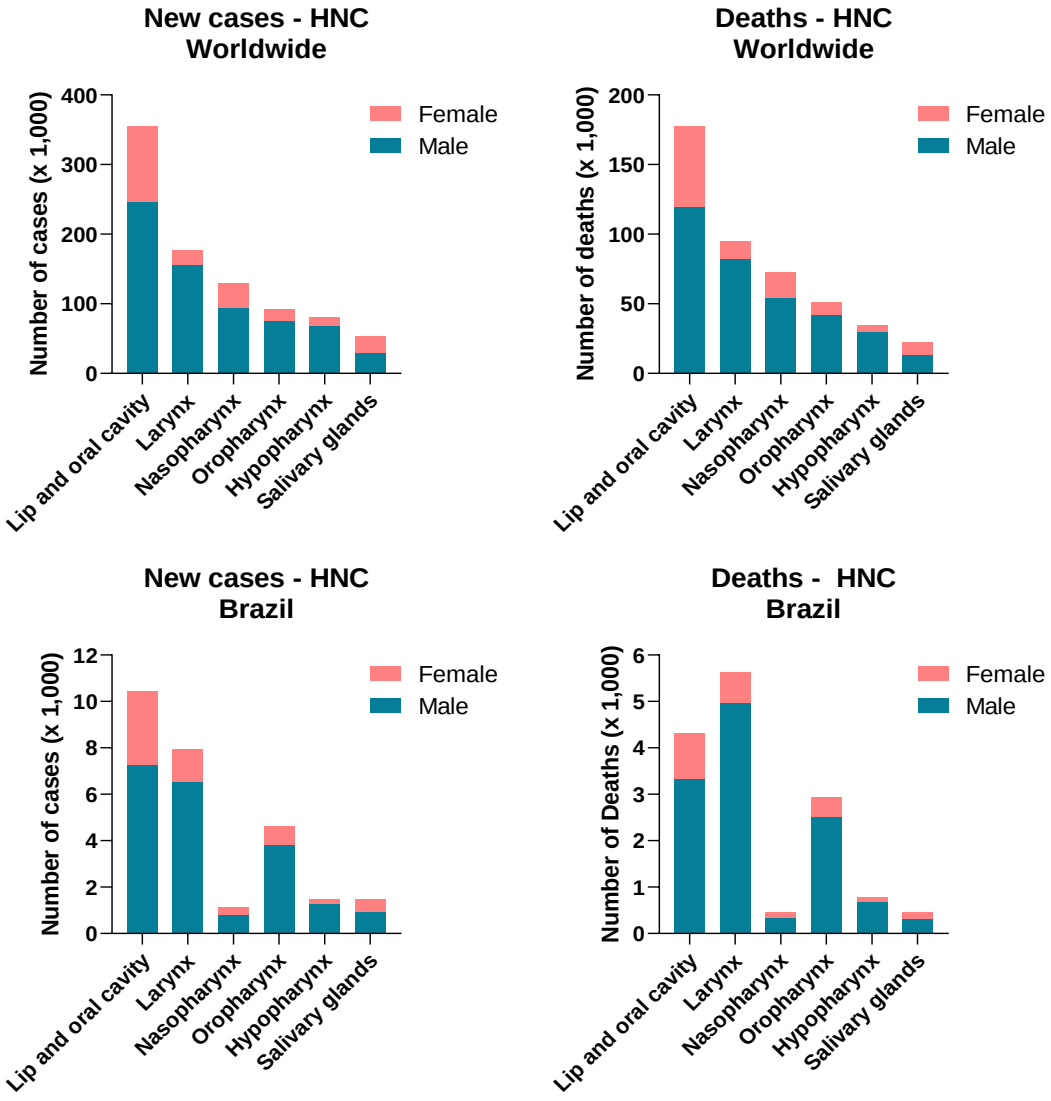


Figure 1. Estimates of new cases HNC and deaths due to the disease in 2018, reported according to sex. Data source: GLOBOCAN 2018, IARC.

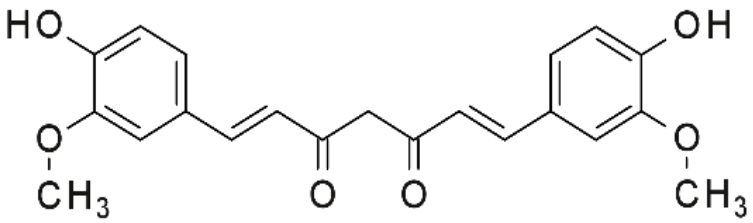
As reported by GCO, the number of new cases of HNC totalized 887,659 worldwide in 2018, and the deaths 453,307. In Brazil, the new cases were 27,137 and the deaths were 14,574. That means that approximately 3% of the new HNC cases and 3.2% of deaths due to HNC were registered in Brazil.

The majority of HNCs are squamous cell carcinoma (HNSCC), to which tobacco and alcohol consumption is the most important etiological factor [2-4]. Betel-quid and areca-nut chewing also increase significantly the risk of HNSCC, which is especially prevalent in the Asia-Pacific region and among immigrants from this area [5, 6]. Infection with oncogenic subtypes of the human papillomavirus (HPV) is associated with the HNSCC carcinogenesis, particularly in the oropharynx, and the HPV+ status is related to an improved prognosis [7].

The treatment of HNSCC patients depends on the stage of the disease, and surgery is still the preferred approach, combined or not with radiotherapy and chemotherapy [4]. Aiming to reduce the morbidity that is inherent to HNSCC therapy and to improve the quality of life of patients, technological developments on the HNSCC therapy have been recently proposed, and evidence on their efficiency and efficacy has been produced. Regarding surgery, examples of such events are the microvascular reconstruction on management of advanced-stage primary HNSCC and the minimally invasive transoral surgical approaches, such as the transoral robotic surgery and the transoral laser microsurgery, on the treatment of early-stage cancers [4]. Radiotherapy benefits from the altered fractionation, intensity-modulated radiotherapy, adaptive radiotherapy, and proton beam therapy modalities, and an increasing number of studies report the advantages of distinct systemic therapy methods, such as different combinations of drugs or their association with other therapeutic modalities, targeted therapy (anti-EGFR therapy), and immunotherapy [4]. The advent of immunotherapy is regarded as the most remarkable development in the HNSCC therapy field [4], with a large body of evidence supporting its effectiveness on recurrent and/or metastatic HNSCC [8].

The research on nutraceuticals, natural diet-derived molecules that exert a potential effect on the control, treatment or prevention of a disease, is part of this effort to produce therapeutic options that are more specific and less detrimental to the patients who undergo cancer treatment. In fact, in 2017, the journal Seminars in Cancer Biology published an entire special issue with reviews on the role of different nutraceuticals in controlling oncogenesis [9].

Curcumin (Figure 2) is a natural polyphenol isolated from turmeric (*Curcuma longa*) rhizome, which has been used for thousands of years in the traditional Chinese and Ayurvedic medicine for wound healing, respiratory problems, and liver and dermatological disorders [10, 11]. It is also used as spice in the preparation of food, especially in Eastern Asia and Latin America [10]. Curcumin, desmethoxycurcumin, and bisdemethoxycurcumin are active molecules identified in turmeric and collectively referred to as curcuminoids [11]. The curcuminoids represent 3-5% of the constitution of turmeric, and among them, curcumin accounts for 77% [11]. Curcumin is practically insoluble in aqueous solutions at neutral and acidic pH, and solubility (and degradation) increases under alkaline conditions [11]. Due to its lipophilic nature, curcumin is soluble in organic solvents such as ethanol and dimethyl sulfoxide (DMSO) [11]. The therapeutic potential of curcumin is hindered by its objectionable pharmacological properties, such as poor bioavailability, solubility, and stability [12]. However, various types of chemical modifications of curcumin have been proposed as improvements to its pharmacokinetic profile and therapeutic properties [13].



Curcumin

Figure 2. Molecular structure of curcumin.

Studies on curcumin have been increasingly published in the past decade (Figure 3), mostly due to its antioxidant, anti-inflammatory, and antitumor effects [14, 11, 15]. The presence of phenol groups in the molecular structure of curcumin (Figure 2) induces destabilization of reactive oxygen species (ROS) and justifies the antioxidant effects [10]. The anti-inflammatory effects derive from the inhibition of cyclooxygenase-2 (COX2) and the nuclear factor kappa B (NF-κB), proinflammatory proteins, and consequent reduction in synthesis of cytokines such as interleukin (IL)-1α, IL-1β, IL-2, IL-6, and IL-10 [16, 10]. The antitumor effects of curcumin will be further discussed in the studies presented in this thesis.

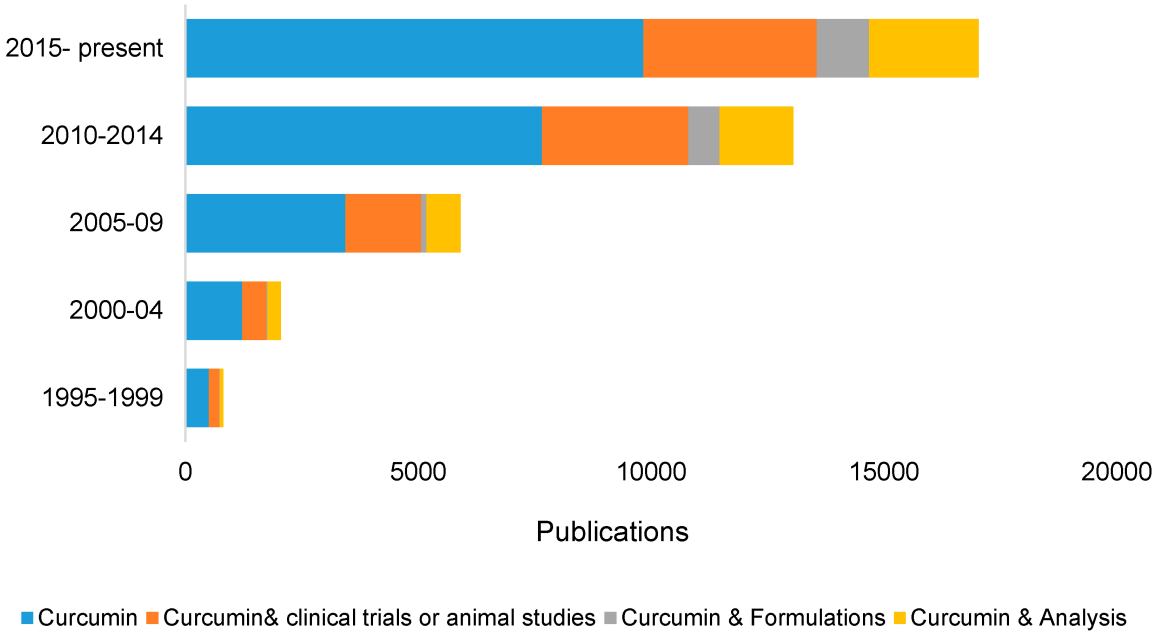


Figure 3. Number of studies on curcumin published between 1995 and 2019. The graph was prepared and published by Kotha and Luthria [11].

Phase I clinical trials with both healthy and cancer patients suggest treatment with curcumin, even in high daily doses, does not result in severe adverse events [17-19]. Additionally, several phase II and III clinical trials are currently under development [10]. A phase III clinical trial (NCT03769766) is being conducted to assess the potential of curcumin in preventing the progression of prostate cancer. Another (NCT02064673) is investigating its effect on recurrence-free survival in prostate cancer patients submitted to radical prostatectomy.

Ongoing phase II studies are assessing the association of curcumin with bevacizumab or the FOLFIRI regimen (folinic acid, fluorouracil, and irinotecan) on progression-free survival in colorectal cancer patients with unresectable metastasis (NCT02439385) and the association of curcumin to paclitaxel in advanced breast cancer (NCT03072992).

The phosphoinositide-3-kinase (PI3K) - protein kinase B (AKT) - mechanistic target of rapamycin (mTOR) signaling pathway controls cellular events such as cell growth and proliferation, metabolic homeostasis, protein and lipid synthesis, cell migration, and cytoskeleton organization [20]. This signaling pathway is described in the literature as markedly overactivated in cancer, which results in cell survival, cytoskeleton rearrangement, invasion, metastasis, and evasion of apoptosis [20].

The PI3K-AKT-mTOR signaling pathway is represented in Figure 4. The binding of growth factors to receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGRF), the insulin receptor (ISR), and the G-protein coupled receptor (GPCR) triggers this pathway. The RTKs recruit to the membrane PI3K, which catalyzes the conversion of phosphatidylinositol-4,5-biphosphate (PIP_2) into phosphatidylinositol-3,-4,-5-triphosphate (PIP_3), which is negatively controlled by the phosphatase and tensin homolog (PTEN). PIP_3 activates AKT and the phosphatidylinositol-dependent kinase 1 (PDK1). Both PDK1 and PIP_3 phosphorylate AKT, which inhibits the tuberous sclerosis complex 1 and 2 (TSC1/2) by phosphorylating TSC2. The inhibition of TSC1/2 increases the activation of the Ras homolog enriched in brain (Rheb) by GTP-binding, which phosphorylates mTOR. mTOR forms different protein complexes (mTORC1 and mTORC2), each with their specific functions. mTORC1 enhances glucose metabolism and promotes lipid, DNA, and protein synthesis, as consequences of the phosphorylation of p70 ribosomal protein S6 kinase (p70 S6K) and 4E-binding protein 1 (4E-BP1), followed by the activation of the eukaryotic translation initiation factor 4E (eIF4E). mTORC2 results in actin cytoskeleton rearrangement and cell migration after phosphorylation of the protein kinase C (PKC) and the Ras homolog family of GTPases (Rho GTPases) and in

inhibition of apoptosis after phosphorylation of AKT and the serum and glucocorticoid-induced kinase (SGK) [20-22].

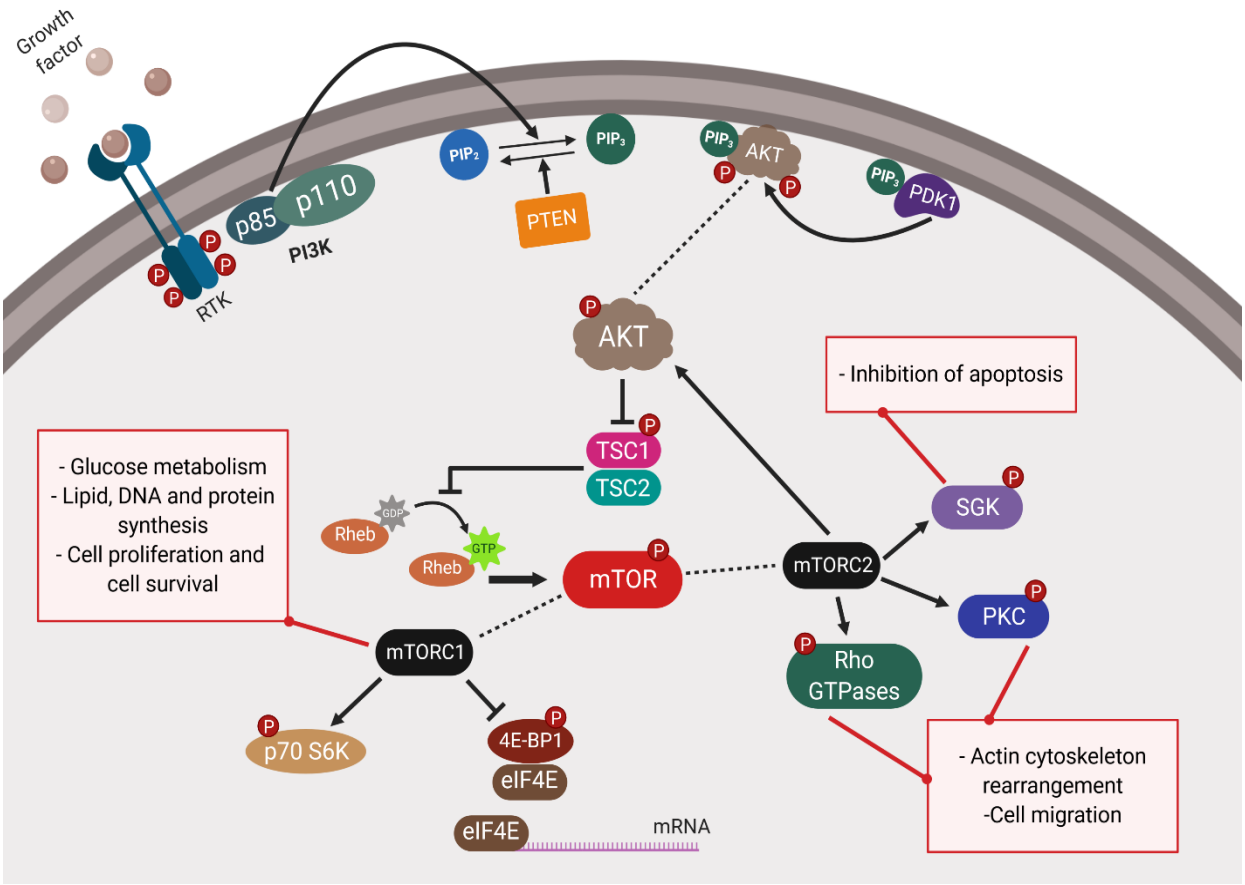


Figure 4. Schematic representation of the PI3K/AKT/mTOR signaling pathway.

Mutagenic events, mainly associated with tobacco and alcohol consumption, are considerably relevant to the HNSCC oncogenesis. In general, whenever the metabolic processing of carcinogens is inadequate, either due to genetic alterations that result in dysfunctional enzymes or because of a high concentration of carcinogens in the organism, the DNA is damaged [23]. That leads to an abnormal protein expression and mechanisms to evade apoptosis, prompt angiogenesis, maximize proliferation, and promote invasion and metastasis [23].

The cell cycle is a physiological process that results in cell proliferation, and that is essential for tissue development and repair. The cell cycle, represented in Figure 5, is characterized by two distinct phases: interphase (phases G_1, S, and G_2) and mitosis (phase M).

The transition from one phase to another is coordinated by complexes formed by a cyclin and a cyclin-dependent kinase (CDK), which are assembled and activated under diverse stimuli. The retinoblastoma protein (pRB) is another critical cell cycle regulator. It binds to transcription factors of the E2 factor (E2F) family, inactivates them, and keeps the cell cycle arrested at phase G_1. The complexes cyclin-CDK then phosphorylate pRB, which releases E2F to take part in the transcription of genes that are vital for the cell cycle progression and cell proliferation [24, 25].

Many cell cycle and cell-cycle-related proteins are overexpressed or overactive in human cancers, particularly cyclin D, cyclin E, CDK4, CDK6, and CDK2 [26]. Research efforts focused on targeting such proteins have been made, with the objective of establishing the cell cycle inhibitors as a possible anticancer strategy [26].

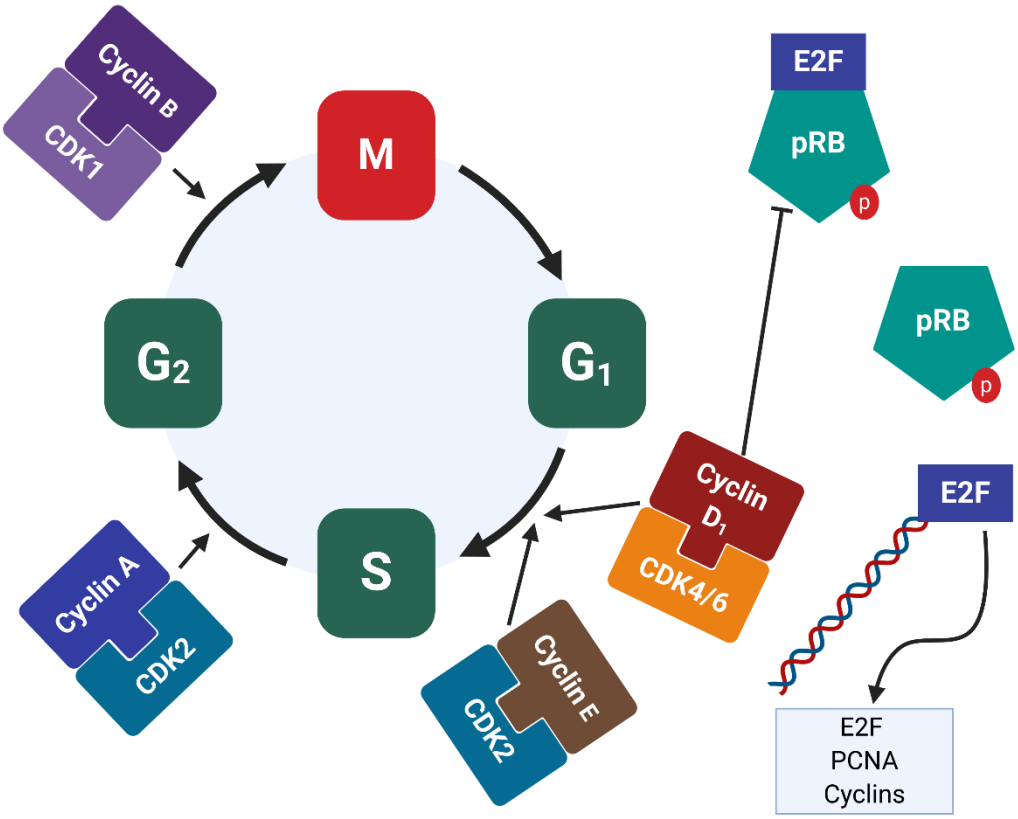


Figure 5. Schematic representation of the cell cycle.

Apoptosis is a controlled and non-inflammatory cell death process that is observed on multicellular organisms from the earliest stage of development to adulthood. It is induced by a variety of intrinsic and extrinsic stimuli, such as irradiation, chemical agents, drugs, hormones,

and inflammation-related proteins [27]. Apoptosis is characterized by biochemical and morphological changes in the cell, such as asymmetry, cell shrinkage, nuclear condensation, DNA fragmentation, and formation of apoptotic bodies [25]. Two main mechanisms trigger apoptosis, and they are represented in Figure 6: the extrinsic and the intrinsic pathways [28]. In both pathways, caspases are the main effector proteins. The initiator caspases, such as caspase-8 and caspase-9, cleave the inactive precursor forms of executioner caspases, such as caspase-3 and caspase-3, which, in their active forms, will lead the cell to an apoptotic cell death.

The extrinsic pathway is initiated by the binding of the Fas ligand (FasL) or the tumor necrosis factor (TNF) to their membrane receptors. The adaptor proteins Fas-associated protein with death domain (FADD) and TNF receptor type 1-associated DEATH domain protein (TRADD) are recruited by their respective receptors, and couple with procaspase-8, forming the death-inducing signaling complex (DISC). After DISC is formed, procaspase-8 is cleaved into its active form, which initiates apoptosis [27, 28]. The intrinsic or mitochondrial pathway is majorly controlled by members of the B-cell lymphoma 2 (Bcl-2) family, which might be either anti- or pro-apoptosis. These proteins coordinate the process by controlling the permeability of the mitochondrial membrane. When proapoptotic proteins are activated, the membrane is permeable, and the cytochrome C is translocated from the mitochondria to the cytosol. Once in the cytosol, cytochrome C forms with the apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 a complex known as apoptosome. After the apoptosome is formed, procaspase-9 is cleaved into its active form [27, 28]. The protein p53, among other functions, activates apoptosis through the intrinsic pathway, especially in circumstances in which an irreversible DNA damage is observed [28].

Evasion of apoptosis as a means to keep the proliferative potential is a hallmark of cancer. The loss-of-function of proapoptotic genes, the reduced expression of proapoptotic proteins such as p53, the Bcl-2-associated X protein (Bax), and Fas, and the increased

expression of anti-apoptotic proteins such as Bcl-2 in HNSCC cell lines, animal models, and patient samples have been described [25, 29-31].

