UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS- GRADUAÇÃO EM ODONTOLOGIA

NÍVEL DOUTORADO

ÁREA DE CONCENTRAÇÃO PATOLOGIA BUCAL

PALOMA SANTOS DE CAMPOS

MODULAÇÃO DO COMPORTAMENTO DE CÉLULAS DE CARCINOMA ESPINOCELULAR ORAL: INFLUÊNCIA DE FATORES QUÍMICOS E FÍSICOS

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Linha de pesquisa: Câncer bucal

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Área de Concentração: Patologia Bucal

Orientador: Prof. Dr. Marcelo Lazzaron Lamers

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Dedico este trabalho a todos aqueles que persistem, porque a persistência realiza o impossível.

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“Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
Quem quer passar além do Bojador
Tem que passar além da dor,
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu.”
Fernando Pessoa

RESUMO

O carcinoma espinocelular oral (CEC) corresponde a 95% dos tumores que acometem a cavidade bucal e apresenta uma taxa de sobrevida em 5 anos de aproximadamente 50%. O tratamento convencional envolve cirurgia, radioterapia e/ou quimioterapia. Todas essas modalidades promovem algum grau de toxicidade e/ou morbidade ao paciente e, em muitos casos, não se mostram totalmente eficientes em impedir a progressão tumoral. Parte deste insucesso clínico se deve à presença de um microambiente tumoral, composto de variantes físicos, químicos e biológicos, o qual é capaz de modular o comportamento das células tumorais, como a migração celular observada durante a metástase. Os compostos que modulam este comportamento, como sais imidazólicos (SI) e polifenois, podem constituir alternativas terapêuticas complementares para melhorar o prognóstico de pacientes. Assim, nessa tese nossa hipótese é que o comportamento agressivo do CEC oral pode ser modulado por elementos físicos do microambiente tumoral e por compostos químicos seletivos. Nesta tese, foram analisados três parâmetros: A) fenótipo migratório das células CEC frente a diferentes durezas do ambiente, B) efeitos de sais imidazólicos sobre o CEC e C) analisar evidências de potenciais compostos para tratamento de câncer. Para o parâmetro A, células de CEC foram tratadas em diferentes graus de rigidez (0.48kPa e 20kPa) na presença/ausência de moduladores da contratilidade celular e submetidos a ensaios de migração. Observou-se que o pré-tratamento em substrato rígido induz maior velocidade de migração celular, o qual se mantém quando a célula é transferida para um ambiente menos rígido. A aquisição desta “memória” mecânica foi dependente da contratilidade celular, indicando potencial terapêutico para prevenir metástases e reincidências. Para o parâmetro B, foi realizado uma triagem de 09 compostos derivados de SI com potencial de afetar o comportamento de células CEC. Observou-se que o composto **C_{16}MImCl** inibiu a proliferação celular e adesão celular, além de induzir a morte celular, apresentando efeitos semelhantes à cisplatina – substância utilizada como quimioterápico em CEC. Para o parâmetro C, foi conduzida uma revisão sistemática para analisar ensaios clínicos sobre o uso de polifenois no tratamento de tumores. Observou-se que os flavonoides, especialmente flavopiridol, mostraram resultados positivos para os tumores não-sólidos (140 pacientes com resposta completa – RC – e 88 com resposta parcial – RP – entre 615 pacientes em 11 ensaios) do que para tumores sólidos (4 pacientes com RC e 21 com PR entre 525 pacientes em 12 ensaios). Em conjunto, conclui-se a importância de avaliar diferentes parâmetros do microambiente tumoral, e assim, compreendendo a interação entre esses componentes pode-se buscar estratégias de tratamento que obtenham maiores taxas de sucesso.

Palavras-chave: Matriz extracelular. Tratamento. Rigidez. Contratilidade. Sal imidazólico. Polifenóis.