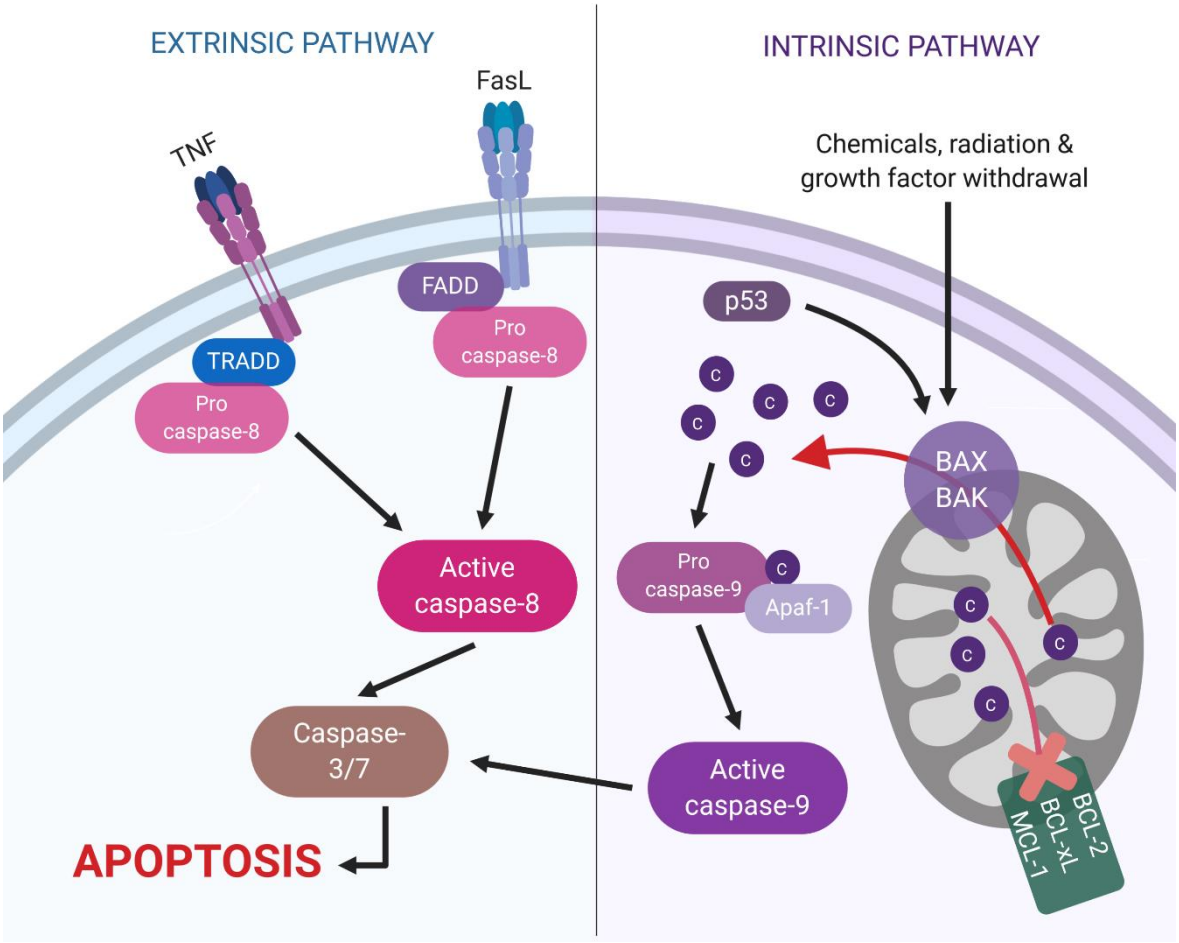


Figure 6. Schematic representation of the extrinsic and intrinsic pathways of apoptosis.

2. OBJECTIVES

In light of the evidence previously presented, the objectives of this thesis are:

1) Outline the state-of-the-art status of *in vitro* and *in vivo* research on curcumin and its effect on head and neck cancer;
2) Provide new insights into the biological effects of curcumin on head and neck carcinoma cell lines (SCC-9 and FaDu);
3) Demonstrate how curcumin modulates the PI3K-AKT-mTOR signaling pathway in head and neck carcinoma cell lines.

3. HYPOTHESES

1) The *in vitro* and *in vivo* studies published in the literature indicate that curcumin reduces cell viability, cell proliferation, and tumor growth on head and neck cancer cell lines and animal models;
2) When used in SCC-9 (tongue carcinoma) and FaDu (oropharynx carcinoma) cell lines, curcumin reduces cell viability, arrests cell cycle, induces apoptosis, and disorganizes the cytoskeleton, all effects that are expected of a drug against cancer and support curcumin as an antitumor agent;
3) Curcumin downregulates the PI3K-AKT-mTOR signaling pathway by reducing the expression of genes and proteins that are related directly or indirectly to it.

4. ARTICLE 1

Title: *In vivo* and *in vitro* effects of curcumin on head and neck carcinoma: A systematic review

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ABSTRACT

BACKGROUND: Head and Neck Squamous Cell Carcinoma (HNSCC) contributes globally to a great number of deaths and morbidity, in spite of new therapeutic strategies. There is a great need for new therapeutic drugs that are significantly effective and less deleterious to the patients' general health. In this sense, the study of phytotherapy is a tendency, with results pointing to the use of phytochemicals as chemo-preventive and adjuvant agents. Therefore, the objective of this systematic review was to investigate the effects of curcumin on proliferation and survival of HNSCC.

MATERIALS AND METHODS: The search was conducted on six databases: Cochrane, LILACS, EMBASE, MEDLINE, PubMed, and Web of Science. *In vitro* and *in vivo* studies that evaluated the effects of curcumin on cell viability, tumor growth, cell cycle or cell death in HNSCC cell lines or animal models were selected.

RESULTS: Out of 525 initially gathered studies, 30 met the inclusion criteria. These studies demonstrated that curcumin induces cytotoxicity, apoptosis (via intrinsic pathway), and cell cycle arrest at G_2/M phase in HSNCC cell lines. It also reduces tumor measurements in animal models. These events were mostly studied with MTT assay, flow cytometry, and expression of cell-cycle- and apoptosis-related proteins.

CONCLUSION: This systematic review demonstrated that curcumin is effective in HNSCC cell proliferation and survival, reinforcing the currently available evidence that curcumin could be an adjuvant drug in HNSCC treatment.

Keywords: curcumin; phytotherapy; head and neck neoplasms; systematic review

INTRODUCTION

Head and neck cancers, comprehended by oral cavity, pharynx and larynx cancers [1], are the sixth most common type of cancer worldwide, being especially incident in the south and southeast of Asia and parts of Europe and South America [2, 3]. Most of the head and neck cancers are squamous cell carcinomas (HNSCC) [4], to which the different forms of tobacco and alcohol consumption are considered the main causes [5, 3]. The human papillomavirus has also been recognized as an etiological factor, mainly for oropharyngeal cancers [6]. According to the National Cancer Institute in the United States, the occurrence of 45,780 new cases of HNSCC was estimated for 2015, as well as 8,650 deaths due to the disease [7].

Treatment of HNSCC is multimodal and involves surgery, radiotherapy, and chemotherapy. In spite of modern surgical techniques and therapeutic strategies such as cetuximab and taxanes, HNSCC mortality rates continue to be high in most countries, leading to an overall 5-year survival rate below 50% [5]. Therefore, new therapeutic strategies that are significantly effective and less deleterious to patients' general health are required [8]. In this sense, targeted-therapy figures as an alternative, targeting proteins that have a considerable role in the oncogenic process [9, 10]. The study of plant- and diet-derived substances is also a tendency, with results pointing to their use as chemo-preventive and adjuvant agents, or even as a source of molecules that could be actively applied as HNSCC treatment [11-13]. One of these alternatives is curcumin, a yellow polyphenol derived from the rhizome of *Curcuma longa*. Studies demonstrate its potential as an anti-inflammatory, antibiotic, and antioxidant agent [14, 15]. Its anticancer activity, comprising the regulation of proliferation, survival, migration, invasion, angiogenesis, and metastasis, has also been reported for several types of cancer [16].

There is a relative number of *in vitro* and *in vivo* published studies that describe the activity of curcumin on HNSCC. However, a systematic review has never been written on this subject. Thus, the objective of this systematic review is to summarize the results of those

studies, especially concerning the effects of curcumin on proliferation and survival of HNSCC.

METHODS

Protocol and Registration

This systematic review was developed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses PRISMA Checklist [17]. The protocol was not registered, given the nature of the investigated population.

Eligibility Criteria

Inclusion Criteria

The inclusion criteria for this systematic review were based on PICOS (Population, Intervention, Comparison, Outcome, and Study Design). We considered *in vitro* and *in vivo* studies in which HNSCCs were evaluated. Cancers of the oral cavity, pharynx, and larynx were regarded as HNSCC [1]. The intervention was curcumin, and no treatment or treatment with other drugs and/or radiotherapy was the comparison. The expected outcomes were: (1) *in vitro* cell proliferation, viability or cytotoxicity or *in vivo* tumor volume or tumor incidence; and (2) apoptosis and/or cell cycle arrest, including analysis of protein expression.

Exclusion Criteria

Studies were excluded if: 1) Curcumin was not used, or used as treatment for conditions other than HNSCC; 2) Curcumin was not tested for both cell viability / cytotoxicity/ tumor growth and apoptosis / cell cycle arrest; 3) Only nasopharyngeal carcinoma cell lines were used; 4) Reviews, letters, personal opinions, book chapters, conference abstracts, and patents; 5) Clinical trials; 6) Written in alphabets other than Latin (Roman) alphabet; 7) Full copy was not available.

Information sources and search strategy

Individual search strategies were designed for each of the following bibliographic databases: Cochrane, LILACS, EMBASE, MEDLINE, PubMed, and Web of Science (Appendix 1). The search strategy used for PubMed was as following: (("oral cancer" or "oral carcinoma" or "oral cancers" or "oral carcinomas" or "OSCC" or "head and neck cancer" or "head and neck carcinoma" or "head and neck cancers" or "head and neck carcinomas" or "HNSCC" or (head and neck cancer[MeSH Terms]) or (head and neck neoplasms[MeSH Terms]) or (cancer of head and neck[MeSH Terms])) and (curcumin or curcuma)). The search included all articles published on or before January 26, 2016, with no time restrictions nor limits. In addition, the reference lists of selected articles were hand-screened for potentially relevant studies that could have been missed during the electronic database search.

Study Selection

Study selection was completed in two phases. In phase one, two authors (GAB and DFR) independently reviewed the titles and abstracts of all references. They selected articles that met the inclusion criteria based on their titles and abstracts. In cases of disagreement, a third author (DXA) intervened. Studies that clearly failed the inclusion criteria were discarded and those whose abstracts did not contain all needed information proceeded to phase two. Reference lists for all included articles were critically assessed by GAB for new articles. In phase two, full articles were read to determine the studies in which cell proliferation, cell viability, cytotoxicity, tumor volume or tumor incidence, and apoptosis or cell cycle arrest were studied. Two authors (GAB and DFR) independently participated in phase two. The third reviewer (DXA) resolved any disagreement. Final selection was always based on the publication full-text.

Data Collection Process and Data Items

One author (GAB) collected the required information from the selected articles. DFR and DXA crosschecked the collected information and confirmed its veracity. Any disagreement was resolved by discussion and mutual agreement. A fourth reviewer (ENSG) was involved as required.

Risk of Bias in Individual Studies

Quality of evidence was methodically assessed with the GRADE tool [18, 19] for *in vitro* studies or the SYRCLE's RoB tool [20] for *in vivo* studies. The GRADE tool was adapted to *in vitro* studies, according to Pavan *et al.* [21], given that no specific quality assessment method was developed for this type of study. For *in vitro* studies, two authors (DFR and DXA) categorized the articles as “high”, “moderate”, “low” or “very low” quality, according to their analysis of each study. For *in vivo* studies, the same two authors scored each item as “yes,” “no,” or “unclear” for each article. When they did not reach a consensus, a third author (GAB) intervened to make a final decision.

Summary Measures

Cell viability, tumor growth, cell cycle, and cell death in HNSCC cell lines and animal models after treatment with curcumin were the main evaluated outcomes.

RESULTS

In phase one, 525 articles were gathered from the databases. After duplicate removal, 236 articles remained. A comprehensive evaluation of titles and abstracts resulted in the exclusion of 152 articles. No additional article was identified from the reference lists of selected studies. A full-text review was conducted on 84 articles retrieved in phase one. This process led

to the exclusion of 54 studies (Appendix 2). In the end, 30 articles were maintained for final analyses [22-51]. A flow chart detailing this process is depicted in Figure 1.

Studies Characteristics

The studies were conducted in ten different countries: Brazil (n=1), China (n=5), Croatia (n=1), India (n=5), Israel (n=1), Italy (n=2), South Korea (n=4), Spain (n=1), Taiwan (n=5), and United States (n=8) [22-25, 29, 33, 46, 47]. All studies were published from 1998 to 2015, in English language.

Studies were divided into two subgroups: *in vitro* (n=28) and *in vivo* (n=10). All *in vitro* studies evaluated cell viability, cell proliferation, or cytotoxicity. For these evaluations, MTT assay was mostly applied, although XTT, MTS, and SRB (sulforhodamine B) assays, colony formation assay, and cell viability kits were also used. Cell cycle arrest was assessed by flow cytometry with propidium iodide (PI) staining. Apoptosis was assessed directly, mostly through flow cytometry with annexin V-FITC and PI staining or apoptosis detection kits, or indirectly, through the analysis of apoptosis-related proteins expression with western blot and immunofluorescence, caspase activity assays, DNA fragmentation, and nuclear morphology.

Most of the *in vivo* data were collected from studies that performed both *in vivo* and *in vitro* experiments, and already fulfilled the inclusion criteria for the *in vitro* data. For this reason, even though the *in vivo* data did not completely meet the criteria, they were still included. All *in vivo* studies evaluated tumor measurements, including tumor growth, volume, weight, multiplicity, and incidence. Studies also assessed apoptosis through western blot, TUNEL assay, and immunohistochemistry.

A summary of the descriptive characteristics of the included studies is presented in Table 1 and the PICOS information for each included study is described in Table 2.

Risk of Bias in Individual Studies

When assessed with GRADE, as seen in Table 3, six studies were graded as of moderate quality and one as of low quality. Three did not report the statistical analysis for considered results, even though the effects, especially concerning cell viability, were described in the text and figures. In one study, the curcumin concentrations used to establish IC_{50} values were not specified, and in another study the experimental design did not fully answer our question, considered that it aimed to evaluate the effects of curcumin and cisplatin on ototoxicity. In two other studies, experiments were conducted only with KB cell line, and in one HEp-2 and 7T, a HEp-2 subline, were the only cell lines used. According to the ATCC, KB and HEp-2 cell lines were originally thought to be derived from HNSCC, but found to be a HeLa (cervical cancer) cell contaminant [52, 53]. Considered their use as HNSCC cell lines by the authors, the aforementioned articles were still reviewed.

All studies that evaluated animals were considered of unclear risk of bias when assessed with SYRCLE's RoB (Table 4). These studies did not fully describe information on allocation, randomization, and blinding, which are required for quality assessment.

Synthesis of Results

In vitro studies

A great variety of HNSCC cell lines were used in the included studies, and CAL-27 (n=5), FaDu (n=4) and KB (n=3) were the most frequent.

Curcumin was used alone and compared to negative control (n=17), or used in association and compared to other treatments (n=11), which were irradiation (n=3), cisplatin (n=2), 5-fluorouracil (n=1), doxirubicin (n=1), EGCG (n=1), resveratrol (n=1), lycopene (n=1), photodynamic therapy (n=1), EF-24 (n=1), and FLLL12 (n=1) (the last two are curcumin analogs). Curcumin doses varied between 0.0001 and 400 μM, being the effective dosage rather variable among studies. IC_{50}, when informed, ranged from 3 to 271.5 μM.

All *in vitro* studies (n=28) described an inhibition of proliferation and cell viability, reaching statistically significant levels in 14. A dose-dependent pattern was observed in most studies (n=19). When curcumin was compared to other treatments, it induced better effects, with the exception of photodynamic therapy and FLLL12, and when combined, the effects were better than with each treatment alone.

Cell cycle arrest was also observed (n=8), mostly at G_2/M phase, as reported by seven studies. The sub-G_1 cell population was monitored in six studies, and half of them described its increase after treatment with curcumin. In the other half, no significant difference was noticed when compared to control. Cell cycle-related proteins, such as cyclin D_1 (n=6), cyclin B (n=1), cyclin A_2 (n=1), cyclin E_2 (n=1), CDK1 (n=1), CDK2 (n=1), and CDC25C (n=1), were evaluated in seven studies and found to have their expression decreased. Both cell cycle arrest and increased sub-G_1 population were considered indicators of apoptosis by the authors.

All studies that evaluated apoptosis directly (n=10) confirmed its occurrence after treatment with curcumin. Apoptosis-related protein expression was thoroughly investigated (n=18). The pro-apoptotic proteins caspase-3 (n=12), caspase-7 (n=1), caspase-8 (n=3), caspase-9 (n=7), PARP (n=8), cytochrome c (n=3), Bax (n=4), AIF (n=2), Endo G (n=2), Apaf-1 (n=1), Bik (n=1) and Bim (n=1) were increased in their active forms, whereas the anti-apoptotic proteins Bcl-2 (n=8), NF-κB (n=5), XIAP (n=2), cIAP2 (n=1) and c-flip (n=1) had their expression decreased. Three studies reported no alteration in Bcl-2 and caspase-3 expression. Nuclear morphology alterations (n=9) and DNA fragmentation (n=5) compatible with apoptosis were also observed.

***In vivo* studies**

Out of ten *in vivo* studies, two adopted as animal model hamsters with DMBA-induced oral cancer and the other eight used mice inoculated with CCL23, CAL-27, SCC1, SCC40, SAS/luc, FaDu, Tu 686 or SALTO cells. One of these also used mice with 4NQO-induced oral

cancer [29].

Curcumin was tested alone and only compared to negative control (n=6), used in association with irradiation (n=2) or resveratrol (n=1), or compared to FLLL12 (n=1). Curcumin concentration varied between 0.6% to 1% when added to standard diet, 2 to 15 mg/100 µL when added to corn oil, and 35 to 200 mg/kg when delivered by gavage. Concerning experiment design, studies were heterogeneous regarding controls, period and regime of treatment, and delivery method.

Nine studies described an inhibition of tumor growth or volume as a consequence of treatment with curcumin, considered significant by some authors (n=4). In studies that associated curcumin with other treatments, the effects were better than each treatment alone. A cell-cycle-related protein, cyclin D_1 (n=1), was down-regulated in curcumin-treated animals. The pro-apoptotic proteins caspase-3 (n=4), caspase-8 (n=1), caspase-9 (n=1), cytochrome c (n=2), and Bax (n=2) were increased in their active form and the anti-apoptotic proteins Bcl-2 (n=3), survivin (n=1) XIAP (n=1) and c-flip (n=1) had their expression decreased.

DISCUSSION

Summary of evidence

Curcuma has been used in culinary as a flavoring, coloring, and preservative agent, and in the traditional Ayurvedic and Chinese medicine for over 6,000 years [24]. Curcumin, one of its constituents, shows beneficial effects to human health, provoking interest in researchers. He *et al.* [54] verified that curcumin might be used for the prevention and treatment of several chronic conditions, such as diabetes, cardiovascular, neurological, and pulmonary diseases. The activity of curcumin against breast, colon and lung cancers, lymphoma, and leukemia has also been reported, and its chemo-preventive and therapeutic properties against oral cancer were experimentally and clinically demonstrated [55-57, 33, 58-60]. These data are supported by the

significant cytotoxicity and reduction of cell viability and tumor measurements observed in the reviewed studies.

Overexpression of EGFR, thus activating downstream signaling pathways such as PI3K/AKT/mTOR and Ras/MAPK, has been associated with the development and progression of HNSCC [5, 61]. These alterations result in induction of the proliferation, survival, angiogenesis, invasion, and metastasis behind the oncogenic process [61]. Overexpression and overactivation of proteins related to these pathways and others, such as NF-κB proteins, cyclins, and Bcl-2, all pro-oncogenic proteins, were observed in several included articles, and in all of them these proteins were found to be inhibited or suppressed by curcumin, pointing to an important effect of curcumin on HNSCC treatment.

Dysregulation of apoptosis is one of the most common molecular events known to be associated with the development of HNSCC, and the capacity of re-establishing its signaling, regulating and inducing it, is considered an essential characteristic of a possible anticancer agent [62]. The induction of cell cycle arrest is also desirable, given that an interruption in the cycle and apoptosis are closely related events [63]. Both apoptosis induction and cell cycle arrest were observed on HNSCC cells lines after curcumin treatment, which corroborates to the use of curcumin as an alternative anticancer agent, or at least it instigates further investigations.

Even though seven out of eight studies that evaluated the cell cycle showed a cell cycle arrest in G_2/M phase, the most investigated cell cycle-related protein was cyclin-D_1, associated with the G_1/S transition [64]. Only one study assessed the expression of Cyclin-B, CDK1, and CDC25C, proteins related to the G_2/M transition [64]. Such observation is important, as to direct future studies on which proteins should be further evaluated.

Apoptosis is triggered by two major mechanisms: the extrinsic and the intrinsic pathways [65]. In both pathways, the caspases play important roles as mediators of these processes. Initiator caspases such as Caspase-8 and Caspase-9, which cleave inactive pro-forms of the executor caspase-3 and caspase-7, were evaluated in eight included studies. Caspase-3

and caspase-7 were evaluated in 12 included studies [23, 28, 34-36, 40-43, 46, 49, 51]. The intrinsic or mitochondrial apoptotic pathway is mostly controlled by members of the Bcl-2 family, which may act as either inhibitors or promoters of apoptosis [65]. Bax, Bik, and Bim are pro-apoptotic proteins from the Bcl-2 family and had their increased expression found in seven included studies. Bcl-2 is an anti-apoptotic member of the Bcl-2 family, and its expression was found to be decreased by curcumin as well. The release of cytochrome c from the mitochondria to the cytosol, thus forming the apoptosome with Apaf-1 and caspase-9 and activating executor caspases, is an important process within the intrinsic pathway [66]. An increased expression of cytochrome c and Apaf-1 after treatment with curcumin was observed in four included studies. With these results it is possible to believe that the apoptosis observed in HNSCC cell lines and animal models might be triggered through the mitochondrial-dependent pathway.

The supra-additive effect of curcumin, when associated with currently prescribed chemotherapy agents, radiotherapy, novel therapeutic options or even other plant-derived substances, has been prompting a variety of studies with different types of cancer [67-69], all resulting in an additive effect when compared to treatments alone. Similar findings were observed when curcumin was associated with EGCG, resveratrol, and photodynamic therapy in HNSCC cell lines and animal models. The association of curcumin with irradiation or chemotherapeutic agents such as cisplatin and 5-Fluorouracil, currently prescribed as therapy for HNSCC, is of considerable importance, and was found to be significantly effective in five reviewed studies.

Although curcumin presents low toxicity to humans and animals [70, 55, 71], a number of limiting factors concerning its clinical use has been reported, especially when it comes to solubility, bioavailability and systemic absorption [72, 15]. Solutions for such limitations have been studied, aiming new drug delivery systems by oral administration, as proposed by three included studies. Lu *et al.* [38] demonstrated better solubility and stability of curcumin when it

was encapsulated in folate-linked liposomes, compared to its free form. Chang *et al.* [40] found that curcumin in nanoparticles induced apoptosis and a considerable cell growth inhibition in HNSCC cell lines, but little cytotoxicity in non-cancerous cell lines, evidencing the specificity of such delivery system. Mazzarino *et al.* [45], though, described better cytotoxic effects with free curcumin than with curcumin loaded in nanoparticles, with respective IC_{50} values of 93.40 µM and 271.5 µM after a 24h-treatment.

Limitations

Limitations regarding clinical application of this review should be considered. With our search strategy, we found eight clinical studies, but none of these evaluated the effects of curcumin on HNSCC directly. There is no standard quality assessment tool specific for *in vitro* studies. Therefore, we adapted the GRADE tool [18] to assess the quality of our selected *in vitro* studies, classifying them through comparable baselines. Furthermore, four articles were excluded in phase two because they were written in Chinese, according to our exclusion criteria, and the full copy of five articles could not be obtained. Nonetheless, if their data were included, our results might have been different, and therefore such exclusions were also considered a limitation.

Conclusion

This systematic review demonstrates that curcumin emerges as a potential promise in the treatment of HNSCC. The results showed that curcumin is effective on HNSCC cell lines, inhibiting cell proliferation and viability and inducing apoptosis (via intrinsic pathway) and cell cycle arrest at G_2/M phase. In addition, curcumin reduced tumor burden in animal models. Altogether, this systematic review supports that curcumin might be potentially used in the HNSCC treatment, and that it should be further analyzed in clinical trials.

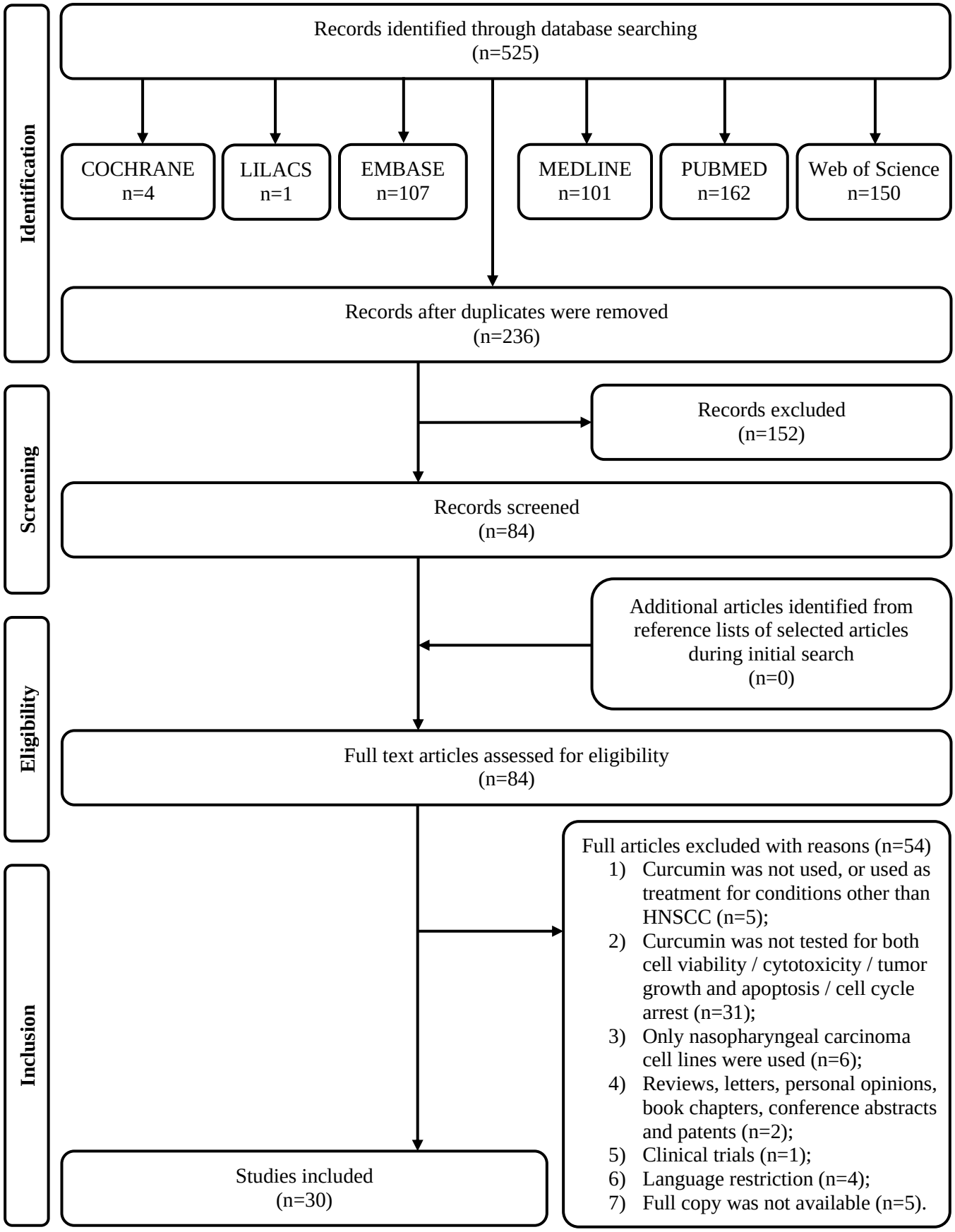

Figure 1. Flow Diagram of literature search and selection criteria adapted from PRISMA [17].

Table 1. Summary of descriptive characteristics of included studies (n=30).

Year	Author	Country	Study Design	Assays	Treatment	Results	Main Conclusions
1998	Khafif *et al.*	USA	*In vitro*	Hoechst 33342 proliferation assay; Cell cycle with ethidium bromide and flow cytometry	Curcumin and/or EGCG	ED_{50} was constant in both control and HNSCC cell lines (3.45 and 5.18 µM). Combination index at ED_{50} was 0.7±0.08 for Curcumin+EGCG, indicating synergism. Cell cycle arrest at G_2/M.	As single agent, curcumin was effective in suppressing growth of both normal and malignant cells. Combination treatment was more effective than each agent alone.
2004	Aggarwal *et al.*	USA	*In vitro*	MTT assay; Cell cycle with propidium iodide (PI) and flow cytometry; Apoptosis with annexin V-FITC and flow cytometry; Western blot	Curcumin	Dose-dependent inhibition of proliferation in all cell lines. Cell cycle arrest at G_1/S (54% in control, 87% at 25 µM) in 24h. Dose-dependent increased apoptosis. Dose-dependent cleavage of caspase-3 and -9 and PARP. Decreased expression of NF-κB, cyclin-D1, and Bcl-2.	Curcumin suppressed proliferation and induced G_1/S phase arrest in all HNSCC cell lines studied, very likely through downregulation of cyclin-D1 and Bcl-2.
2005	LoTempio *et al.*	USA	*In vitro* and *in vivo*	*In vitro*: MTT assay; Cell sorting and apoptosis with PI and annexin V-FITC and flow cytometry; Immuno-fluorescence; Western Blot *In vivo*: Tumor measurement	Curcumin; Curcumin injections and topical paste (*in vivo*)	*In vitro*: Significant dose-dependent cytotoxicity in all cell lines (P<0.0001). Increased cell death (27-33%, depending on the cell line) including early apoptosis (6-17%), at 50 µM. Decreased expression of NF-κB and cyclin-D1. *In vivo*: Curcumin injections had little to no effect on tumors. Saline/curcumin paste inhibited tumor growth.	Curcumin treatment resulted in growth inhibition, which was due to downregulation of the anti-apoptotic NF-κB. *In vivo*, topical applications of saline/curcumin paste were more efficient than injections.
2006	Sharma *et al.*	India and USA	*In vitro*	MTT assay; Cell cycle with PI and flow cytometry	Curcumin	Dose-dependent decrease in viability. IC_{50} was 25-50 µM, depending on the cell line. Increase in sub-G_1 population (14.4% in control, 75.1% in treatment).	Curcumin induced cell death by apoptosis in HNSCC cells, as observed by a sub-G_1 peak in cell cycle analysis.
2008	Garg *et al.*	India	*In vivo*	Tumor measurements; Western blot; TUNEL assay	Curcumin	Decrease in tumor incidence (by 45%), tumor burden (from 700 to 300 mm^3 i.e. by 43%) and tumor multiplicity (by 51%). Decreased latency period of DMBA-induced SCC (10 weeks in control, 12 weeks in treatment). Significant decrease in expression of PCNA and Bcl-2 and increase in Bax and Caspase-3 ($P \leq 0.05$).	Curcumin decreased the DMBA-induced tumor burden and multiplicity and enhanced the latency period of SCC tumors. Higher Bax/Bcl-2 ratio and apoptotic index suggested apoptotic cell death.
2009	Khafif *et al.*	Israel	*In vitro* and *in vivo*	*In vitro*: XTT assay; Cell cycle with PI and flow cytometry *In vivo*: Tumor measurements	Curcumin and/or Irradiation	*In vitro*: Dose-dependent inhibition of viability (at 15 µM, P<0.05). IC_{50} was 15-22 µM, depending on the cell line. Better results when treatments were combined (P<0.05). Minor effects on sub-G_1 population with treatments alone. Additive effect on sub-G_1 population with combination. *In vivo*: No significant decrease in neither tumor volume nor tumor weight with treatments alone. Combined treatment reduced tumor weight (25%, P=0.09) and tumor sizes (15%, P=0.23).	Curcumin inhibited HNSCC cell growth and enhanced effect of radiation *in vitro*. Combined treatment showed a trend towards improvement in tumor weight compared to curcumin or irradiation alone.

2009	Lin *et al.*	Taiwan	*In vitro*	MTT assay; apoptosis evaluation with ApopNexin FITC detection kit; DNA fragmentation; Caspase activity assay; Western blot	Curcumin	Significant dose-dependent decrease in survival and proliferation ($P<0.05$). Dose-dependent increased apoptosis. Significant DNA fragmentation increase at 25 µM ($P<0.05$). Significant decrease in procaspase-3 and increase in activity of caspase-3 ($P<0.05$).	Curcumin could inhibit cell proliferation and irreversibly induce apoptosis in HNSCC cells.
2010	Clark *et al.*	USA	*In vitro* and *in vivo*	*In vitro*: MTS assay; Cell cycle with PI and flow cytometry; Western blot *In vivo*: Tumor measurements	Curcumin	*In vitro*: Significant inhibition of HNSCC cells in comparison with normal cells at 10 µM and after 72h ($P<0.05$). Significant cell cycle arrest at G_2/M with one cell line at 10 µM and at G_1/S with two other cell lines at 20 µM ($P<0.001$). No increase in sub-G_1 population. Decreased cyclin-D1 ($P\leq0.01$). No cleaved caspase-3. *In vivo*: Significant reduction in xenograft tumor volume at 5mg (days 0-16, $P=0.02$) and at 15mg (day 5, $P=0.01$. day 8, $P=0.02$). No significant difference at 10mg. Pretreatment suppressed growth of xenograft tumor ($P<0.01$) at 15mg.	Curcumin inhibited cell proliferation but did not induce apoptosis in the HNSCC cell lines studied. *In vivo* growth inhibitory effects were noted at earlier time points, which suggests that curcumin inhibits the grafting of tumor cells. It delayed carcinogenesis, indicating its potential for chemoprevention.
2010	Lin *et al.*	Taiwan	*In vitro* and *in vivo*	*In vitro*: SRB assay; Cell cycle with PI and flow cytometry *In vivo*: Optical imaging with luciferase gene transfection; Bioluminescence imaging (BLI)	Curcumin	*In vitro*: IC_{50} was about 3 µM. Dose-dependent increase in sub-G_1 population. Significant cell cycle arrest at G_2/M (33.58% in control, 47.72% at 3 µM, 49.93% at 5 µM and 39.83% at 7 µM, $P<0.05$). *In vivo*: Significant decrease in tumor volume on the 26^{th} day ($P<0.01$). No difference among different drug-treated groups. No significant body weight change in mice with or without treatments.	The inhibitory effects of curcumin on the growth of HNSCC cells and of the tumor were significant.
2010	Shin *et al.*	South Korea	*In vitro*	Viability assay with the cell counting Kit-8; Nuclear morphology with Hoechst 33342	Curcumin	Strong dose-dependent inhibition of proliferation. IC_{50} was 8.37±1.13 µM. Nuclear morphological features compatible with apoptosis at 10 µM.	Curcumin induced inhibition of proliferation and possibly apoptosis.
2011	Ip *et al.*	Taiwan	*In vitro*	Viability assay with PI and flow cytometry; Cell cycle with PI and flow cytometry; DAPI staining; Western blot	Curcumin	Dose-dependent decrease in viability in 24h ($P<0.05$ at 15 and 20 µM) and 48h ($P<0.05$ at 5, 10, 15, and 20 µM). Marked cell cycle arrest at G_2/M phase (20.26%, compared to control). Increased sub-G_1 population (16.02%). Decrease in expression of cyclin-B, CDK1, CDC25C, Bcl-2, and XIAP. Increase in AIF and Endo G.	Curcumin delayed cell cycle progression, and the observed decreased viability might be justified by cell cycle arrest and apoptosis.
2011	Liao *et al.*	China and the USA	*In vitro*	MTT assay; Colony formation assay; ELISA apoptosis detection kit; Apoptosis with PI and annexin V-FITC and flow	Curcumin	Significant cell growth inhibition (5 µM in 48h, $P<0.05$; 5 µM in 72h and 7.5 µM in 48 and 72h, $P<0.01$). Significant reduction in colony formation ($P<0.01$). Dose-dependent apoptosis (14% in control, 26% at 5 µM after 48h. Cell cycle arrest at G_2/M (5.5% in control, 13.6% at 5 µM).	Curcumin elicited a dramatic effect on growth inhibition and induction of apoptosis in HNSCC cells, besides inducing a G_2/M phase arrest.

				cytometry; Cell cycle with PI and cytometry; Western blot		Decreased expression of Bcl-2 and cyclin-D1.	
2012	Ahn *et al.*	South Korea	*In vitro*	MTT assay; Morphological analysis with Hoechst 33342 and PI; Western blot	Curcumin and/or photo-dynamic therapy	Dose- and time-dependent decrease in cell viability. Nuclear morphological features compatible with apoptosis. Increased cleaved caspase-3 and -9, cleaved PARP and cytochrome c. Effects were enhanced when treatments were combined.	Photodynamic therapy associated with curcumin enhanced cytotoxic and apoptotic effects in HNSCC cells via mitochondria-dependent apoptosis.
2012	Jeon *et al.*	South Korea	*In vitro*	MTT assay; DAPI staining; DNA fragmentation; Western blot; Caspase activity assay with PhiPhiLux-G_1D_2	Curcumin; EF-24, curcumin analog	No effect observed at 0.1-10 µM. Significant inhibition of viability at 30 and 100 µM ($P<0.001$). IC_{50} was 30.61±2.83 µM. Nuclear morphology compatible with apoptosis. DNA ladder formation at 50 µM. Increased cleaved caspase-3, -7 and -9.	Curcumin inhibited growth of HNSCC cells by activating apoptosis, possibly through caspases-3, -7 and -9-dependent processes.
2012	Kim *et al.*	South Korea	*In vitro*	MTT assay; Cell cycle with PI and flow cytometry; Western blot; Morphological analysis	Curcumin	Significant inhibition of viability at 10, 20, and 40 µM ($P<0.05$). Morphological features compatible with apoptosis at 20 µM. No increase in sub-G_1 population at 10 µM. Time-dependent increase in cleaved PARP and caspase-3 at 10 µM.	Curcumin potently induced cell death in the absence of a high ratio of apoptotic cells, implying that it may activate another cell death pathway, such as autophagy.
2012	Kumar *et al.*	India	*In vivo*	Tumor measurements; Western blot; Immunohistochemical staining; TUNEL assay	Curcumin	Inhibition of tumor growth. Decreased tumor multiplicity (by 40-47%) and tumor burden (by 55-63%). Increased Bax and decreased Bcl-2 (significantly in 4 weeks, $P<0.05$). Increase in caspase-3 and -9 and cytochrome c and decreased survivin, PCNA, and cyclin-D1 ($P<0.05$).	Curcumin decreased tumor burden and multiplicity, due to enhanced apoptosis, as revealed by increased Bax/Bcl-2 ratio and apoptotic index and decreased proliferation or both.
2012	Lu *et al.*	China	*In vitro*	MTT assay; Apoptosis with PI and annexin V-FITC and flow cytometry	Free curcumin; Curcumin-loaded liposomes	Dose-dependent inhibition. IC_{50} was 14.5µg/mL for free curcumin, 16.3µg/mL for folate-receptor-targeted liposomal curcumin (F-CUR-L) and 29.5µg/mL for non-targeted liposomal curcumin (CUR-L). Time-dependent apoptosis (mean rate: 46.7%) with F-CUR-L.	F-CUR-L enhanced solubility of curcumin and could specifically target a folate-receptor-positive tumor cell line.
2013	Camacho-Alonso *et al.*	Spain	*In vitro*	MTT assay; ELISA apoptosis detection kit	Curcumin or lycopene and/or irradiation	Dose-dependent cytotoxicity, especially evident at 5.5 and 6.75 µM. Better results for curcumin than for lycopene. Apoptosis observed with or without irradiation, although more intense when treatments were combined.	Curcumin increased cytotoxic activity, and combination with irradiation exerted synergic effect.
2013	Chang *et al.*	Taiwan	*In vitro*	MTT assay; Morphological analysis; DAPI staining; Western blot; Caspase-3 and -9 colorimetric assay	Curcumin-loaded nano-particles	Significant dose- and time-dependent viability decrease at 20, 40, and 80 µM ($P<0.001$). Morphological features compatible with apoptosis. No viability impact and morphological changes in control cell lines ($IC_{50}>80$ µM). Dose-dependent nuclear condensation. Increase in cleaved caspase-3 and -9, cytochrome c, Apaf-1, AIF, Endo G, and Bax. Downregulated Bcl-2.	Curcumin nanoparticles caused anti-proliferative effects in a dose- and time-dependent manner, but little toxicity to normal cells. It induced apoptosis, possibly through the intrinsic signaling pathway.

2013	Rak *et al.*	Croatia	*In vitro*	MTT assay; Comet assay; Nuclear morphology with acridine orange and ethidium bromide; Apoptosis with PI and annexin V-FITC and flow cytometry; Western blot	Curcumin	Dose-dependent inhibition of cell growth for all cell lines. Nuclear morphology and staining compatible with apoptosis. Apoptosis (approx. 40% compared to control at 25 µM). Cleavage of PARP and caspase-3. No alteration in Bcl-2 expression.	Curcumin exhibited potential in treatment of HNSCC cell lines, also inducing apoptotic cell death.
2014	Chiang *et al.*	Taiwan	*In vitro* and *in vivo*	*In vitro*: MTT assay; Colony formation assay; DNA fragmentation; Western blot *In vivo*: Tumor measurements; Bioluminescent imaging (BLI); Western blot	Curcumin and/or irradiation	*In vitro*: Significant time- and dose-dependent decrease in viability ($P<0.01$). IC_{50} was about 30 µM. DNA laddering with 48h. Increased cleaved caspase-3 and -8 and cytochrome c. Decreased Bcl-2, XIAP, and c-flip. These effects were enhanced when treatments were combined. *In vivo*: Significant inhibition of tumor growth ($P<0.01$). Increased cleaved caspase-3 and -8 and cytochrome c. Decrease in Bcl-2, XIAP, and c-flip. These effects were enhanced when treatments were combined ($P<0.01$).	Curcumin inhibited cell proliferation and induced apoptosis via mitochondrial-dependent and independent pathways. It also sensitized cells to radiation and enhanced its antitumor effect.
2014	Masuelli *et al.*	Italy	*In vitro* and *in vivo*	*In vitro*: SRB assay; Cell cycle with PI and flow cytometry; Western blot *In vivo*: Tumor measurements	Curcumin and/or resveratrol	*In vitro*: Significant dose-dependent cell growth reduction for all cell lines ($P<0.001$). Combined treatment was more effective than curcumin alone ($P<0.05$ at 6.25-12.5 µM). Significant increase in sub-G_1 population ($P<0.001$ at 25 µM). Significant cleavage of PARP-1 ($P<0.01$) and increased Bax/Bcl-2 ratio ($P<0.01$), due to Bcl-2 downregulation. Variations in protein expression were more intense with combined treatment ($P<0.05$). Reduced levels of procaspase-9 and -8 ($P<0.001$) and cleavage of caspase-3 and -8. *In vivo*: Both treatments decreased tumor volume by the fourth week. Combined treatment was more effective than curcumin alone by the eighth/ninth week ($P=0.0092$ when treatment started before tumor challenge. $P=0.046$ when started with tumor challenge).	Curcumin inhibited proliferation and induced apoptosis in HNSCC cells, and the combination with resveratrol potentiates these effects. Curcumin was able to delay tumor growth and prolong survival significantly. Resveratrol potentiated these effects. Combined treatment was more potent when administered before tumor challenge.
2014	Zhen *et al.*	China	*In vitro*	MTT assay; Cell cycle with PI and flow cytometry	Curcumin	Dose-dependent inhibition of proliferation. Cell cycle arrest at G_2/M at 10 and 40 µM ($P<0.05$).	Curcumin could inhibit proliferation and block cells at G_2/M phase.
2015	Anisuzzaman *et al.*	USA	*In vitro* and *in vivo*	*In vitro*: SRB assay; Apoptosis with PI and annexin V-Phycoerythrin and flow cytometry *In vivo*: Tumor measurements	Curcumin; FLLL12, curcumin analog	*In vitro*: IC_{50} was 4.53-17.42 µM for curcumin, depending on the cell line. IC_{50} was 0.35-1.55 µM for the analog. FLLL12 was 10-24-fold more potent than curcumin. Time- and dose-dependent apoptosis (10-15 µM curcumin or 1-3 µM FLLL12 – 80% apoptosis in 48h). *In vivo:* The analog decreased tumor volume when compared to control and curcumin ($P<0.05$).	FLLL12 inhibited proliferation and tumor growth and induced apoptosis. It was more potent than curcumin both *in vitro* and *in vivo.*

2015	Fetoni *et al.*	Italy	*In vitro*	DAPI staining; Western blot; TUNEL assay	Curcumin and/or cisplatin	Dose-dependent cytotoxicity – cell loss of 20-70% at 0.5-6.75 µM in 48 and 72h – with curcumin alone. When associated with cisplatin, cell loss was of 30-90% at the same experimental conditions. Nuclear morphology compatible with apoptosis. Increase in marked apoptotic cells of 15-20%. Increased Bax. With cisplatin, apoptotic cells were 35-40%.	Curcumin alone induced antiproliferative and cytotoxic effects and increased pro-apoptotic activity. As an adjuvant to cisplatin therapy, both antiproliferative and apoptotic effects were enhanced.
2015	Hu *et al.*	China	*In vitro* and *in vivo*	*In vitro*: MTT assay; Apoptosis with PI and annexin V-FITC and flow cytometry *In vivo*: Tumor measurements; Immunofluorescence	Curcumin	*In vitro:* Significant dose-dependent decrease in cell viability ($P<0.01$). Significant cleavage of PARP ($P<0.01$). Significant apoptosis (17.2-21.6% when compared to control at 7 and 10 µM, $p<0.05$). Blocked NF-κB activity; Cleavage of caspase-3, -8 and -9. *In vivo:* Significant reduction in tumor growth ($P<0.05$); Increase in cleaved caspase-3 positive cells. No toxicity to mice in concentrations up to 200 mg/kg.	Curcumin inhibited proliferation and induced apoptosis in HNSCC cell lines. Additionally, it reduced tumor growth and increased the presence of cleaved caspase-3 *in vivo.*
2015	Mazzarino *et al.*	Brazil	*In vitro*	MTT assay; Nuclear morphology with acridine orange and ethidium bromide	Free curcumin; Curcumin-loaded nano-particles	Significant reduction of viability at 100 µM and 48h ($P<0.05$). In 24h, IC_{50} was 93.40±4.26 µM for free curcumin and 271.5±1.17 µM for curcumin-loaded nanoparticles (Cur-NPs). In 48h, IC_{50} 75.21±3.25 µM for free curcumin and 260.3±8.35 µM for Cur-NP. Effects were better with free curcumin. Nuclear morphology and staining compatible with apoptosis after 24h.	Curcumin-loaded nanoparticles coated with chitosan decreased cell viability significantly by inducing apoptosis. Even so, results were better for free curcumin.
2015	Mishra *et al.*	India	*In vitro*	MTT assay; EMSA; Western blot	Curcumin	Dose-dependent reduction of viability (<30% at 100 µM). Downregulation of Bcl-2, cIAP2, and NF-κB. Upregulation of Bax.	Curcumin was capable of inducing apoptosis in HPV16-infected HNSCC cells.
2015	Sivananth am *et al.*	India	*In vitro*	SRB assay; Colony formation assay; Nuclear morphology with DAPI; Cell cycle with PI and flow cytometry; Western blot	Curcumin and/or 5-fluorouracil, doxorubicin or cisplatin	Dose-dependent growth inhibition. IC_{50} was 10.12 µM. Curcumin combined with 5-FU, doxorubicin, or cisplatin enhanced cell growth inhibition. Associated with 5-FU or doxorubicin, curcumin decreased expression of Bcl-2, cyclin E2, cyclin D1, and CDK2 and increased Bax, cleaved caspase-3, and PARP. With doxorubicin, cell cycle arrest at G_2/M and downregulation of cyclin A2 and B1. With 5-FU, cell cycle arrest at G_1/S.	Curcumin, when associated with doxorubicin or 5-fluorouracil, exerts important cytotoxicity in HNSCC cells, along with cell cycle arrest and apoptosis.
2015	Xi *et al.*	China and the USA	*In vitro*	Viability assay with the cell counting kit-8; Colony formation assay; DNA fragmentation; Western blot	Curcumin	Significant reduction in cell viability ($P<0.05$), with IC_{50} about 12.5 µM in 24h. Significant colony formation reduction ($P<0.05$). DNA fragmentation consistent with apoptosis. Increased cleaved caspase-3 and -9 and PARP, Bik and Bim. Inhibition of cyclin-D1. No difference in Bcl-x, Bcl-w, Mcl-1, and Bcl-2.	Curcumin could inhibit HNSCC cell growth in a dose and time-dependent manner. It induced apoptosis by activating the intrinsic mitochondrial-mediated pathway.