ABSTRACT

Oral squamous cell carcinoma corresponds to 95% of tumors that affect the oral cavity and has a 5-year survival rate of approximately 50%. Conventional treatment involves surgery, radiotherapy and or chemotherapy. All of these modalities promote some degree of toxicity and or morbidity to the patient and, in many cases, are not totally efficient in preventing tumor progression. Part of this clinical failure is due to the presence of a tumor microenvironment, composed of physical, chemical and biological variants, which is able to modulate behavior of tumor cells, such as cell migration observed during metastasis. Additionally, compounds that modulate this behavior, such as Imidazolium Salts (IS) and polyphenols, can constitute complementary therapeutic alternatives to improve the prognosis of patients. Our hypothesis is that the aggressive behavior of oral squamous cell carcinoma can be modulated by physical elements of the tumor microenvironment and by selective chemical compounds. In this thesis, three parameters were analyzed: A) migratory phenotype of tumor cells against different stiffness environmental, B) effects of Imidazolium Salts on oral cancer and C) evidence analysis of potential compounds for cancer treatment. For parameter A, OSCC cells were treated at different stiffness degree (0.48kPa and 20kPa) in the presence / absence of cellular contractility modulators and subjected to migration tests. It was observed that pre conditioning on stiff substrate induces a higher rate of cell migration, which is maintained when the cell is transferred to soft environment. The acquisition of this mechanical “memory” was dependent to cellular contractility, indicating therapeutic potential to prevent metastases and recurrences. For parameter B, five IS-derived compounds were screened with the potential to affect the behavior of OSCC cells. It was observed that the compound C_{16}MImCl inhibited cell proliferation and cell adhesion, in addition to induces cell death, presenting effects similar to cisplatin - a substance used as a chemotherapeutic agent in oral cancer. For parameter C, a systematic review was conducted to analyze clinical trials that use polyphenols in the treatment of tumors. It was observed that flavonoids, especially flavopyridol, showed positive results for non-solid tumors (140 patients with complete response - CR - and 88 with partial response - PR - among 615 patients in 11 trials) than for solid tumors (4 patients with CR and 21 with PR among 525 patients in 12 trials). Together, this data demonstrate the importance to evaluate different parameters of tumor microenvironment, and thus, understanding the interaction between these components, we will can look for treatment strategies with greater success rates.

Keywords: Oral squamous cell carcinoma. Extra cellular matrix. Treatment. Stiffness. Contractility. Imidazolium salt. Polyphenols.

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Antecedentes e justificativa

bFGF	Fator básico de crescimento de fibroblasto
CAF	fibroblasto associado ao tumor/ *cancer-associated fibroblasts*
CEC	carcinoma espinocelular oral
DNA	ácido desoxirribonucleico
ECM	matriz extracelular/ extracellular matrix
EMT	transição epitélio-mesênquima/ *epithelial-to-mesenchymal transition*
G3BP2	*Ras GTPase-activating protein binding 2*
HIF	*Fator inductor de hipóxia*
HPV	papiloma vírus humano
IGF-1	Fator de crescimento tipo insulina
INCA	Instituto Nacional de Câncer José Alencar Gomes da Silva
JNK	c-Jun N-terminal kinases
MET	transição mesênquima-epitélio/ *mesenchymal-to-epithelial transition*
MMP	metaloproteinase de matriz
OMS	Organização Mundial de Saúde
Pa	Pascal
PDGFR	receptor de fator de crescimento derivado de plaquetas
TAZ	*WW domain-containing transcription regulator protein 1*
TGF-α	T*ransforming growth factor*
TGF-β	fator de crescimento transformante beta
TME	microambiente tumoral/ tumor microenvironment
UV	ultravioleta
VEGF	fator de crescimento vascular endotelial
YAP	*yes-associated protein*

Artigo científico 1

μM	micromolar
2D	bidimensional
3D	tridimensional
AKT	protein kinase B (PKB)/ serine/threonine-specific protein kinase
ANOVA	analysis of variance
CO_2	carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
FAK	focal Adhesion Kinase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPPG	Grupo de Pesquisa e Pós-Graduação
GSK3β	glycogen synthase kinase 3 beta
HCPA	Hospital de Clínicas de Porto Alegre
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$Inv^H/E:N^L$	low differentiated/low E-cadherin:N-cadherin ratio (SCC-25)
$Inv^L/E:N^H$	high differentiated/ high E-cadherin:N-cadherin ratio (CAL27)
kPa	kilopascal
KRAS	kirsten rat sarcoma viral oncogene homolog
LPA	Lysophosphatidic acid
MII	the non-muscle type II myosin
mM	millimolar
OSCC	oral squamous cell carcinoma
PA	polyacrylamide
PAAGs	polyacrylamide hydrogels
pH	potential for hydrogen
Rac-1	small (~21 kDa) signaling G protein
Rho/ROCK	rho-associated protein kinase

RhoGTPase	family of small (~21 kDa) signaling G proteins
RNA	ribonucleic acid
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
Snail1	transcription factor
TAE	tumor adjacent epithelia
TWIST	transcription factor
UCSD	University of California San Diego
UV	ultraviolet light
Zeb1	transcription factor
β-catenin	catenin beta-1 protein