Table 2. Description of PICOS for included studies.

Studies	**P**	**I**			**C**	**O**	**S**
	Cell Line / Animal Model	**Intervention**	**Treatment time**	**Dose**			
Khafif *et al.* 1998	1483; NOE	Curcumin; Curcumin + EGCG; EGCG; Ethanol (Control)	120h	1-100 µM	√	√	√
Aggarwal *et al.* 2004	MDA 1986; Tu 686; Tu 167; MDA 686LN; JMAR C42	Curcumin; DMSO (Control)	24-144h	1-50 µM	√	√	√
LoTempio *et al.* 2005	UM-SCC1; UM-SCC14A; CAL-27; HEp-2; nude mice inoculated with HEp-2 and CAL-27 cells	Curcumin; DMSO (Control)	8h; up to 5 weeks (*in vivo*)	50-400 µM; 50-250 µM/L injections (*in vivo*); 1g/mL topical paste (*in vivo*)	√	√	√
Sharma *et al.* 2006	AMOS-III; SCC38	Curcumin	24-120h	0.0001-100 µM	√	√	√
Garg *et al.* 2008	DMBA-induced cancer in male Syrian hamsters	Curcumin; Standard Laboratory Diet (Control)	15 before, during and 12 weeks after induction	1% in standard laboratory diet	√	√	√
Khafif *et al.* 2009	SCC1; SCC9; KB; CD-1 nude mice inoculated with SCC1	Curcumin; Curcumin + Irradiation; Irradiation; Standard Laboratory Diet (*in vivo* control)	72h; 14 days prior to inoculation and after (*in vivo*)	5-50 µM; 1-6 Gy; 0.6% in standard laboratory diet (*in vivo*)	√	√	√
Lin *et al.* 2009	Detroit 562	Curcumin	24-48h	25-100 µM	√	√	√
Clark *et al.* 2010	FaDu; SCC25; SCC40; SCC066; SCC114; SCC116; PCI13; PCI15a; PCI30; OKF6; BALB/c mice inoculated with SCC40; 4NQO-induced cancer in CBA/Caj mice	Curcumin; Corn oil (*in vivo* control)	24-72h; 5 times a week, up to 20 weeks (*in vivo*)	10-40 µM; 5-15mg/100µL (*in vivo*)	√	√	√
Lin *et al.* 2010	SAS; NOD/ SCID mice inoculated with SAS/*luc*	Curcumin; NaOH (*in vivo* control)	24h; every 1-3 days (*in vivo*)	1-30 µM; 35-100 mg/kg (*in vivo*)	√	√	√

Shin *et al.* 2010	YD-10B	Curcumin	24h	1-100 µM	√	√	√
Ip *et al.* 2011	SCC4	Curcumin; DMSO (Control)	24-48h	1-20 µM	√	√	√
Liao *et al.* 2011	CAL-27	Curcumin; DMSO (Control)	24-72h	2.5-7.5 µM	√	√	√
Ahn *et al.* 2012	AMC-HN3	Curcumin; Curcumin + Photo-dynamic therapy (PDT); PDT	6h	25 µM	√	√	√
Jeon *et al.* 2012	KB	Curcumin; EF-24; DMSO (Control)	24h	0.1-100 µM	√	√	√
Kim *et al.* 2012	YD10B	Curcumin; DMSO (Control)	24h	1-40 µM	√	√	√
Kumar *et al.* 2012	DMBA-induced cancer in male Syrian hamsters	Curcumin; Standard Laboratory Diet (Control)	Up to 4 weeks after induction	1% in standard laboratory diet	√	√	√
Lu *et al.* 2012	KB	Free curcumin; Curcumin-loaded liposomes; Empty liposomes	48h	IC_{50} - 14.5-29.5 µg/mL	√	√	√
Camacho-Alonso *et al.* 2013	PE/CA-PJI15	Curcumin; Lycopene; Curcumin + Irradiation; Lycopene + Irradiation Irradiation; DMSO (Control)	24-72h	3-6.75 µM; 1-5 Gy	√	√	√
Chang *et al.* 2013	CAR; HGF; OK	Curcumin-loaded nanoparticles; Empty nanoparticles	24-48h	10-80 µM	√	√	√
Rak *et al.* 2013	HEp-2; 7T	Curcumin; DMSO (Control)	72h	10-40 µM	√	√	√
Chiang *et al.* 2014	SAS; NOD/SCID mice inoculated with SAS/*luc*	Curcumin; Curcumin + Irradiation; Irradiation; DMSO (Control)	24-48h; daily for 3 weeks (*in vivo*)	10-50 µM; 2-12 Gy; 70 mg/kg (*in vivo*)	√	√	√
Masuelli *et al.* 2014	CAL-27; SCC15; FaDu; BALB/c mice inoculated with SALTO	Curcumin; Curcumin + Resveratrol; Resveratrol; DMSO (*in vitro* control); Corn oil or water (*in vivo* control)	48h; daily, 2 weeks before and 3 times a week after inoculation (*in vivo*)	6-50 µM; 2 mg/100µL (*in vivo*)	√	√	√
Zhen *et al.* 2014	SCC25	Curcumin; DMSO (Control)	24-48h	10-80 µM	√	√	√
Anisuzzaman *et al.* 2014	Tu 212; Tu 686; MDA686LN; PCI13; SqCCY1; UM-22B; nude mice inoculated with Tu 686	Curcumin; FLLL12; DMSO (Control)	24-72h; 5 days a week, for up to 3 weeks (*in vivo*)	Not described for IC_{50} calculation; 50 mg/kg (*in vivo*)	√	√	√

Fetoni *et al.* 2015	PE/CA-PJ15	Curcumin; Curcumin + Cisplatin; Cisplatin; DMSO (Control)	24-72h	0.5-6.75 µM	√	√	√
Hu *et al.* 2015	FaDu; CAL-27; nude mice inoculated with FaDu	Curcumin	6-48h; daily for 4 weeks (*in vivo*)	7-10 µM; 200 mg/kg (*in vivo*)	√	√	√
Mazzarino *et al.* 2015	SCC9	Free curcumin; Curcumin-loaded nanoparticles; Empty nanoparticles	24-72h	1-100 µM	√	√	√
Mishra *et al.* 2015	93VU147T	Curcumin	24-72h	10-100 µM	√	√	√
Sivanantham *et al.* 2015	NT8e	Curcumin; Curcumin + 5-Fluorouracil (5-FU); Curcumin + Doxorubicin (DOX); Curcumin + Cisplatin (CIS); 5-FU; DOX; CIS	48h	1-25 µM;	√	√	√
Xi *et al.* 2015	FaDu; CAL-27	Curcumin; DMSO (Control)	16-72h	1.7-25 µM	√	√	√

P: Population; I: Intervention; C: Control; O: Outcomes; S: Study (RCT or Comparable baselines); √ - Yes.

1483 – Retromolar Trigone SCC; 7T – Carboplatin resistant HEp-2 subline; 93VU147T – Oropharynx SCC; AMC-HN3 – HNSCC; AMOS-III – Mouth Floor SCC; CAL-27 – Tongue SCC; CAR – CAL-27 cisplatin resistant subline; Detroit 562 – Pharynx SCC; FaDu – Oropharynx SCC; HEp-2 – HeLa contaminant; JMAR C42 – Floor Mouth SCC; KB – HeLa contaminant; MDA 1986 – Cervical Node ;Metastasis of Tongue SCC; MDA 686LN – Lymph Node Metastasis of Tongue SCC; NT8e – Oral cavity SCC; PCI13 – HNSCC; PCI15a – HNSCC; PCI30 – HNSCC; PE/CA-PJ15 – Tongue SCC; SALTO – Salivary Gland Cancer; SAS – Tongue SCC; SCC066 – HNSCC; SCC1 – Oral Cavity SCC; SCC114 – Mouth Floor SCC; SCC116 – HNSCC; SCC15 – Tongue SCC; SCC25 – Tongue SCC; SCC38 – HNSCC; SCC4 – Tongue SCC; SCC40 – Tongue SCC; SCC9 – Tongue SCC; SqCCY1 – Oral cavity SCC; Tu 167 – Mouth Floor SCC; Tu 212 – Hypopharynx SCC; Tu 686 – Base of Tongue SCC; UM-22B – Lymph node metastasis of hypopharynx cancer; UM-SCC1 – HNSCC; UM-SCC14A – HNSCC; YD10B – Tongue SCC; HGF – Human Gingival Fibroblasts; NOE – Normal Oral Epithelial; OKF6 – Oral Keratinocytes; and OK – Oral Keratinocytes.

Table 3. Risk of bias in individual studies according to GRADE criteria.

Author	Study Limitation	Inconsistency	Indirect-ness	Imprecision	Publication Bias	Dose effect	Overall Quality
Khafif *et al.* 1998	√	√	√	Unclear[1]	√	√	+++
Aggarwal *et al.* 2004	√	√	√	Unclear[1]	√	√	+++
LoTempio *et al.* 2005	√	√	√	√	√	√	++++
Sharma *et al.* 2006	√	√	√	√	√	√	++++
Khafif *et al.* 2009	√	√	√	√	√	√	++++
Lin *et al.* 2009	√	√	√	√	√	√	++++
Clark *et al.* 2010	√	√	√	√	√	√	++++
Lin *et al.* 2010	√	√	√	√	√	√	++++
Shin *et al.* 2010	√	√	√	√	√	√	++++
Ip *et al.* 2011	√	√	√	√	√	√	++++
Liao *et al.* 2011	√	√	√	√	√	√	++++
Ahn *et al.* 2012	√	√	√	√	√	√	++++
Jeon *et al.* 2012	√	√	X[2]	√	√	√	+++
Kim *et al.* 2012	√	√	√	√	√	√	++++
Lu *et al.* 2012	√	√	X[2]	√	√	√	+++
Camacho-Alonso *et al.* 2013	√	√	√	√	√	√	++++
Chang *et al.* 2013	√	√	√	√	√	√	++++
Rak *et al.* 2013	√	√	X[3]	√	√	√	+++
Chiang *et al.* 2014	√	√	√	√	√	√	++++
Masuelli *et al.* 2014	√	√	√	√	√	√	++++
Zhen *et al.* 2014	√	√	√	√	√	√	++++
Anisuzzaman *et al.* 2015	√	√	√	√	Unclear[4]	√	+++

Fetoni *et al.* 2015	√	√	X[5]	Unclear[1]	√	√	++
Hu *et al.* 2015	√	√	√	√	√	√	++++
Mazzarino *et al.* 2015	√	√	√	√	√	√	++++
Mishra *et al.*	√	√	√	√	√	√	++++
Sivanantham *et al.* 2015	√	√	√	√	√	√	++++
Xi *et al.* 2015	√	√	√	√	√	√	++++

[1]Statistical analysis for considered results is not shown; [2]KB was the only cell line used; [3]Hep-2 and 7T were the only cell lines used; [4]The curcumin concentrations used to establish the IC_{50} values were not described; [5]The experimental design did not fully answer our question – Evaluation of effects of curcumin and cisplatin on ototoxicity. Grade Factors: √, No Serious Limitations; X, Serious Limitations; Unclear, Unable to rate item based on available information. For Overall Quality of Evidence: + very low; ++ low; +++ moderate; ++++ high.

Table 4. Risk of bias in individual studies according to SYRCLE's RoB criteria.

Item	LoTempio *et al.* 2005	Garg *et al.* 2008	Khafif *et al.* 2009	Clark *et al.* 2010	Lin *et al.* 2010	Kumar *et al.* 2012	Chiang *et al.* 2014	Masuelli *et al.* 2014	Anisuzzaman *et al.* 2015	Hu *et al.* 2015
1) Was the allocation sequence adequately generated and applied?	U	U	U	U	U	U	U	U	U	U
2) Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3) Was the allocation to the different groups adequately concealed?	U	U	U	U	U	U	U	U	U	U
4) Were the animals randomly housed during the experiment?	U	Y	Y	U	U	Y	Y	U	U	U
5) Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	U	U	U	U	U	U	U	U	U	U
6) Were animals selected at random for outcome assessment?	U	U	U	U	U	U	Y	U	U	U
7) Was the outcome assessor blinded?	U	U	U	U	U	U	U	U	U	U
8) Were incomplete outcome data adequately addressed?	U	U	U	U	U	U	U	U	U	U
9) Are reports of the study free of selective outcome reporting?	U	U	U	U	U	U	U	U	U	U
10) Was the study apparently free of other problems that could result in high risk of bias?	U	U	U	U	U	U	U	U	U	U

Y=yes, N=no, U=unclear

Appendix 1. Database search.

Database	Search
Cochrane (December 6, 2015 Update: January 29, 2016)	(("oral cancer" OR "oral carcinoma" OR "oral cancers" OR "oral carcinomas" OR OSCC OR "head and neck cancer" OR "head and neck carcinoma" OR "head and neck cancers" OR "head and neck carcinomas" OR HNSCC) OR (MeSH descriptor: [Head and Neck Neoplasms] explode all trees)) AND (curcumin OR curcuma)
LILACS (December 6, 2015 Update: January 29, 2016)	(("câncer oral" OR "carcinoma oral" OR "câncer de cabeça e pescoço" OR "carcinoma de cabeça e pescoço" OR "neoplasmas de cabeça e pescoço" OR HNSCC OR OSCC OR "carcinoma de células escamosas" OR "neoplasias bucais" OR "neoplasias de cabeça e pescoço") OR ("cáncer oral" OR "carcinoma oral" OR "cáncer de cabeza y cuello" OR "carcinoma de cabeza y cuello" OR "neoplasias de cabeza y cuello" OR "carcinoma de células escamosas" OR "cáncer oral" OR "neoplasias de cabeza y cuello")) AND (curcuma OR curcumina)
MEDLINE (December 6, 2015 Update: January 29, 2016)	(("Head and Neck Neoplasms" or Mouth Neoplasms) OR (Mandibular Neoplasms) OR ((head and neck neoplasms).mp.) OR (Tongue Neoplasms or Palatal Neoplasms) OR ("oral carcinoma*".mp.) OR ("oral cancer*".mp.) OR ("head and neck cancer*".mp.) OR ("head and neck carcinoma*".mp.)) AND ((Curcumin) OR (curcumin.mp.) OR (curcuma.mp.))
PubMed (December 6, 2015 Update: January 29, 2016)	(("oral cancer" OR "oral carcinoma" OR "oral cancers" OR "oral carcinomas" OR "OSCC" OR "head and neck cancer" OR "head and neck carcinoma" OR "head and neck cancers" OR "head and neck carcinomas" or "HNSCC" OR (head and neck cancer[MeSH Terms]) OR (head and neck neoplasms[MeSH Terms]) OR (cancer of head and neck[MeSH Terms])) AND (curcumin OR curcuma))
Web of Science (December 6, 2015 Update: January 29, 2016)	(“oral cancer" OR "oral carcinoma" OR "oral cancers" OR "oral carcinom OR OSCC OR "head and neck cancer" OR "head and neck carcinoma" O "head and neck cancers" OR "head and neck carcinomas” OR HNSCC O "head and neck neoplasms") AND (curcumin OR curcuma)
EMBASE (January 29, 2016)	("oral cancer" OR "oral carcinoma" OR "oral cancers" OR "oral carcinomas" OR OSCC OR "head and neck cancer" OR "head and neck carcinoma" OR "head and neck cancers" OR "head and neck carcinomas" OR HNSCC) AND (curcumin OR curcuma)

Appendix 2. Excluded articles and reasons for exclusion (n=54).

Authors	Reason for exclusion
1. Aggarwal *et al.* (2007)	2
2. Azuine et Bhide (1994)	2
3. Balakrishnan *et al.* (2010)	2
4. Basak *et al.* (2015)	1
5. Chakravarti *et al.* (2006)	2
6. Chang *et al.* (2010)	2
7. Chen *et al.* (2011)	6
8. Cohen *et al.* (2009)	2
9. D'Ambrosio *et al.* (2000)	7
10. de Paiva Goncalves *et al.* (2015)	2
11. Duarte *et al.* (2010)	2
12. Dudas *et al.* (2013)	2
13. Elattar et Virji (2000)	7
14. Giudice (2012)	2
15. Gosangari et Watkin (2012)	2
16. Hann *et al.* (2013)	2
17. Jiang *et al.* (2001)	7
18. Jin *et al.* (2013)	2
19. Khafif *et al.* (2005)	2
20. Krishnaswamy *et al.* (1998)	2
21. Kumar *et al.* (2010)	4
22. Kumar *et al.* (2014)	1
23. Kuo *et al.* (2011)	3
24. Lee *et al.* (2015)	2
25. Lee *et al.* (2013)	5
26. Li (a) *et al.* (2002)	6
27. Li (b) *et al.* (2002)	1
28. Lin (a) *et al.* (2012)	2
29. Lin (b) *et al.* (2012)	2
30. Lopez-Jornet *et al.* (2011)	2
31. Manoharan *et al.* (2009)	2
32. Manoharan *et al.* (1996)	7
33. Meyer *et al.* (2011)	2
34. Reiter *et al.* (2012)	1
35. Rinaldi *et al.* (2002)	2
36. Roomi *et al.* (2015)	1
37. Sakagami *et al.* (2000)	7
38. Singh (a) *et al.* (2014)	2
39. Singh (b) *et al.* (2014)	2
40. Spingarn *et al.* (1998)	2
41. Tsang *et al.* (2012)	2
42. Tuttle *et al.* (2012)	2
43. Wang (a) *et al.* (2008)	2
44. Wang (b) *et al.* (2008)	6
45. Wang (a) *et al.* (2011)	3

46. Wang (b) *et al.* (2011)	3
47. Wang *et al.* (2014)	3
48. Weir et Hague (2002)	4
49. Wong *et al.* (2010)	3
50. Wu *et al.* (2014)	3
51. Xiao *et al.* (2014)	2
52. Yan *et al.* (2005)	2
53. Yang *et al.* (2006)	6
54. Yu *et al.* (2013)	2

1) Curcumin was not used, or used as treatment for conditions other than HNSCC;
2) Curcumin was not tested for both cytotoxicity / tumor growth and apoptosis / cell cycle arrest;
3) Only nasopharyngeal carcinoma cell lines were used;
4) Reviews, letters, personal opinions, book chapters, conference abstracts, and patents;
5) Clinical trials;
6) Written in alphabets other than Latin alphabet;
7) Full copy was not available.

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5. ARTICLE 2

Title: Curcumin downregulates the PI3K-AKT-mTOR pathway and inhibits growth and progression in head and neck cancer cells

Running/short title: *In vitro* effects of curcumin in HNSCC cells

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Conflict of interest

The authors have no conflict of interest.

ABSTRACT

Curcumin, a polyphenol isolated from the rhizome of *Curcuma longa*, has been thoroughly studied in the past years due to its antioxidant, antimicrobial, and anti-inflammatory properties. This study aimed to evaluate the biological effects of curcumin on HNC (Head and Neck Cancer) cell lines, and how it modulates the PI3K-AKT-mTOR signaling pathway. Dose-response curves for curcumin were established with FaDu (hypopharynx carcinoma), SCC-9 (tongue carcinoma), and HaCaT (keratinocytes) cell lines and IC_{50} values were calculated. The cells were treated at IC_{50} concentrations and submitted to flow cytometry assays, to investigate cell cycle and cell death, and to phalloidin+FITC staining, to assess cytoskeleton organization. Also, qPCR array and western blot assays were performed to analyze gene and protein expression. Curcumin reduced cell viability in a dose-dependent and selective manner, induced cell death on SCC-9 cells, and arrested cell cycle at phase G_2/M on both SCC-9 and FaDu cells. A disorganized cytoskeleton and modifications on cell morphology were observed. Furthermore, curcumin downregulated the PI3K-AKT-mTOR signaling pathway by altering the expression of key genes and proteins. All those findings might be interrelated, and they highlight the promising therapeutic potential of curcumin to inhibit HNC growth and progression and to modulate the PI3K-AKT-mTOR signaling pathway.

Keywords: Curcumin, head and neck cancer, cell cycle, cell death, cytoskeleton, PI3K-AKT-mTOR pathway

INTRODUCTION

Curcumin, a natural polyphenol isolated from *Curcuma longa*, has been extensively studied in the past years, mostly due to its antioxidant, antimicrobial, and anti-inflammatory properties [1-3]. Such effects might be explained by its ability to interact with different transcription factors, inflammatory mediators, and protein kinases, and therefore modulate signaling pathways that are linked to several conditions [1, 4, 5].

As reported by the GLOBOCAN 2018, the global incidence for oral cavity, oropharynx, and hypopharynx cancers combined was estimated at 528,359 in 2018 [6]. Head and neck squamous cell carcinoma (HNSCC), the most frequent of the head and neck cancers (HNC), has as main risk factor the use of tobacco, associated or not to alcohol, as well as the infection with oncogenic subtypes of human papillomavirus (HPV) [7]. Treatment depends on the stage of the disease, and in most cases, surgery is the preferred approach, to which radiotherapy and chemotherapy might be combined [7]. The morbidity associated with the treatment of HNC is still a concern, despite remarkable advances in the field, such as targeted therapy, immunotherapy, and technological developments in surgery and radiotherapy [7].

The phosphoinositide-3-kinase (PI3K) - protein kinase B (AKT) - mechanistic target of rapamycin (mTOR) signaling pathway regulates many cellular processes that ultimately relate to cell growth and proliferation [8]. This signaling pathway is found deregulated in cancer, characterized by an overexpression/hyperactivation of its effector proteins and alterations (amplification and mutation) on genes that encode those proteins [8]. This deregulation results in cell survival, cytoskeleton rearrangement, invasion, metastasis, and evasion of apoptosis [8].

In a systematic review regarding the effects of curcumin on *in vitro* and *in vivo* HNC models, curcumin was reported to significantly reduce cell viability and tumor growth, inhibit cell proliferation, and induce cell cycle arrest and apoptosis [9]. Recent studies showed that curcumin inhibited the activity of the mTOR complex 1 (mTORC1) on erythroleukemia cell

lines [10] and significantly decreased the expression and phosphorylation of AKT and mTOR on lung cancer [11] and ovarian cancer cells [12]. It also reduced the phosphorylated levels of the downstream effectors ribosomal protein S6 kinase (p70 S6K) and 4E-binding protein 1 (4E-BP1) on ovarian cancer cells [12].

Therefore, the objectives of this study were to provide new biological insights into the effects of curcumin on two different HNC cell lines (SCC-9 and FaDu) and to demonstrate how it modulates the PI3K-AKT-mTOR signaling pathway.

MATERIALS AND METHODS

Curcumin

Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA – Reference: #08511). Stock solution was prepared by diluting 10mg in DMSO to the concentration of 25mM, and stored, protected from light, at -80ºC until it was used.

Cell cultures

Three human cell lines were used for the experiments: SCC-9 (tongue squamous cell carcinoma), FaDu (hypopharynx squamous cell carcinoma) and HaCaT (human keratinocytes). SCC-9 cells were cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). FaDu and HaCaT wells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. Cells were incubated in 5% CO_2 at 37°C. Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay – Dose-response curves

Cells were seeded into 96-well plates at a density of 5×10^3cells/well, incubated overnight and treated with curcumin (50, 25, 10, 5, 2.5, 1.25 or 0.75µM) for 24 hours. After treatment, 10µL of MTT solution (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) at 5mg/mL were added to each well, followed by incubation for 4h at 37°C. Medium was aspirated, and 100µL of acidified isopropanol were added to dissolve formazan crystals. Absorbance was measured at 570nm in a DTX 800 reader (Beckman Coulter, CA, USA).

The half-maximal inhibitory concentrations (IC_{50}) were estimated based on the dose-response curves, and the Tumor Selectivity Index (TSI) was calculated according to the formula: $TSI = \frac{IC_{50}\ Control\ cell(HaCaT)}{IC_{50}\ Tumor\ cell(SCC-9\ or\ FaDu)}$. Further experiments were performed with curcumin at IC_{50} concentrations.

Cell cytometry – Cell cycle and cell death

For the cell cycle assay, cells were seeded into 6-well plates at a density of 2×10^5cells/well and incubated overnight. SCC-9 and FaDu cells were incubated in medium without FBS for 24 and 48 hours before treatment, respectively. Curcumin was diluted in medium with FBS and added to the wells. After 24 hours of treatment, cells were collected, fixated on 70% ethanol and kept at -20ºC until the assay. Cells were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) at 50µg/mL and assessed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A minimum of 10,000 events were analyzed for each sample.

For the cell death assessment, SCC-9 and FaDu cells were seeded into 6-well plates at a density of 3.5×10^5cells/well and incubated overnight. After treatment, cells were collected,

washed with PBS and centrifuged three times to remove the excess of curcumin, and resuspended in culture medium. The CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Life Technologies, Carlsbad, CA, USA – Reference: #C10427) was applied to the cells, according to the manufacturer's instructions. The gate selection was performed with cells that were treated with curcumin but not stained with the CellEvent kit, so that the fluorescence inherent to curcumin would not confound the readings. The samples were assessed on a BD Accuri C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A minimum of 10,000 events were analyzed for each sample.

Phalloidin assay – Cytoskeleton organization and morphology

SCC-9 and FaDu cells were seeded over glass coverslips placed on the bottom of 12-well plates at a density of 1.6×10^5cells/well and incubated overnight. After a 24-hour treatment, cells attached to the coverslips were fixated in 4% paraformaldehyde for 10 minutes and washed with PBS. Cells were permeabilized with 0.5% Triton X-100 for 10 minutes and washed again. Afterward, cells were incubated with Fluorescein Phalloidin (1:200) (Invitrogen, Carlsbad, CA, USA) for 50 minutes, protected from light. After washing, the coverslips were mounted with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were captured with an Axio Imager.M2 microscope (Zeiss, Oberkochen, Germany).

qPCR array

SCC-9 and FaDu cells were cultured on $10cm^2$ dishes and incubated overnight. After treatment, cells were washed, and RNA was extracted and purified with TRIzol (Invitrogen, Carlsbad, CA, USA) + chloroform (0.2mL/1mL TRIzol) and the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Samples were

treated with the Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA) and stored at -20ºC. RNA was quantified and had its quality assessed on a NanoVue Plus spectrophotometer (GE Health Care, Little Chalfont, UK). A total of 2µg RNA was diluted to the concentration of 0.1µg/µL, submitted to the reverse transcription reaction using the SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA, USA), and stored at -20ºC. cDNA samples were diluted to the concentration of 12.5ng/µL, and 5µL (12.5ng cDNA) were mixed to 5µL TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) per reaction. This PCR reaction mix was added to each well of custom TaqMan Array 96-well FAST plates (Applied Biosystems, Foster City, CA, USA), that were designed for this experiment. The plates were submitted to the PCR on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the thermal cycle conditions suggested by the TaqMan MasterMix protocol.

Western blot

SCC-9 and FaDu cells were plated and incubated overnight. After treatment, they were washed, lysed, and centrifuged. The protein fraction was collected, quantified, and stored at -80ºC. Samples (30µg denatured proteins) were loaded in a 10% acrylamide gel and electrophoresed at 100V. The proteins were then transferred to a PVDF membrane, which was blocked with a 5% blocking solution (milk albumin) for one hour and incubated overnight with the primary antibodies (Cell Signaling, Danvers, MA, EUA; Santa Cruz Biotechnology, Dallas, TX, USA) (Supplementary Table 1) at 4ºC. After incubation, the membrane was washed and incubated with the secondary antibody (Abcam, Cambridge, UK; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at 4ºC. After washing, the membrane was covered with Amersham ECL Prime (GE Healthcare, Little Chalfont, UK) and images were acquired with a

chemiluminescence imager (Amersham Imager 600, GE Health Care, Little Chalfont, UK). Photos of the full membranes are presented in Supplementary Materials.

Statistical analyses

Considering the gaussian distribution of data, Kruskal-Wallis test and Dunn's test were applied to cell viability results. The IC_{50} values for each cell line were estimated through a non-linear regression on the cell viability dose-response curves. Mann Whitney test was used on flow cytometry data. Student's T-test was used to compare the measurements of the nucleus and cytoplasm. The software GraphPad Prism 8 (San Diego, CA, USA) was used for the analyses and graphs. A P value inferior to 0.05 was considered significant.

RESULTS

Curcumin reduced cell viability in a dose-dependent and selective manner

Aiming to establish IC_{50} values for each cell line, we first conducted an MTT cell viability assay. Cells were treated with curcumin in different concentrations (0.75-50µM) so that dose-response curves could be defined.

As observed in Figure 1, a reduction in cell viability was caused by concentrations higher than 2.5µM on the tumor cell lines (SCC-9 and FaDu) or 10µM on the keratinocytes cell line (HaCaT). However, the difference to vehicle-treated cells was only significant with the 50µM concentration on SCC-9 (median±range: 45.4%±43.7-54.2%) and HaCaT (median±range: 48.2%±44.9-54.8%) cells and with the 25µM and 50µM concentrations on FaDu cells (median±range: 48.2%±43.7-58.5 and 42.7%±37.8-43.9, respectively). Curcumin induced a dose-dependent effect on the three cell lines, though FaDu and SCC-9 cell lines tend to be responsive to curcumin in lower concentrations than HaCaT cells (Figures 1A, C, and E).

Based on the dose-response curves, IC_{50} values were established for each cell line (Figures 1B, D, and E). The FaDu cell line resulted in the lowest IC_{50} value (24.8µM), while the HaCaT cell line resulted in the highest (47.8µM). The SCC-9 cell line was in-between, with an IC_{50} value of 40.9µM.

TSIs were calculated to define the selectivity of curcumin to the tumor cell lines. A TSI higher than 1 indicates that the treatment more cytotoxic to the tumor cells than to control cells. Curcumin was selective to SCC-9 (TSI=1.15) and especially to FaDu (TSI=1.91), which demonstrates the potential of curcumin as a selectively cytotoxic agent.

Curcumin induced cell death on SCC-9 cells and cell cycle arrest on FaDu cells

Considering that a cell cycle arrest is associated with cellular stress and may result in cell death [13], we further investigated the effects of curcumin on the cell cycle distribution of HNC cells. The number of cells undergoing necrosis (cells with a permeable cell membrane and positive to SYTOX AAdvanced) or apoptosis (cells with active caspase 3/7) was also investigated.

Curcumin significantly reduced the number of SCC-9 cells that were on phase S (1.4-fold decrease; median±range: 13.4%±12.1-15.2% curcumin *vs.* 20.1%±18.9-20.62% vehicle), while increasing the cell population on phase G_2 (1.4-fold increase; median±range: 19.1%±17-20.1% curcumin *vs.* 13.4%±12.9-14.9% vehicle) (Figure 2A-E). Such significant accumulation of cells on phase G_2 indicates that curcumin induced a G_2/M cell cycle arrest, by impeding cells to proceed from interphase to mitosis (Figure 2F). A significant 2.7-fold increase in the number of SCC-9 cells undergoing necrosis was observed after treatment with curcumin (median±range: 44%±40.9-47.8% curcumin *vs.* 16.4%±15-20.2% vehicle) (Figure 2G). Curcumin also increased the number of apoptotic SCC-9 cells (3.7-fold increase; median±range: 2.8%±2.6-4% curcumin *vs.* 0.75%±0.6-0.8% vehicle), even though this

difference was not significant (Figure 2G). Additionally, the western blots presented in Figure 2H indicate that curcumin lead to the cleavage of procaspase-3 into its active form, caspase-3, a protein that works as an apoptosis effector.

In Figure 2I a schematic representation of these findings is presented. It evidences that the 50% reduction in cell viability that was observed after treating SCC-9 cells with curcumin at IC_{50} concentration is explained by both a cell cycle arrest and the induction of cell death. Cell death, especially necrosis, might be more relevant to justify the effects of curcumin on cell viability, considering that it was more intense than the differences observed in the cell cycle.

Curcumin at the IC_{50} concentration resulted in a significant 3-fold increase in the number of FaDu cells on phase G_2 (median±range: 37.8%±37.4-41.4% curcumin *vs.* 12.9%±11.8-13.8% vehicle), while it also caused a 1.6-fold reduction in the cell population on phase G_1 (median±range: 43.1%±38.4-50.6% curcumin *vs.* 67%±65.5-67.5% vehicle) (Figure 3A-E). Curcumin significantly induced an accumulation of cells on phase G_2, more intensely than on SCC-9 cells, and it indicates a G_2/M cell cycle arrest (Figure 3F). Although curcumin increased considerably the number of FaDu cells undergoing apoptosis (25-fold increase; median±range: 4.5%±3.6-5.4% curcumin *vs.* 0.18%±0.11-0.42% vehicle) and necrosis (1.9-fold increase; median±range: 17.9%±16.4-19.8% curcumin *vs.* 9.4%±8.9-10.1% vehicle), this difference was not statistically significant (Figure 3G). No difference in the levels of procaspase-3 or caspase-3 was observed (Figure 3H).

In Figure 3I a schematic representation of these results is presented. It suggests that the 50% reduction in cell viability that is observed after treating FaDu cells with curcumin at IC_{50} concentration is mainly explained by a significant cell cycle arrest, even though curcumin tends to induce cell death as well.

Curcumin modified the cytoskeleton organization and cell morphology

We also assessed the effects curcumin has on FaDu and SCC-9 cells regarding the actin cytoskeleton organization and cell morphology. It is known that a well-orchestrated remodeling of actin filaments in the cytoskeleton is fundamental to cell motility and migration, differentiation, and proliferation [14, 15]. Mutations and aberrant expression of actin and other cytoskeletal proteins are associated with chemotherapy resistance and metastasis in cancer [14, 15]. The disruption of the cytoskeleton organization is associated with decreased cell migration and might result in apoptosis [15], which are features of interest for a possible cancer therapy agent.

Vehicle-treated cells had their cytoskeleton organized in well-defined f-actin filament networks, consistently distributed across the cytoplasm (Figure 4A-B and G-H). Especially on FaDu cells, the filaments were organized as stress fibers, mostly oriented parallel to the long axis of the cell (Figure 4H). No morphological alteration was observed in vehicle-treated cells.

Curcumin resulted in an evident disruption of cytoskeleton organization on both cell lines, characterized by the loss of the f-actin networks and a diffuse and granular distribution of actin throughout the cytoplasm (Figure 4C-D and I-J). On SCC-9 cells, it was possible to observe peripherical depositions of actin, with a granular and concentrated aspect (Figure 4D). Morphologically, curcumin induced rounding and flattening of cells, as well as micronucleation and multinucleation in some cells. Curcumin caused a statistically significant reduction in the cytoplasm diameter of SCC-9 cells (mean±SD: 47.5µM±12.4µM curcumin *vs.* 53.9µM±16µM vehicle) and in the nucleus diameter of both SCC-9 (mean±SD: 15.3µM±2.6µM curcumin *vs* 20.7µM±3.1µM vehicle) and FaDu cells (mean±SD: 16.3µM±1.6µM curcumin *vs* 18.1µM±2.6µM vehicle) (Figure 4E-F and K-L).

Curcumin altered the expression of genes related to the PI3k-AKT-mTOR signaling pathway

The PI3K-AKT-mTOR signaling pathway plays an essential role in keeping the cells growing and proliferating, migrating, and even invading [8]. Knowingly overactivated in the context of oral carcinogenesis, this signaling pathway is associated with the increased cell proliferation, evasion of apoptosis, and metastasis, all hallmarks of cancer [8]. A qPCR array was performed to investigate the changes curcumin might induce in the expression of genes that are related to the PI3K-AKT-mTOR pathway, considering that such changes might relate to the effects on cell viability, cell cycle, cell death, and cytoskeleton organization previously described.

As observed in Figure 5A, a noticeable number of genes had their expression altered by curcumin. A selection of genes whose expression changed the most (fold change >2 or <0.5) is depicted in Figure 5B-C. In SCC-9 cells, the changes in the expression of *PRKCG*, *EGF*, *PLD1*, *RPS6KA1*, *DDIT4*, *RAC1*, *EGFR*, *IRS1*, *STRADB*, *EIF4E*, *ULK1*, *PTEN*, *PRKAA1*, *RICTOR*, and *A1BG* genes were of considerable importance. In FaDu cells, a markedly increased expression of the *PRKCG*, *PRKAG2*, *VEGFC*, and *PRKAB1* genes was noticed.

The gene with the most considerable change in expression on both cell lines was *PRKCG*, that had its expression increased 13.2-fold on SCC-9 and 8-fold on the FaDu cell line (Figure 4D). An increased expression of *EGF* (11.3-fold change) (Figure 5E) and a decreased expression of *PLD1* (0.089-fold change) (Figure 5F), the second and third most altered genes on SCC-9 cells, were also noteworthy. Other genes that had their expression importantly decreased by curcumin were *EGFR* (Figure 5G), *PTEN* (Figure 5H) (both 0.18-fold and 0.5-fold changes on SCC-9 and FaDu cell lines respectively), and *RPS6KA1* (Figure 5I) (0.17-fold change on SCC-9).

Curcumin downregulated the expression of PI3K-AKT-mTOR signaling pathway proteins

The expression of key proteins to the PIK3-AKT-mTOR signaling pathway and their phosphorylation status were assessed through western blot assays, which are presented in Figure 6.

Curcumin reduced the expression of most proteins on both cell lines (Figure 6). Oncogenic proteins such as PI3K (p110α), AKT, and mTOR were downregulated by curcumin on SCC-9 and FaDu cells, as well as the phosphorylated forms of AKT and mTOR. Surprisingly, the expression of the PTEN protein, a tumor suppressor protein, was also negatively regulated by curcumin on both cell lines. The downstream effector p70 S6K was not evidently altered by curcumin under the experimental conditions described for this study, yet p85 S6K was downregulated on SCC-9 cells.

DISCUSSION

The therapeutic potential of curcumin and other curcuminoids has been thoroughly described, even though concerns regarding their stability, solubility, and bioavailability have been raised, chiefly motivated by the lack of successful results from controlled clinical trials, as reviewed by Nelson *et al.* [16]. Yet, recent double-blind controlled clinical trials indicate that curcumin improved glycemic factors, hepatic function, and serum cortisol levels in overweight patients [17, 18], aided lowering cholesterol in hypercholesteremic patients [19], and decreased triglycerides levels and inflammation in diabetic patients [20]. Additionally, there has been an effort to overcome limitations to the clinical use of curcumin, such as its poor bioavailability and solubility. Strategies to improve its delivery (e.g., nanoparticles or liposomes) and to design derivatives and analogs with better therapeutic properties have been developed [21].

Remodeling of the cytoskeleton is fundamental for cells to progress through the cell cycle, with specific changes in contractility, protrusion, and adherence being required in each phase [22]. Therefore, a disruption in the cytoskeleton organization, such as observed on the HNC cells, might be related to the cell cycle arrest on phase G_2/M that was also caused by curcumin. Even though apoptosis was not clearly identified in our study, as previously mentioned, it is associated with cytoskeleton disorganization and cell cycle arrest [15]. In fact, Chen *et al.* [23] reported that curcumin induced on the cytoskeleton of lung adenocarcinoma cells very similar effects to the ones we observed in our study, and the authors linked the cytoskeleton disruption to apoptosis. Nuclear aberrations such as micronucleation and multinucleation are considered signs of genotoxicity, and they are associated with apoptosis [24]. Treatment with curcumin resulted in a reduction in the nucleus diameter, observed on both cell lines, as well as micronucleation and multinucleation.

In their active forms, the Ras homolog family of GTPases (Rho GTPases) are responsible for coordinating the assembly and organization of the actin cytoskeleton by inducing the generation of contractile forces (actin stress fibers), actin polymerization (membrane protrusion), and ultimately initiate a directed cell movement [15]. *RAC1* and *CDC42*, genes that encode two important Rho GTPases, had their expression reduced by curcumin, which could be associated with the cytoskeleton disorganization.

The deregulation of the PI3K-AKT-mTOR signaling pathway is pivotal in human oncogenesis, and as a major effector of this signaling pathway, mTOR is widely implicated in cell transformation, proliferation, and survival [8]. On SCC-9 and FaDu cells, curcumin decreased the levels of total and phosphorylated mTOR. Additionally, curcumin importantly reduced the expression of *RICTOR* (rapamycin-insensitive companion of mTOR – RICTOR), a constituent of mTORC2, on SCC-9 cells. Although curcumin did not alter the expression of the downstream effector p70 S6K nor of its genes (*RPS6KB1* and *RPS6KB2*), it markedly reduced the expression of the p85 S6K protein and its gene *RPS6KA1* (0.17-fold change) on SCC-9 cells. Also noteworthy was the reduced expression of *EIF4E* (0.18-fold change)

(eukaryotic translation initiation factor 4E - eIF4E), a transcription factor activated by mTORC1, on SCC-9 cells.

Curcumin induced a noticeable increase in the expression of *EGF* (11.3-fold change), which encodes the epidermal growth factor (EGF), one of the growth factors that trigger the PI3K-AKT-mTOR pathway [8]. *EGFR* (epidermal growth factor receptor – EGFR), on the other hand, was greatly decreased (0.18-fold change).

Surprisingly, curcumin induced the downregulation of PTEN and reduced the expression of *PTEN* (0.18- and 0.5-fold change on SCC9 and FaDu cells, respectively). That is unexpected, considered that the transcriptional, posttranscriptional or posttranslational negative regulation of PTEN is associated with a predisposition to carcinogenesis, and a reduced expression of PTEN, even to a minimal degree, is enough for tumor initiation [25, 26]. In contrast to our results, curcumin increased the expression of PTEN on breast, lung, and pancreatic cancer cell lines, which was associated with a reduced expression of the posttranscriptional regulators miRNA-19 and -21 [27].

Even though the protein kinase C (PKC) family has a myriad of functions in different cell types and tissues, it has been traditionally associated with cell transformation and cancer development [28, 29]. Based on this assumption, many PKC inhibitors underwent clinical trials, even though their success was limited [29]. Recent studies suggest that higher levels of PKCα are positively correlated to improved survival in pancreatic adenocarcinoma patients [30] and that PKCβII is considerably downregulated in colorectal cancer tissues [31]. Antal *et al.* [32] reported that the loss-of-function mutations in PKC are the most frequent. Altogether, these results indicate that PKC isoforms act as tumor suppressors, and that the efforts on cancer therapy should focus on restoring PKC activity, and not on inhibiting it [29]. Even so, it is essential to note that the function of PKC on cancer seem to be tissue- or cell-type specific, and that the expression of PKC isoforms is highly variable [29].

PKCγ was believed to be expressed exclusively in neuronal tissues, and its role in oncogenesis is not clear enough [28]. Even though studies regarding the function of PKCγ in tissues other than neuronal and in the development of cancer are scarce, a few have reported it is expressed in colon cancer cells [33, 34], prostate cancer cells [35], and in the retina [36]. Dowling *et al.* [37] report that silencing *PRKCG*, the gene that encodes PKCγ, increased cell proliferation and adhesion in colon cancer cells. Of all genes that we investigated, *PRKCG* was the one with the most altered expression on both cell lines. Curcumin increased its expression 13.2-fold on SCC-9 cells and 8-fold on FaDu, which are considerable changes. Even though it is possible to claim that curcumin increases the expression of *PRKCG*, the role of PKCγ on SCC-9 and FaDu cells remains elusive.

Phospholipase D (PLD) is an enzyme that is extensively found in a variety of organisms. In humans, the isoforms PLD1 and PLD2 catalyze the production of phosphatidic acid [38]. It binds to mTOR and displaces the mTOR interacting protein (DEPTOR), a mTORC1 endogenous inhibitor, which results in mTORC1 activation and stabilization [39, 40]. Increased expression, subcellular mislocalization, and altered catalytic activity of PLD1 and PLD2 have been linked to a variety of cancer types [38]. Our results indicate that curcumin reduced the expression of *PLD1* and *PLD2* on both cell lines. The reduction of *PLD1* expression was particularly remarkable on SCC-9 cells (0.089-fold change), which suggests that a reduced expression of PLD1 might have aided the reduction in mTOR activity.

In conclusion, we provide evidence that curcumin downregulates the PI3K-AKT-mTOR signaling pathway on SCC-9 and FaDu cell lines. This study indicates that curcumin is a promising therapeutic agent due to its potential to inhibit HNC growth and progression. Such effects could be related to the reduced cell viability, cytoskeleton disorganization, cell cycle arrest, and cell death that were observed after treatment with curcumin.

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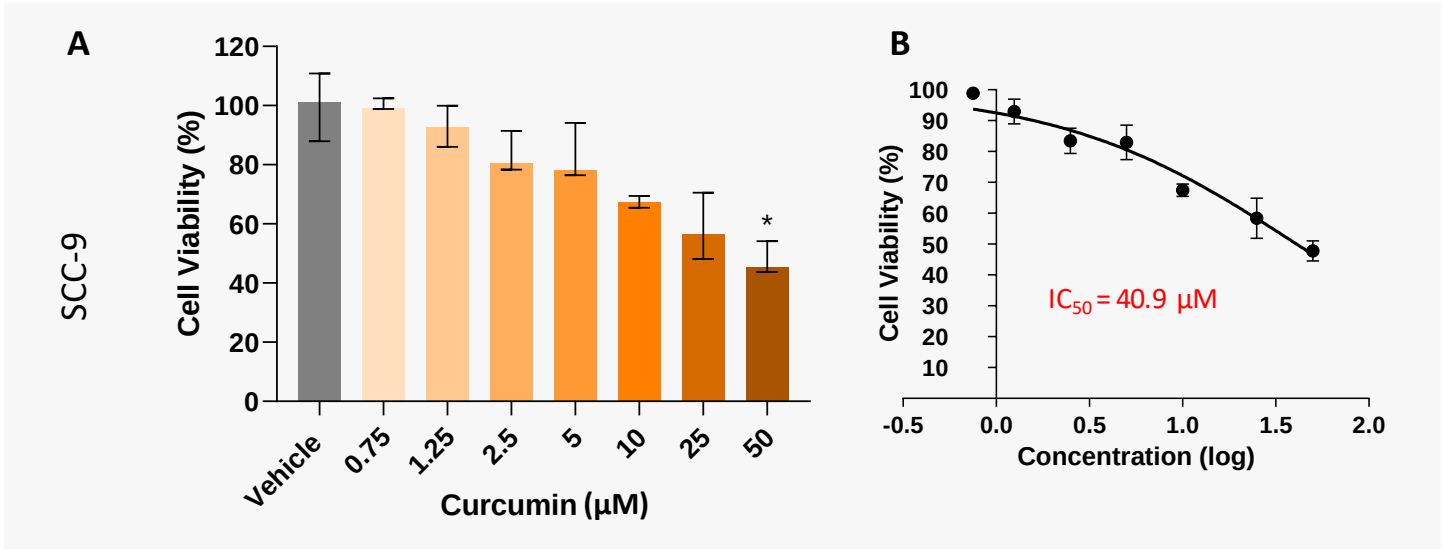