Artigo científico 2

2D	bidimensional
3D	tridimensional
7AAD	7-aminoactinomycin D
ANOVA	analysis of variance
C_{10}MImCl	1-*n*-decyl-3-methylimidazolium chloride
C_{16}Im	neutral imidazole
$C_{16}M_2$ImCl	1-*n*-hexadecyl-2,3-dimethylimidazolium chloride
$C_{16}M_2$ImMeS	1-*n*-hexadecyl-2,3- dimethylimidazolium methanesulfonate
C_{16}MImCl	1-*n*-hexadecyl-3-methylimidazolium chloride
C_{16}MImMeS	1-*n*-hexadecyl-3-methylimidazolium methanesulfonate
C_{16}PyrCl	pyridinium chloride
C_{18}MImCl	1-*n*-octadecyl-3-methylimidazolium chloride
C_4MImCl	1-*n*-butyl-3-methylimidazolium chloride
CAL27	high differentiated/low invasive OSCC
Cdk4	cyclin Dependent Kinase 4
CDP	cis-platinum
Cl^-	chloride ion
CPD	cumulative Population Double
CyQUANT® NF	Cell Proliferation Assay Kit

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
ESI	electrospray ionization
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
G0/G1	cell cycle phases
HCC	hepatocellular carcinoma
HEK-293	human epithelial kidney
IC50	half maximal inhibitory concentration
ICT	induction chemotherapy
I-ITFSI	imidazolium-(trifluoromethanesulfonyl) imide (TFSI) salt - iodo radiolabeled
IS	Imidazolium salts
MeS^-	methanesulfonate ion
MHz	megahertz
MHz	megahertz
mmol	milimol
MS	mass spectrometry
nm	nanometer
NMR	nuclear magnetic resonance
OSCC	oral squamous cell carcinoma
PARP	poly (ADP-ribose) polymerase
PE	phycoerythrin
ppm	parts per million
RCT	radiochemotherapy
SCC-25	low differentiated/high invasive OSCC
SCC-9	low differentiated/high invasive OSCC
SMMC-7721	human hepatocarcinoma cell line
SRB	sulforhodamine B
TPP1	imidazolium salt with triphenylphosphonium substituent

Artigo científico 3

AML	acute myeloid leukemia
Ara-C	cytosine arabinoside
Car	carboplatin
CI	continuous intravenous infusion
Cis	cisplatin
CLL	chronic lymphocytic leukemia
CR	complete response
Cyt	cytosine
Daun	danorubicin
DFS	disease-free survival
Doc	docetaxel
EFS	event-free survival
Erlo	erlotinib
FAA	flavone acetic acid
Gemc	gemcitabine
IV	intravenous therapy
IVB	intravenous bolus infusion
JADAD	scale for quality assessment of selected clinical trials
Lyc	lycopene
MCL	mantle cell lymphoma
mg/m^2	milligrams per square meter
Mit	mitoxantrone
mo	months
NCI	National Cancer Institute
OS	overall survival
Pac	paclitaxel
PD	progressive disease
PDX	phenoxodiol
PFS	progression-free survival
PR	partial response

Ras	rat sarcoma vírus
RECIST	Criteria—Response Evaluation on Solid tumors
rIL-2	recombinant interleukin-2
SD	stable disease
TFS	treatment-free survival
TTP	time to progression
UK	United Kingdom
USA	United States of America
WHO	World Health Organization Criteria

SUMÁRIO

1. ANTECEDENTES E JUSTIFICATIVA

Câncer é descrito como um grupo de neoplasias malignas, com elevado nível de heterogeneidade, que se caracteriza pelo crescimento anormal das células que adquirem o potencial de proliferar e invadir tecidos adjacentes. Segundo a Organização Mundial da Saúde (OMS) o câncer é a segunda principal causa de morte no mundo, com uma estimativa de 18.1 milhões de casos no ano de 2018, além de 9.6 milhões de mortes para o mesmo período (BRAY; FERLAY; SOERJOMATARAM; SIEGEL *et al.*, 2018). Atualmente, é o principal problema de saúde pública no mundo e cerca de 70% das mortes ocorrem em países com renda média ou baixa. Para o Brasil, a estimativa aponta que ocorrerão 625 mil casos novos de câncer por ano no período de 2020-2022 (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2019).