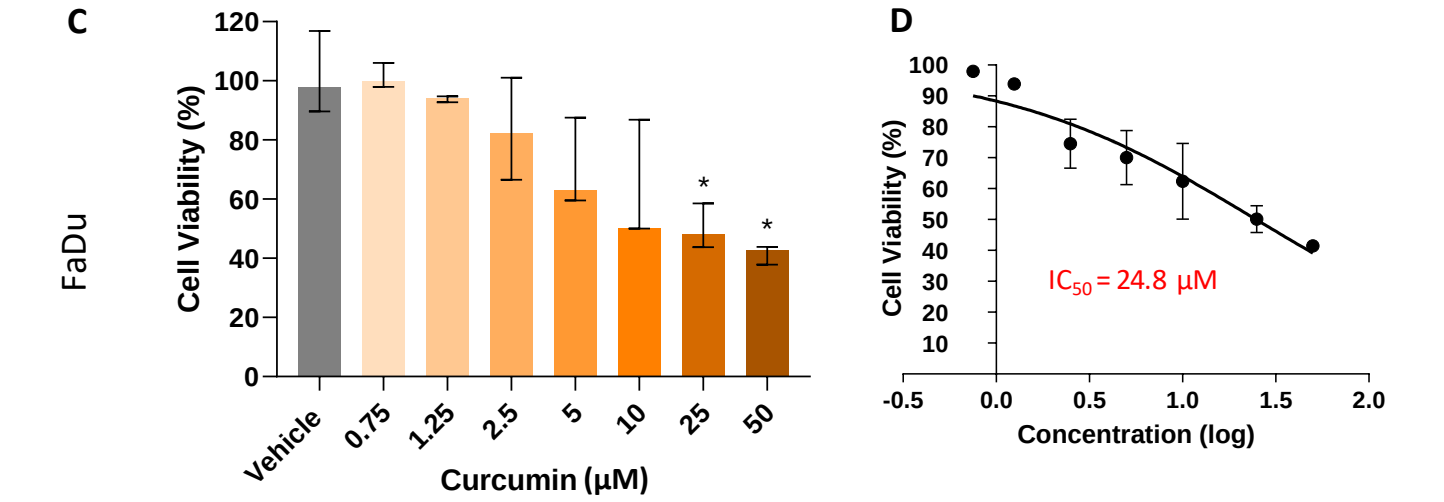

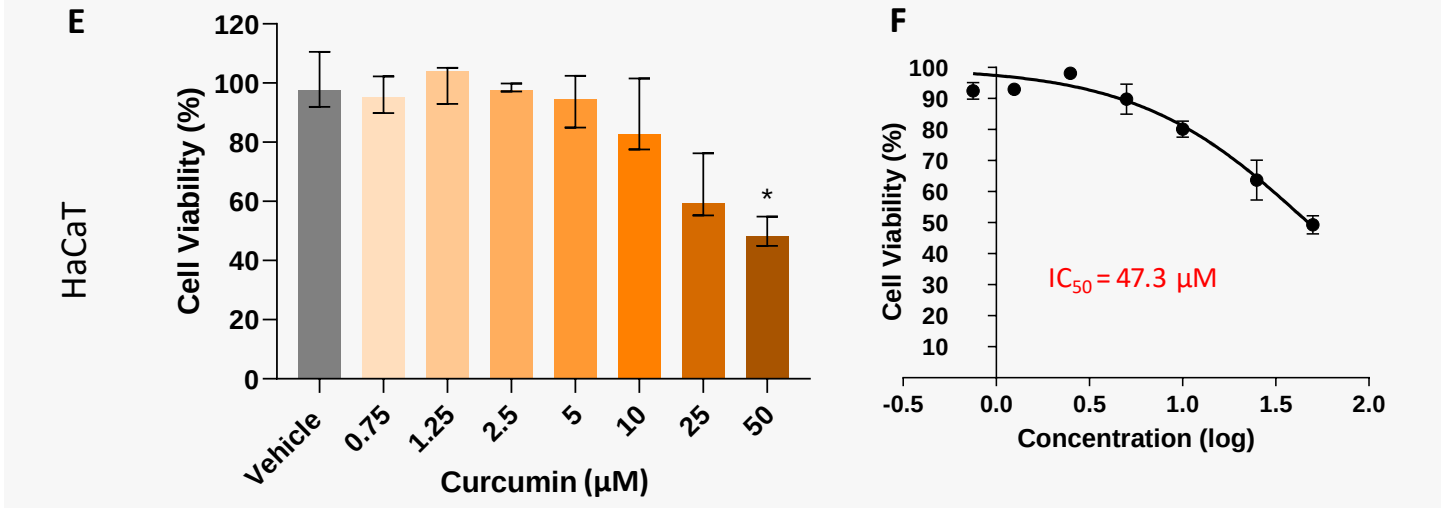

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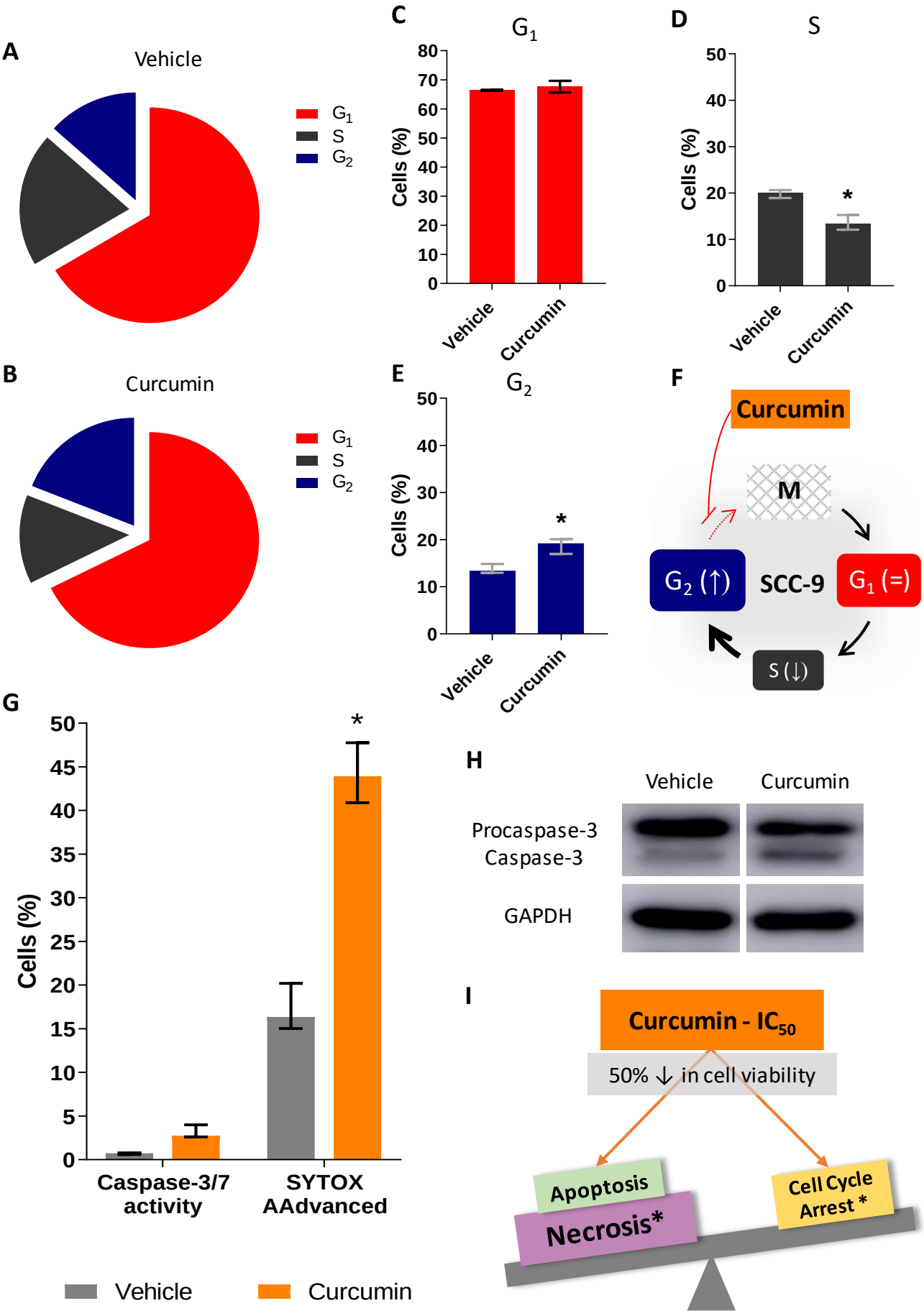
$$\text{Tumor Selectivity Index (TSI)} = \frac{IC_{50}\ HaCaT}{IC_{50}\ Tumor\ cell\ line}$$

Cell Line	TSI
SCC-9	1.15
FaDu	1.91

Figure 1: Effects of curcumin on HNC cells viability. Kruskal-Wallis test and Dunn's test were applied to the cell viability data, and the IC_{50} values were calculated after a non-linear regression on the dose-response curves. Graphs A, C, and E are representations of medians±range. Graphs B, D, and F are representations of means±SD. **A-B)** Results for the SCC-9 cell line: **A.** Curcumin reduced cell viability in a dose-dependent manner, and such reduction was considered significant at [50μM] (45.4% cell viability, when compared to vehicle-treated cells); **B.** Dose-response curve and IC_{50} (40.9μM). **C-D)** Results for the FaDu cell line: **C.** Curcumin also reduced cell viability in a dose-dependent manner, with significant difference at [25μM] and [50μM] (cell viability values of 48.2% and 42.7% respectively, when compared to vehicle-treated cells); **D.** Dose-response curve and IC_{50} (24.8μM). **E-F)** Results for the HaCaT cell line: **E.** Curcumin resulted in a dose-dependent reduction in cell viability, and the difference to vehicle-treated cells was considered significant at [50μM] (cell viability of 48.2%); **F.** Dose-response curve and IC_{50} (47.3μM). **G.** Tumor Selectivity Indexes (TSIs) were calculated for the tumor cell lines (SCC-9 and FaDu). TSIs higher than 1 indicated that curcumin was selective to the tumor cell lines when compared to the keratinocyte cell line (HaCaT). * = $P < 0.05$.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G2 (↑)
SCC-9
G1 (=)
S (↓)
G
Cells (%)
50
45
40
35
30
25
20
15
10
5
0
*
Caspase-3/7 activity
SYTOX AAdvanced
Vehicle
Curcumin
H
Vehicle
Curcumin
Procaspase-3
Caspase-3
GAPDH
I
Curcumin - IC50
50% ↓ in cell viability
Apoptosis
Necrosis*
Cell Cycle Arrest *

Figure 2: Effects of curcumin on cell cycle and cell death for the SCC-9 cell line. Mann Whitney test was applied to all flow cytometry results. Bars are representative of medians±range. **A**-**B)** Distribution of vehicle-treated (A) and curcumin-treated (B) cells on the cell cycle: **A.** Most of vehicle-treated cells were on phase G_1 (red - 66.5%) and to a lesser extension on phases S (dark grey - 20.1%) and G_2 (blue - 13.4%); **B.** Most curcumin-treated cells remained on phase G_1 (red - 67.7%), and a smaller number of cells were on phases S (dark grey - 13.4%) and G_2 (blue – 19.1%). **C-E)** Comparison between cell populations on phases G_1, S, and G_2 treated with either vehicle or curcumin: **C.** No difference was observed between the populations of vehicle-treated and curcumin-treated cells on phase G_1; **D.** Curcumin significantly reduced the number of cells on phase S; **E**. Curcumin significantly increased the number of cells on phase G_2. **F)** Summary of the effects of curcumin on the cell cycle of SCC-9 cells: Curcumin reduced the number of cells on phase S and increased the number of cells on phase G_2, which suggests it induced a cell cycle arrest on the G_2/M transition. **G)** Cell death profile after treatment with either vehicle (light grey) or curcumin (orange): Even though curcumin increased the number of cells with active caspase-3/7 in comparison to vehicle (from 0.75% to 2.8%), such difference was not significant. Curcumin significantly increased the number of necrotic cells when compared to vehicle (from 16.4% to 44%), considering the nuclear staining with SYTOX AAdvanced. **H)** Protein levels of procaspase-3 (upper blots) and its active form, caspase-3 (lower blots): Curcumin reduced the levels of procaspase-3 and increased caspase-3, which suggests it induced the cleavage of procaspase-3 into caspase-3 in SCC-9 cells. Images of the full membranes are presented as Supplementary Materials. **I)** Summary of cell cycle and cell death results on the SCC-9 cell line: Curcumin resulted in significant cell cycle arrest and cell death on the SCC-9 cell line. Cell death, especially necrosis, might be of more relevance than the cell cycle arrest to justify the effects on cell viability (50% reduction, caused by treatment with curcumin at IC_{50} concentration). * = $P < 0.05$.

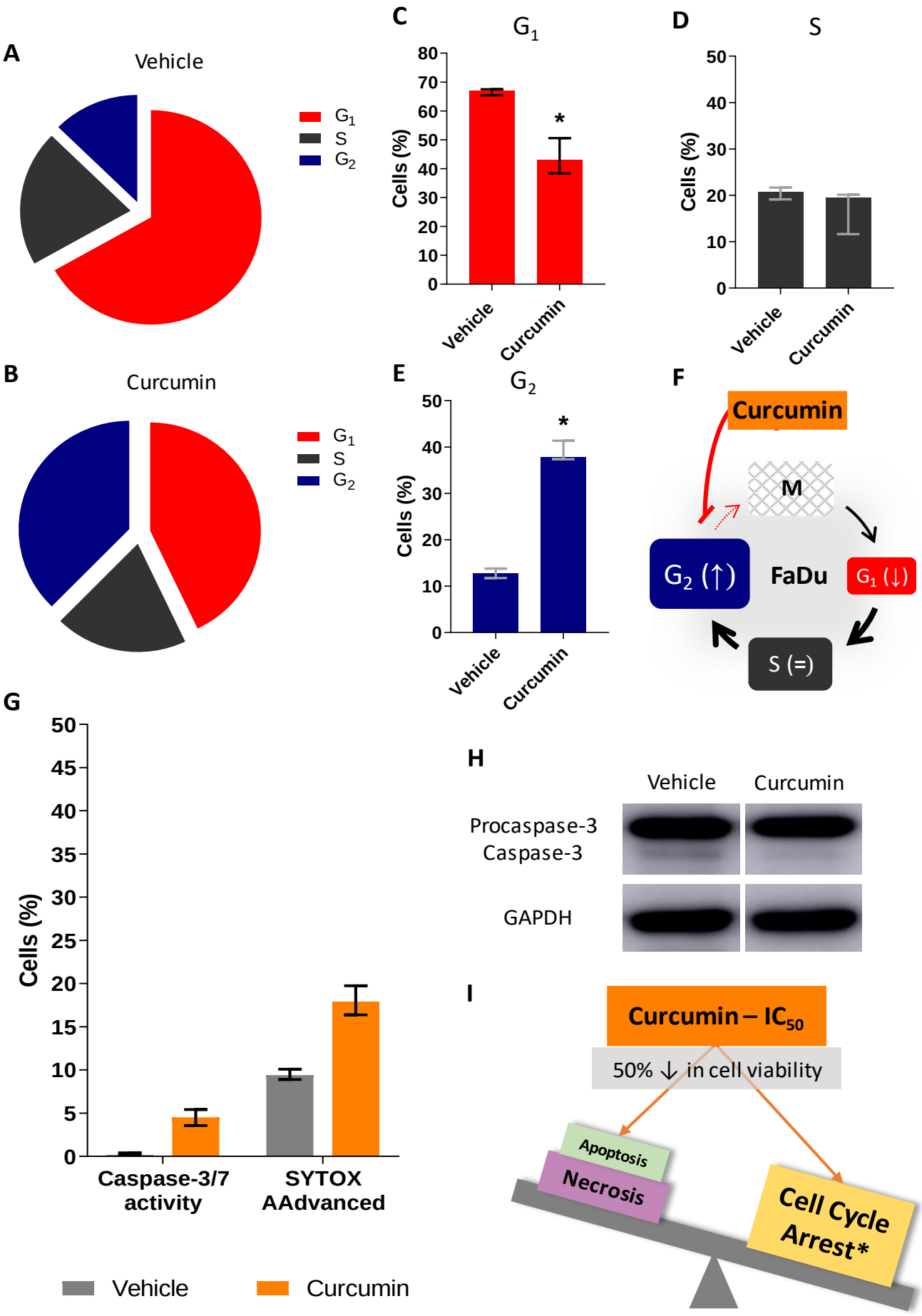
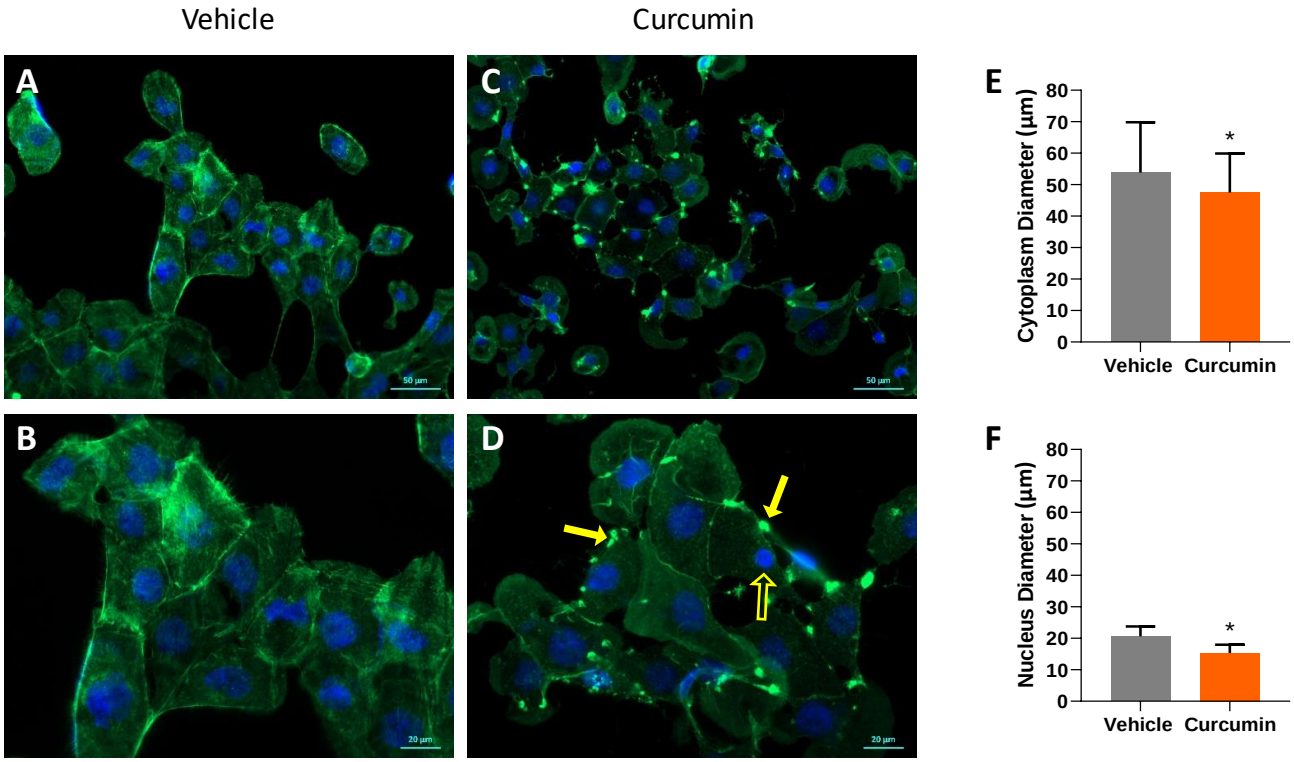

A
Vehicle
G1
S
G2
B
Curcumin
G1
S
G2
C
G1
Cells (%)
Vehicle
Curcumin
D
S
Cells (%)
Vehicle
Curcumin
E
G2
Cells (%)
Vehicle
Curcumin
F
Curcumin
M
G2 (↑)
FaDu
G1 (↓)
S (=)
G
Cells (%)
Caspase-3/7 activity
SYTOX AAdvanced
Vehicle
Curcumin
H
Vehicle
Curcumin
Procaspase-3
Caspase-3
GAPDH
I
Curcumin – IC50
50% ↓ in cell viability
Apoptosis
Necrosis
Cell Cycle Arrest*

Figure 3: Effects of curcumin on cell cycle and cell death for the FaDu cell line. Mann Whitney test was applied to all flow cytometry results. Bars are representative of medians±range. **A-B)** Distribution of vehicle-treated (A) and curcumin-treated (B) cells on the cell cycle: **A.** Most of vehicle-treated cells were on phase G_1 (red - 67%) and to a lesser extension on phases S (dark grey - 20.5%) and G_2 (blue - 12.9%). **B.** The distribution of curcumin-treated cells in the cell cycle was more homogeneous: most cells remained on G_1 (red – 43.1%), and a smaller number of cells were on phases S (dark grey – 19.3%) and G_2 (blue – 37.8%). **C-E)** Comparison between cell populations on phases G_1, S, and G_2 treated with either vehicle or curcumin: **C.** Curcumin significantly reduced the number of cells on phase G_1; **D.** No difference was observed between the populations of vehicle-treated and curcumin-treated cells on phase S; **E.** Curcumin significantly increased the number of cells on phase G_2. **F)** Summary of the effects of curcumin on the cell cycle of FaDu cells: Curcumin reduced the number of cells on phase G_1 and considerably increased the number of cells on phase G_2, which suggests it induced an important cell cycle arrest on the G_2/M transition. **G)** Cell death profile after treatment with either vehicle (light grey) or curcumin (orange): Even though curcumin increased the number of apoptotic (caspase-3/7 positive) and necrotic (SYTOX Aadvanced-stained) cells, such differences were not significant. **H)** Protein levels of procaspase-3 (upper blots) and its active form, caspase-3 (lower blots): Curcumin did not alter the levels of procaspase-3 and caspase-3 in the FaDu cells. Images of the full membranes are presented as Supplementary Materials. **I)** Summary of cell cycle and cell death results on the FaDu cell line: Curcumin resulted in significant cell cycle arrest and was not found to induce necrosis nor apoptosis on the FaDu cell line. The cell cycle arrest justifies the effects on cell viability (50% reduction, caused by treatment with curcumin at IC50 concentration). * = $P < 0.05$.

SCC-9 cells



FaDu cells

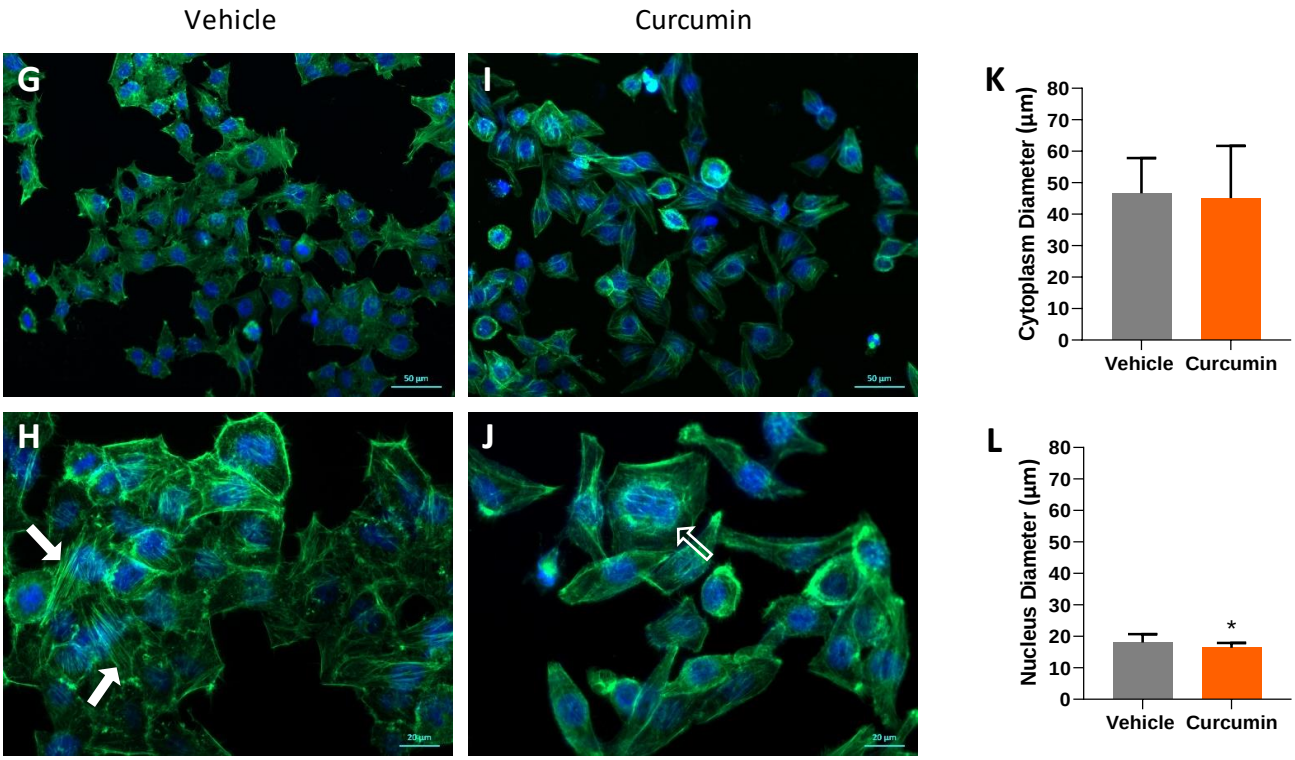


Figure 4: Effects of curcumin on cytoskeleton organization and cell morphology. Student's T-test was applied to the morphometric data (E, F, K, and L). Bars are representative of means±SD. Green: Phalloidin+FITC; Blue: DAPI. A, C, G and I – 20x magnification (scale bars = 50µm); B, D, H and J – 40x magnification (scale bars = 20µm). **A-B)** Vehicle-treated SCC-9 cells: actin filaments are organized in a distinct network, distributed homogeneously throughout the cell cytoplasm. Cytoplasm and nucleus are morphologically normal, as expected. **C-D)** Curcumin-treated SCC-9 cells: When compared to vehicle-treated cells, modifications on the cell morphology and the actin filaments organization are noticeable. Curcumin induced rounding and flattening of the cells. Actin is distributed in a diffuse and granular pattern in the cytoplasm, and the microfilaments organization is lost. Granular depositions of actin were observed in the periphery of cells (D - solid yellow arrows), as well as micronucleations (D – open yellow arrow). **E-F)** Cytoplasm (E) and nucleus (F) diameter measurements: Curcumin induced a significant reduction in the diameter of both cytoplasm (from 53.9µm to 47.5µm) and nucleus (from 20.7µm to 15.3µm) of SCC-9 cells. **G-H)** Vehicle-treated FaDu cells: actin filaments are clearly organized into stress fibers (H - solid white arrow), oriented parallel to the long axis of the cells and distributed throughout the cell cytoplasm. Cytoplasm and nucleus are morphologically normal. **I-J)** Curcumin-treated FaDu cells: When compared to vehicle-treated cells (G-H), it is possible to observe that the actin filaments organization was kept to a lesser degree, yet in some cells, a complete disorganization of the cytoskeleton is noticed. Curcumin induced rounding and flattening of the cells, that acquire a circular/ovoid appearance. Multinucleation (J – open white arrow) is observed. **K-L)** Cytoplasm (K) and nucleus (L) diameter measurements: Curcumin induced a significant reduction in the nucleus diameter (from 18.1µm to 16.3µm) of FaDu cells. No difference in the cytoplasm diameter was noticed. * = $P < 0.05$.

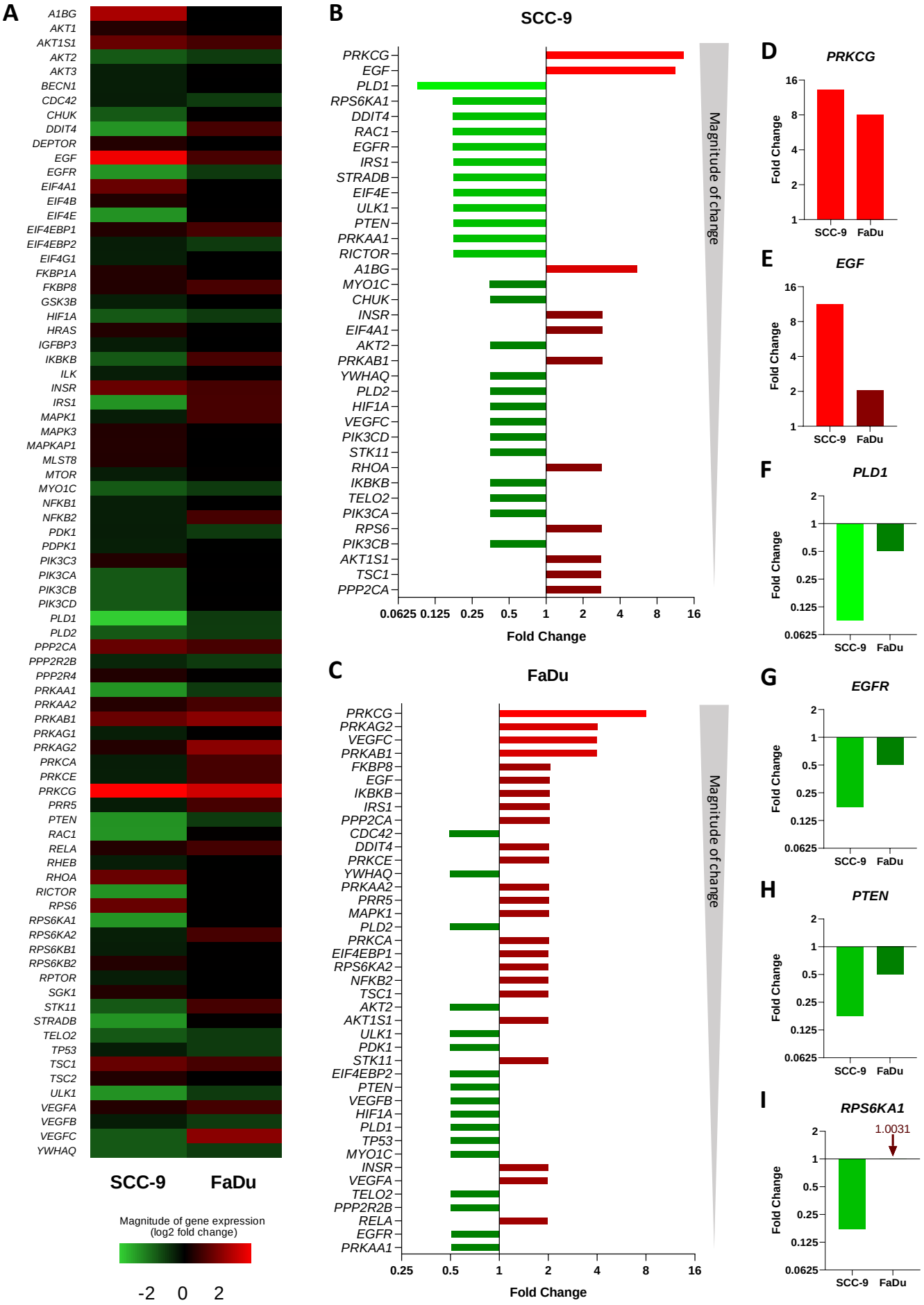

A
A1BG
AKT1
AKT1S1
AKT2
AKT3
BECN1
CDC42
CHUK
DDIT4
DEPTOR
EGF
EGFR
EIF4A1
EIF4B
EIF4E
EIF4EBP1
EIF4EBP2
EIF4G1
FKBP1A
FKBP8
GSK3B
HIF1A
HRAS
IGFBP3
IKBKB
ILK
INSR
IRS1
MAPK1
MAPK3
MAPKAP1
MLST8
MTOR
MYO1C
NFKB1
NFKB2
PDK1
PDPK1
PIK3C3
PIK3CA
PIK3CB
PIK3CD
PLD1
PLD2
PPP2CA
PPP2R2B
PPP2R4
PRKAA1
PRKAA2
PRKAB1
PRKAG1
PRKAG2
PRKCA
PRKCE
PRKCG
PRR5
PTEN
RAC1
RELA
RHEB
RHOA
RICTOR
RPS6
RPS6KA1
RPS6KA2
RPS6KB1
RPS6KB2
RPTOR
SGK1
STK11
STRADB
TELO2
TP53
TSC1
TSC2
ULK1
VEGFA
VEGFB
VEGFC
YWHAQ
SCC-9
FaDu
Magnitude of gene expression
(log2 fold change)
-2 0 2
B
SCC-9
PRKCG
EGF
PLD1
RPS6KA1
DDIT4
RAC1
EGFR
IRS1
STRADB
EIF4E
ULK1
PTEN
PRKAA1
RICTOR
A1BG
MYO1C
CHUK
INSR
EIF4A1
AKT2
PRKAB1
YWHAQ
PLD2
HIF1A
VEGFC
PIK3CD
STK11
RHOA
IKBKB
TELO2
PIK3CA
RPS6
PIK3CB
AKT1S1
TSC1
PPP2CA
Magnitude of change
0.0625 0.125 0.25 0.5 1 2 4 8 16
Fold Change
C
FaDu
PRKCG
PRKAG2
VEGFC
PRKAB1
FKBP8
EGF
IKBKB
IRS1
PPP2CA
CDC42
DDIT4
PRKCE
YWHAQ
PRKAA2
PRR5
MAPK1
PLD2
PRKCA
EIF4EBP1
RPS6KA2
NFKB2
TSC1
AKT2
AKT1S1
ULK1
PDK1
STK11
EIF4EBP2
PTEN
VEGFB
HIF1A
PLD1
TP53
MYO1C
INSR
VEGFA
TELO2
PPP2R2B
RELA
EGFR
PRKAA1
Magnitude of change
0.25 0.5 1 2 4 8 16
Fold Change
D
PRKCG
Fold Change
16 8 4 2 1
SCC-9 FaDu
E
EGF
Fold Change
16 8 4 2 1
SCC-9 FaDu
F
PLD1
Fold Change
2 1 0.5 0.25 0.125 0.0625
SCC-9 FaDu
G
EGFR
Fold Change
2 1 0.5 0.25 0.125 0.0625
SCC-9 FaDu
H
PTEN
Fold Change
2 1 0.5 0.25 0.125 0.0625
SCC-9 FaDu
I
RPS6KA1
1.0031
Fold Change
2 1 0.5 0.25 0.125 0.0625
SCC-9 FaDu

Figure 5: Effects of curcumin on the expression of genes that are related to the PI3K-AKT-mTOR signaling pathway (qPCR array results). Green indicates that curcumin reduced the gene expression when compared to the vehicle-treated samples. Red indicates that curcumin increased expression. Color values indicate the intensity of the effect curcumin exerted on the gene expression (the lighter the color, the more intense the effect was). Values are expressed as fold changes. **A)** Heat map depicting the changes curcumin induced in the expression profile of all genes that were assessed on the PCR array. Genes are presented alphabetically. **B-C)** Selection of the genes that were altered the most (fold change >2 or <0.5) on SCC-9 (B) and FaDu (C) cells: **B)** Curcumin considerably increased the expression of *PRKCG* (13.2-fold change) and *EGF* (11.3-fold change) and decreased the expression of *PLD1* (0.089-fold change) on SCC-9 cells. *RPS6KA1, DDIT4, RAC1, EGFR, IRS1, STRADB, EIF4E, ULK1, PTEN, PRKAA1,* and *RICTOR* were also substantially decreased (≈ 0.175-fold change), and *A1BG* increased (5.5-fold change). **C)** Curcumin increased *PRKCG* (8-fold change) considerably on FaDu cells, as well as *PRKAG2, VEGFC,* and *PRKAB1* (≈ 4-fold change). **D-F)** Comparison of the expression on SCC-9 and FaDu cells of the most altered genes after treatment with curcumin: Curcumin induced a sizeable increased expression of *PRKCG* (D) on both SCC-9 and FaDu cell lines, while such a remarkable change on the expression of *EGF* (E) and *PLD1* (F) was only observed on the SCC-9 cell line. **G-I)** Other genes that were importantly altered by curcumin: Curcumin reduced the expression of *EGFR* (G) and *PTEN* (H) on both cell lines and reduced the expression of *RPS6KA1* on SCC-9 cells.

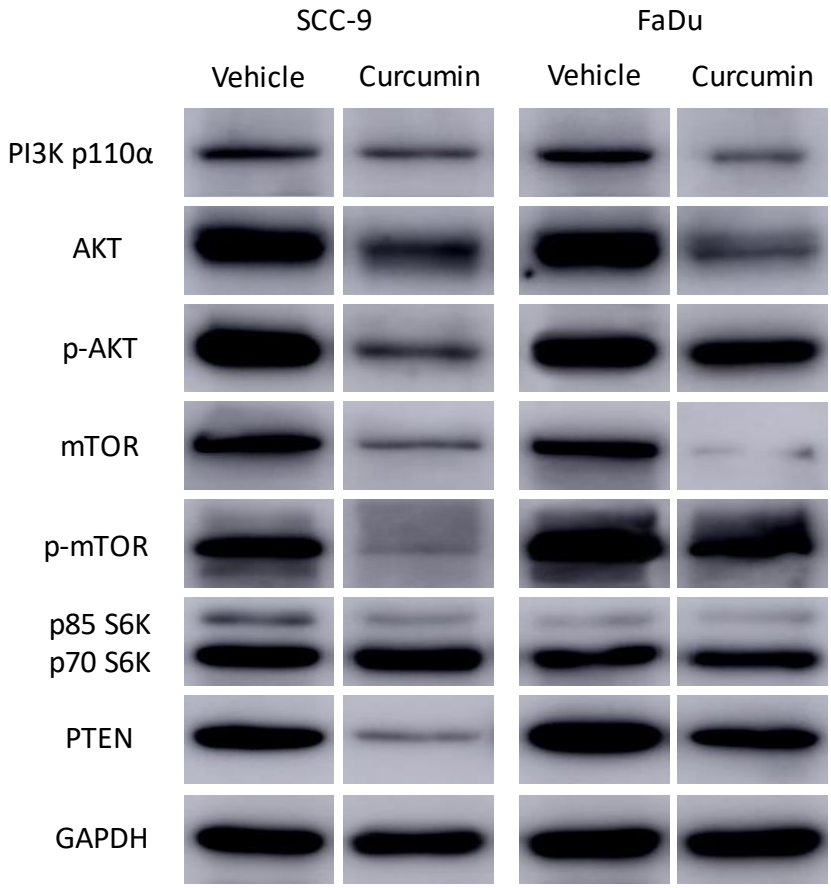


Figure 6: Effects of curcumin on the expression of proteins that are related to the PI3K-AKT-mTOR signaling pathway. Curcumin reduced the levels of PI3K, AKT, mTOR and p85 S6K, all oncogenic proteins, on both SCC-9 and FaDu cell lines. A reduction in the phosphorylated form of AKT (p-AKT) and mTOR (p-mTOR) was also observed, especially on the SCC-9 cell line. Interestingly, PTEN, a tumor suppressor protein, was downregulated by curcumin in both cell lines. Images of the full membranes are presented as Supplementary Materials.

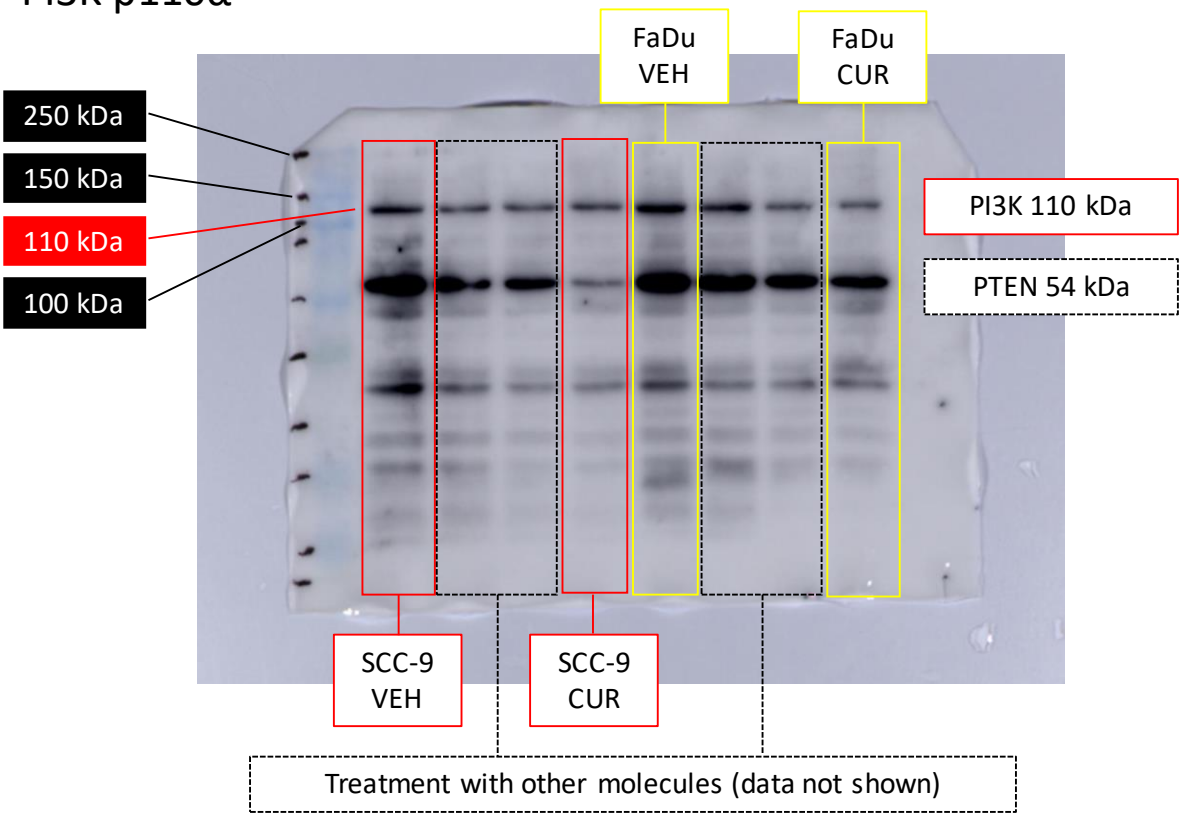
Supplementary Table 1. Antibodies used for western blot.

Antibody	Dilution	Reference
Anti-PI3K p110α Rabbit mAb	1:1000	#4249 – Cell Signaling
Anti-AKT Rabbit mAb	1:1000	#4691 – Cell Signaling
Anti-phospho-AKT (Ser473) Rabbit mAb	1:1000	#4060 – Cell Signaling
Anti-mTOR Rabbit mAb	1:1000	#2983 – Cell Signaling
Anti-phospho-mTOR (Ser2448) Rabbit mAb	1:1000	#5536 – Cell Signaling
Anti-p70S6K Rabbit pAb	1:1000	#9202 – Cell Signaling
Anti-PTEN Rabbit mAb	1:1000	#9559 – Cell Signaling
Anti-Caspase-3 Rabbit pAb	1:500	#7148 – Santa Cruz
Anti-GAPDH Mouse mAb	1:3000	#47724 – Santa Cruz
Goat anti-rabbit	1:5000	#6721 – Abcam
Goat anti-mouse	1:6000	#2005 – Santa Cruz

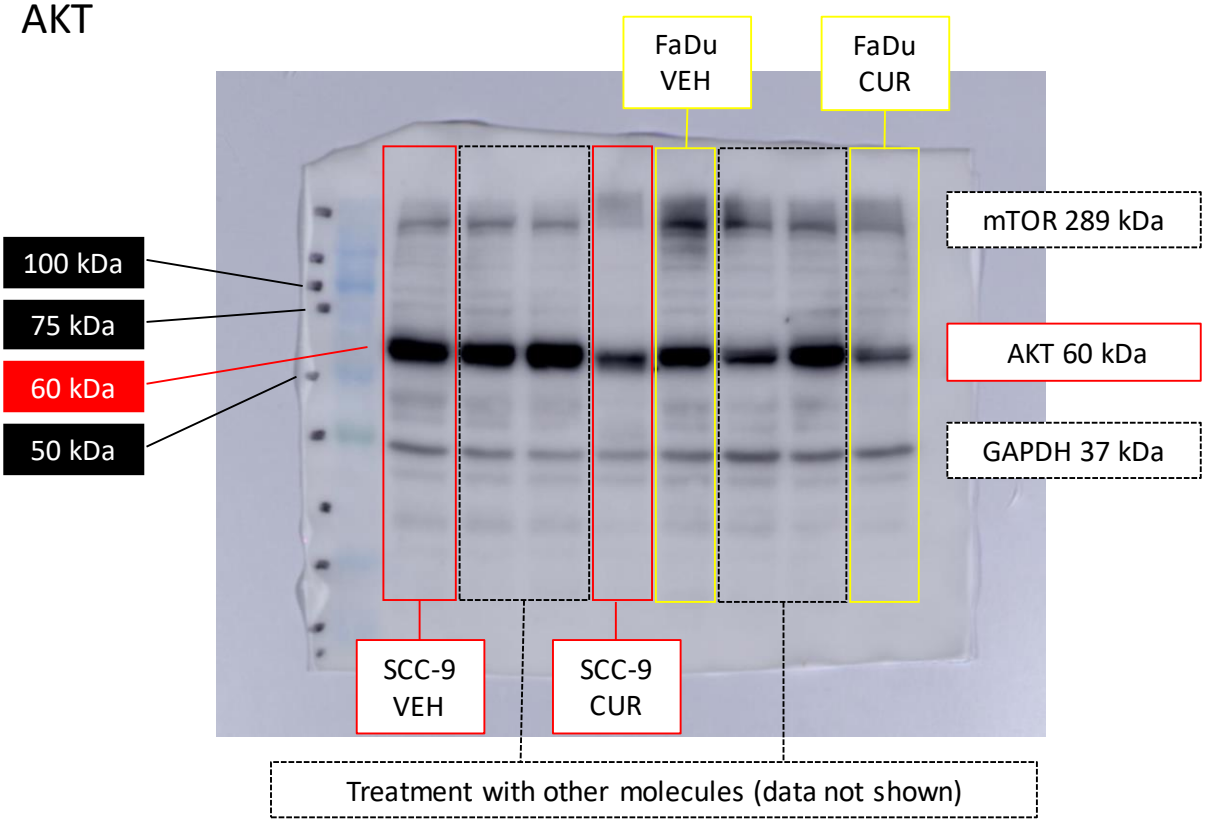
mAB : Monoclonal Antibody; pAB: Polyclonal Antibody

Supplementary Material – Photos of original western blot membranes

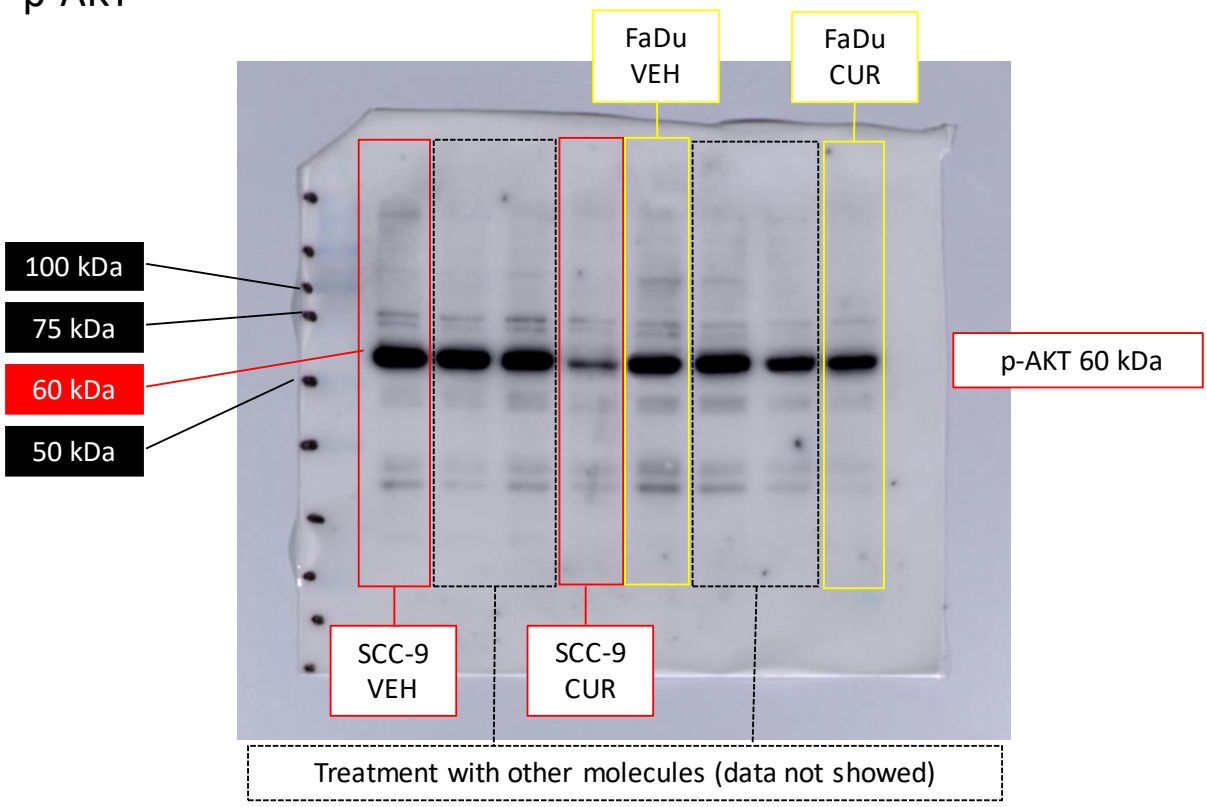
PI3K p110α



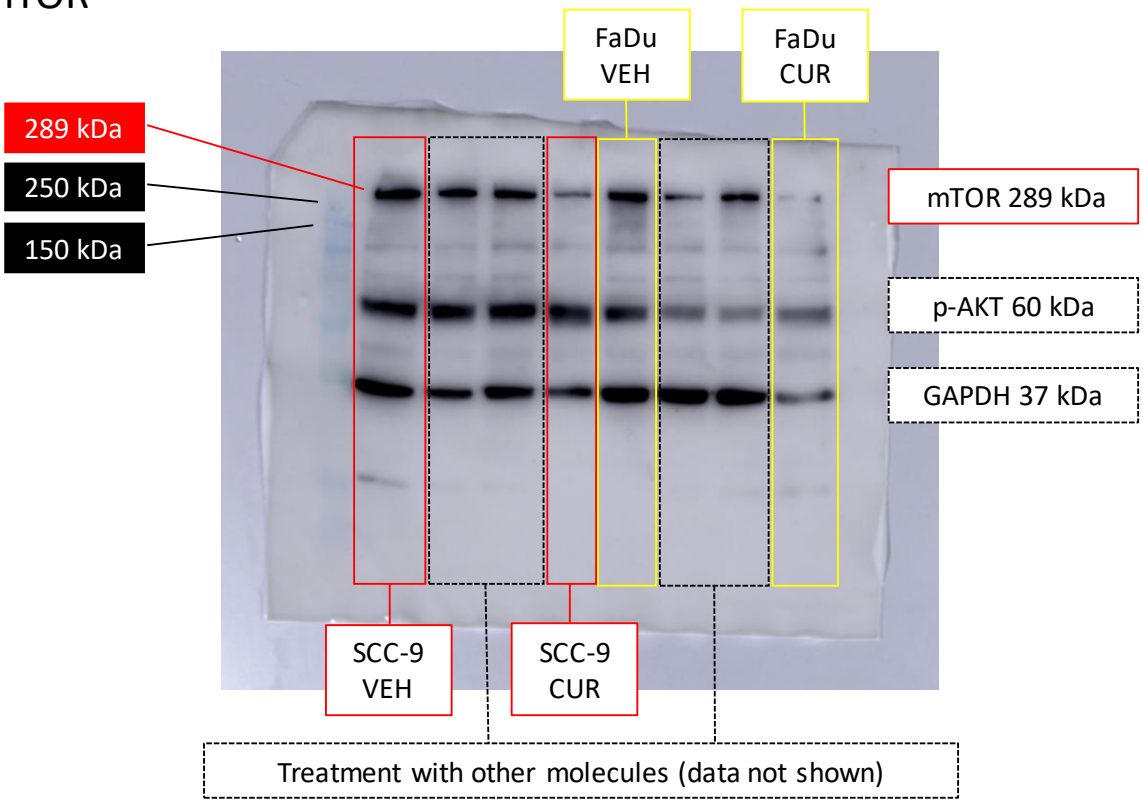
AKT



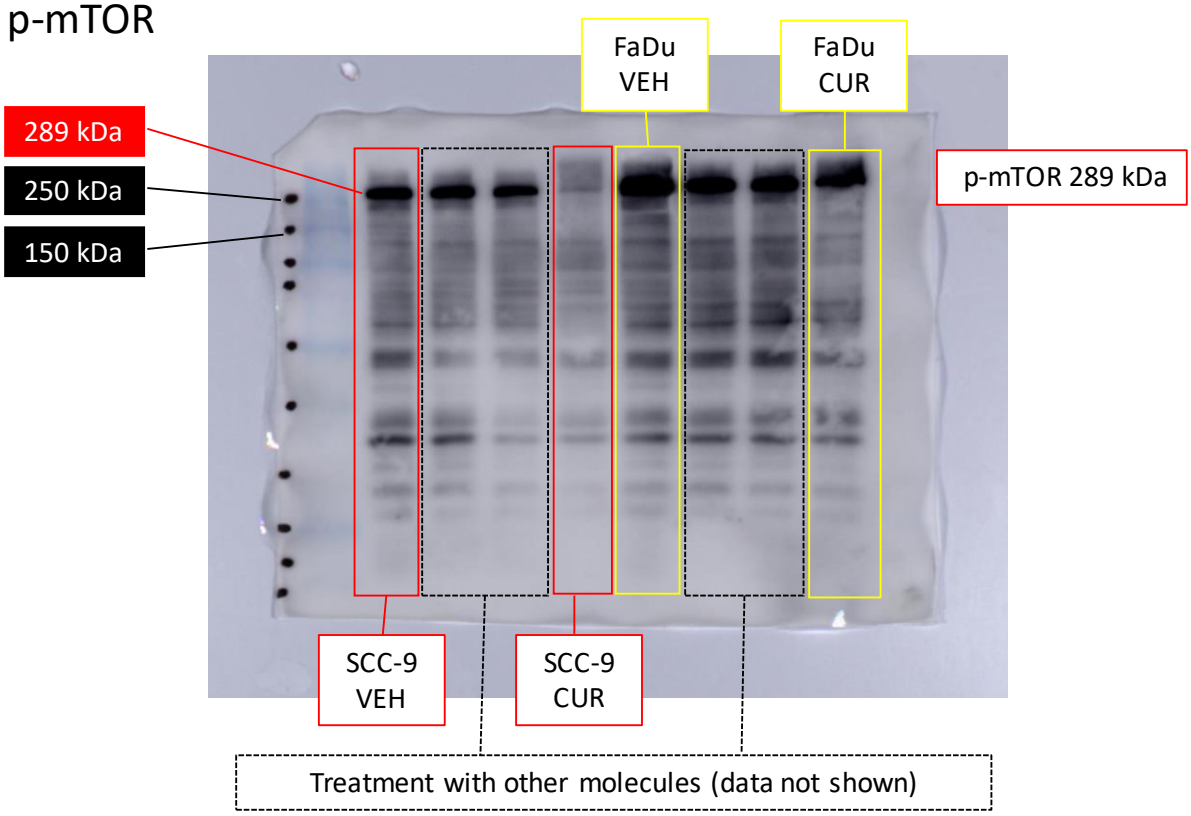
p-AKT


FaDu VEH
FaDu CUR
100 kDa
75 kDa
60 kDa
50 kDa
p-AKT 60 kDa
SCC-9 VEH
SCC-9 CUR
Treatment with other molecules (data not showed)

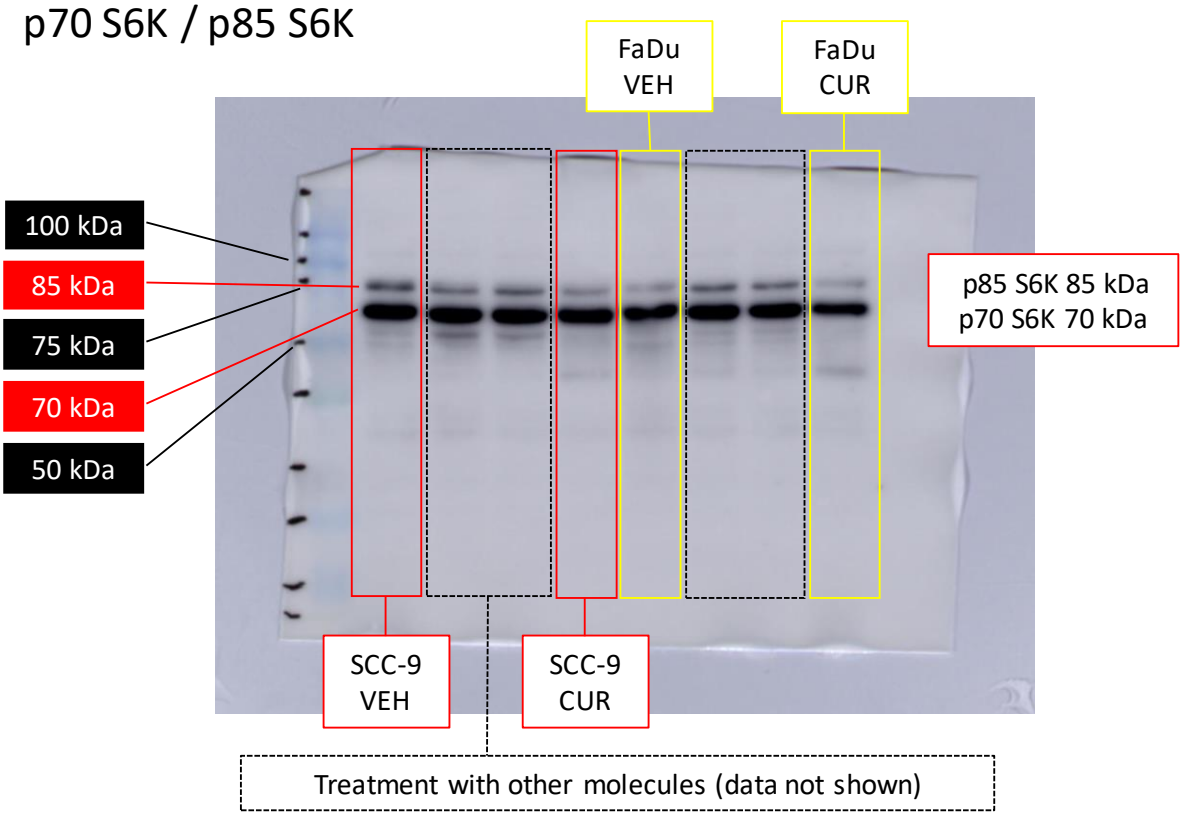
mTOR


FaDu VEH
FaDu CUR
289 kDa
250 kDa
150 kDa
mTOR 289 kDa
p-AKT 60 kDa
GAPDH 37 kDa
SCC-9 VEH
SCC-9 CUR
Treatment with other molecules (data not shown)

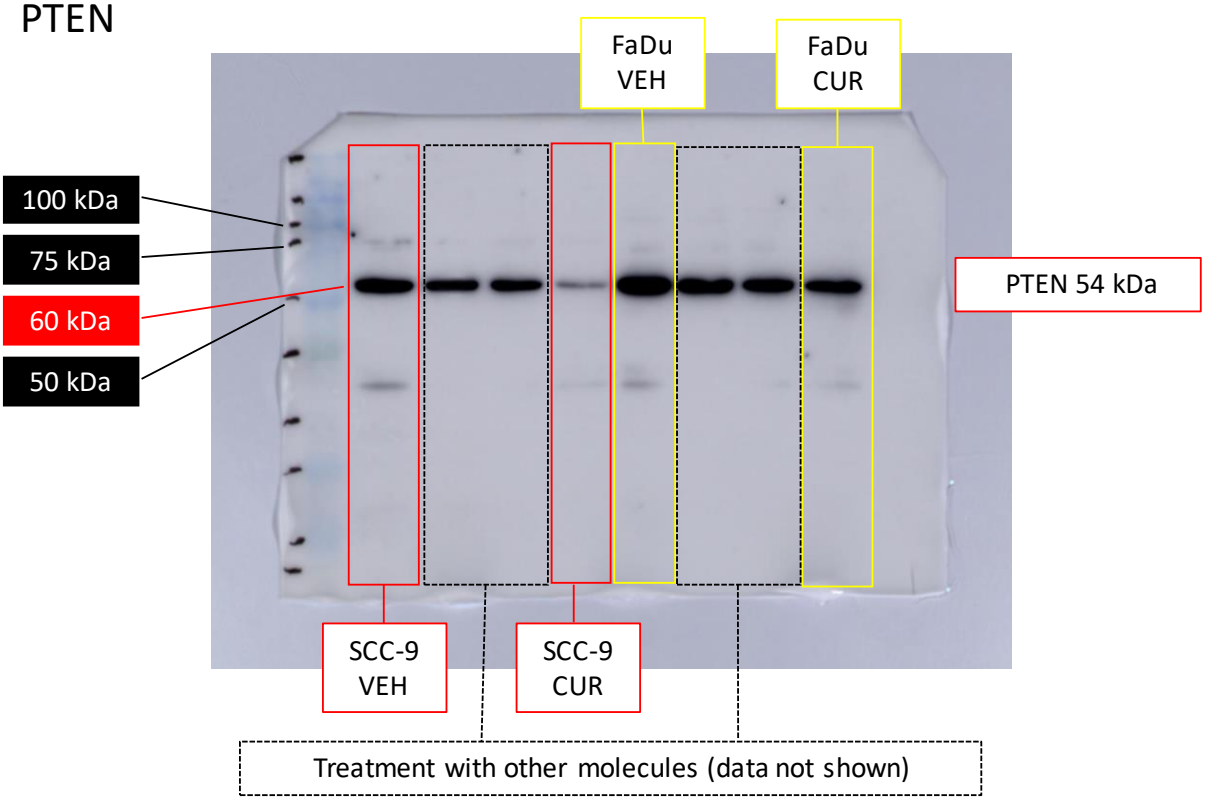
p-mTOR



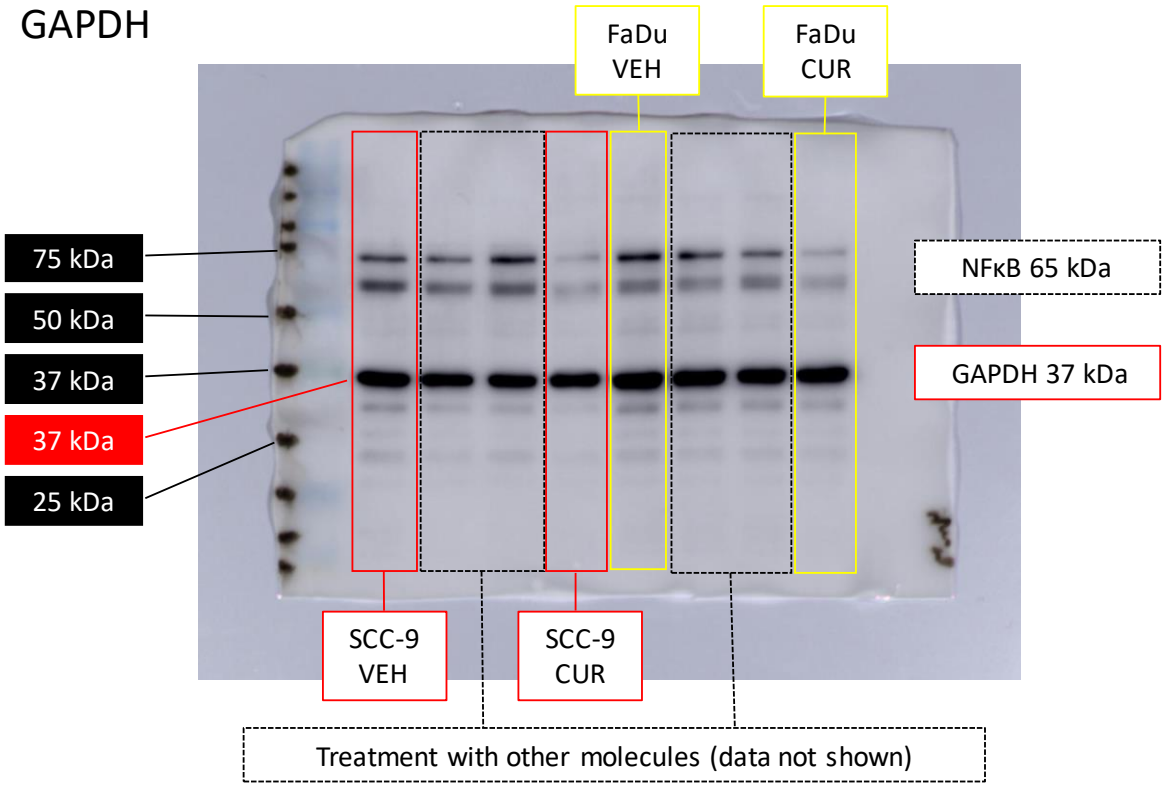
p70 S6K / p85 S6K



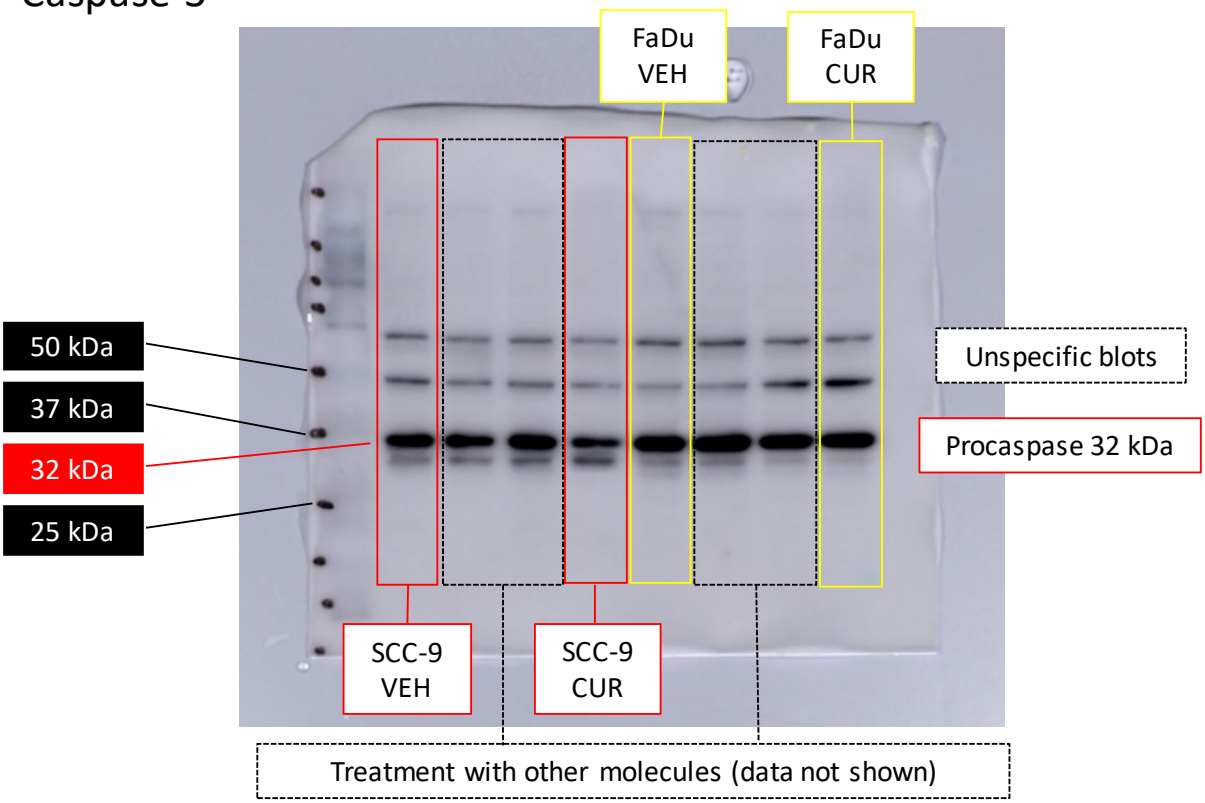
PTEN


FaDu VEH
FaDu CUR
100 kDa
75 kDa
60 kDa
50 kDa
PTEN 54 kDa
SCC-9 VEH
SCC-9 CUR
Treatment with other molecules (data not shown)

GAPDH


FaDu VEH
FaDu CUR
75 kDa
50 kDa
37 kDa
37 kDa
25 kDa
NFκB 65 kDa
GAPDH 37 kDa
SCC-9 VEH
SCC-9 CUR
Treatment with other molecules (data not shown)

Caspase-3


FaDu VEH
FaDu CUR
50 kDa
37 kDa
32 kDa
25 kDa
Unspecific blots
Procaspase 32 kDa
SCC-9 VEH
SCC-9 CUR
Treatment with other molecules (data not shown)

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6. DISCUSSION

Turmeric has aroused interest in scientists since the 19th century, when Vogel and Pelletier [32] reported in 1815 for the first time the chemical analysis of the *safran des Indes*, as turmeric was known, and proposed a method for extracting a dye from turmeric, which they named *curcumine*. Already in 1881, Jackson and Menke [33] reviewed ten previously published articles and research notes on the chemical properties of the molecule, becoming the authors of the first review on curcumin. In spite of the medicinal use of turmeric by Ayurvedic and traditional Chinese medicine for millennia, the powder was first used in the occidental world as a dye for wool, cotton, and silk in the 18th century [34]. Schraufstatter and Bernt [35] in 1949 were the first to report the biological effects of curcumin scientifically, showing that it has a marked activity against *Staphylococcus aureus*, *Salmonella paratyphi*, *Trichophyton gypseum*, and *Mycobacterium tuberculosis*. The anti-inflammatory and antioxidant properties of curcumin, its characteristic features, were first described in 1973 and 1976, by Srimal and Dhawan [36] and Sharma [37], respectively. Kuttan *et al.* [38] were the first to report in 1985 the anticancer effect of curcumin, suggesting that it induces substantial cytotoxicity in lymphoma and leukemia cells and in a lymphoma animal model. Today, a simple search on PubMed with the entry 'curcumin' delivers 13,800 results, an indication that extensive research has been conducted on curcumin.

We demonstrated in our systematic review that curcumin is cytotoxic to a variety of HNSCC cell lines and reduced the tumor burden in HNSCC animal model. It also induced a G_2/M cell cycle arrest and apoptosis, possibly through the intrinsic pathway. We were also able to define with this systematic review how the research on curcumin and its effects on HNSCC is being conducted. That was done by determining the methods that each reviewed article applied to produce their results, the concentrations they used and IC_{50} they established, and the different formulations of curcumin and associations with other drugs or molecules. Having such

ground information defined is essential for the decision as to where the next steps on this field should be directed.

In comparison to the impressive amount of scientific literature on curcumin, the number of systematic reviews is relatively small. A search on PubMed with the entry ‘curcumin AND “systematic review”’ results in only 89 studies, that review diverse biological effects of curcumin on various conditions and circumstances. Previously to the publication of our study, no systematic review on curcumin and cancer had been published. Afterward, a systematic review concluded that curcumin inhibits proliferation and induces apoptosis in specific subpopulations of glioblastoma, after reviewing 19 *in vitro* and *in vivo* studies [39]. Another systematic review reported that curcumin induced on breast cancer cells a G_2/M cell cycle arrest, which was associated with the inhibition of histone deacetylases (HDACs) 1 and 2 and subsequent increased acetylation of histones H3 and H4, upregulation of p21, and inhibition of cyclin B1, cyclin D1, and CDK1 [40].

In spite of every possible post-translational modifications on histones, the acetylation of lysine residue is possibly the most frequently studied [41]. The acetylation is promoted by histone acetyltransferases (HATs), that transfer the acetyl functional group from the acetyl-Coenzyme A (acetyl-CoA) to the ε-amino group of lysine residues [41]. The acetylation neutralizes the positive charge inherent to histones and reduces their interaction with the negatively charged chromatin, which becomes open and accessible to transcription factors [42, 41]. The abnormal activity and expression of both HATs and HDACs have been reported on different types of cancer, and such a scenario has prompted the investigation of several HAT and HDAC inhibitors and their anticancer potential [42, 41].

Other than the articles included in the systematic review previously discussed [40], different studies on the effects of curcumin or analogs on the activity of HDACs have demonstrated it downregulates the expression of HDAC1, 2, 4 ,6 and 11 on hepatocellular

carcinoma cells, which was associated with an inhibition of cell proliferation [43], inhibits the expression of HDAC8 on the cervical cancer HeLa cell line [44], decreases HDAC3 and total HDAC activity on prostate cancer cells [45], and reduces the levels of HDAC4, 5, 6 and 8 on a colorectal adenocarcinoma cell line [46]. In fact, the multiplicity of effects curcumin has on different diseases and biological events might be justified by its ability to regulate epigenetic mechanisms [47]. The reduction of cell viability, G_2/M cell cycle arrest, cytoskeleton disorganization, and PI3K-AKT-mTOR pathway downregulation, observed on SCC-9 and FaDu cell lines treated with curcumin in our study, could be related to HDAC inhibition or other form of epigenetic modulation, and further studies are necessary to elucidate this hypothesis.

Differently from the significant apoptosis-inducing effects of curcumin on HNSCC cell lines that are reported in our systematic review, the increase in the number of SCC-9 and FaDu cells undergoing apoptosis (active caspase-3/7) was not significant in our original research. Even so, the western blot assay suggested curcumin induced the activation of caspase-3 on the SCC-9 cell line. One should consider that the reference gates for the cytometry assay were established with cells that were treated with curcumin and not stained with the fluorescent markers. The protocol was thus defined because curcumin imprints a fluorescence signature on the cells, that is detected in the same channel as the caspase-activity marker [48]. By establishing the gates as previously mentioned, we considered as positive only cells that had a fluorescence signal higher than the curcumin-imprinted one. Therefore, the number of apoptotic cells might have been underestimated in the cytometry assay, since truly positive cells might have been masked by curcumin's fluorescence and were not accounted.

The G_2/M cell cycle arrest induced by curcumin on HNSCC cells is not necessarily a novelty, as we report on our systematic review. However, our original research is the first to describe it on the SCC-9 and FaDu cell lines.

7. CONCLUSIONS

1) Based on our systematic review, curcumin reduced cell viability and tumor growth and induced G_2/M cell cycle arrest and apoptosis in several head and neck cancer cell lines and animal models;
2) On SCC-9 and FaDu cell lines specifically, curcumin reduced cell viability in a dose-dependent and selective manner, induced cell death and G_2/M cell cycle arrest, and modified the cytoskeleton organization and cell morphology;
3) Curcumin also downregulated genes and proteins that are related to the PI3K/AKT/mTOR signaling pathway, which might be associated with the reduction in cell viability, the cytoskeleton disorganization, the cell cycle arrest, and the cell death profile that were observed on SCC-9 and FaDu cells after treatment with curcumin.

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