As neoplasias malignas são caracterizadas por um alto grau de complexidade. Para melhor compreensão da sua diversidade, foram caracterizadas as seguintes capacidades biológicas adquiridas durante o desenvolvimento da doença: 1- manutenção da capacidade proliferativa ilimitada, 2- manutenção da sinalização proliferativa, 3- escape dos supressores de crescimento, 4- resistência a morte celular, 5- indução de angiogênese e, 6- ativação da invasão tecidual e metástase (HANAHAN; WEINBERG, 2000). Proliferação, sobrevivência e invasão são conceitos clássicos relacionados à tumorigênese, no entanto, outras características são descritas como facilitadoras durante este processo como: 1- instabilidades genômica, 2- promoção de inflamação, 3- reprogramação do metabolismo energético e, 4- evasão do sistema imune. Além das células tumorais, os tumores compreendem diversas células recrutadas, que compõem o microambiente tumoral, contribuindo para a aquisição das características que facilitam a progressão da doença (HANAHAN; WEINBERG, 2011).

1.1 Carcinoma espinocelular oral

1.1.1 Epidemiologia do carcinoma espinocelular oral

O carcinoma espinocelular oral (CEC) é o tipo de tumor mais comum da cavidade bucal, representando cerca de 90% dos tumores malignos dessa região, os locais acometidos variam nos estudos previamente reportados, mas os sítios mais comumente afetados são língua e assoalho de boca (JOHNSON; JAYASEKARA; AMARASINGHE, 2011; PIRES; RAMOS; OLIVEIRA; TAVARES *et al.*, 2013; SCHMIDT JENSEN; JAKOBSEN; MIRIAN; CHRISTENSEN *et al.*, 2019). Segundo dados da OMS, no mundo, em 2018 foram estimados 246 mil novos casos para os tumores da cavidade bucal (BRAY; FERLAY; SOERJOMATARAM; SIEGEL *et al.*, 2018). Em relação ao Brasil, o número de casos novos esperados para 2020, será de 11.180 casos para homens e de 4.010 para mulheres (INCA, 2019), sendo que pacientes do sexo masculino são mais acometidos, entre a quinta e sexta década de vida. De acordo com a Sociedade Americana de Câncer, os dados mostram que a incidência do câncer de boca continua aumentando (SIEGEL; MILLER; JEMAL, 2020). Os dados mostram que o número de casos vem aumentando e a taxa de sobrevida não foi alterada significativamente, assim é importante conhecer os dados epidemiológicos sobre esse tipo de tumor, os fatores de risco associados, além do perfil molecular e do paciente acometido pela doença para que se possa planejar e buscar formas de tratamento mais resolutivas.

1.1.2 Aspectos clínicos e histopatológicos

O tumor da cavidade bucal é caracterizado clinicamente por uma placa branca ou vermelha não removível a raspagem, geralmente ulcerada, com evolução por mais de duas semanas, possui bordos irregulares, elevados e endurecidos (Figura 1)(CHI; DAY; NEVILLE, 2015). Estudos tem mostrado que taxa de sobrevida para esses pacientes ainda é muito baixa, entre 40 e 63% em 5 anos. Esta variação é explicada pelo estadiamento clínico do tumor durante o processo diagnóstico, porque tumores em estágio mais avançados estão relacionados a um pior prognóstico (KAMANGAR; DORES; ANDERSON, 2006; MONTERO; PATEL, 2015; SCULLY; BAGAN, 2009).

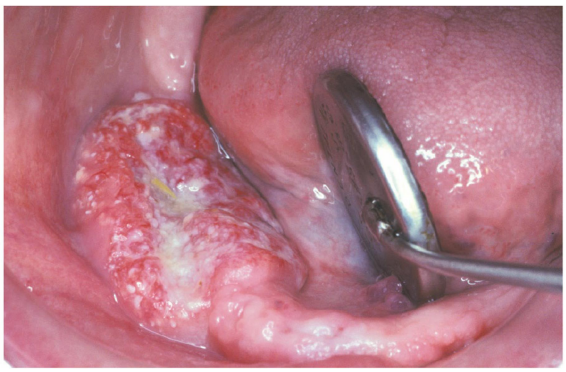

Figura 1: Carcinoma Epidermoide. Lesão exofítica com superfície e irregular possui (NEVILLE; DAMM; ALLEN; CHI, 2016).

O CEC oral é histopatologicamente (Figura 2) caracterizado por ilhas e cordões de células epiteliais malignas que invadem o tecido conjuntivo, a invasão é representada pela descontinuidade do epitélio neoplásico através da membrana basal em direção ao tecido conjuntivo subjacente. Células epiteliais individuais, lençóis ou ilhas de células proliferam para o interior do tecido conjuntivo, sem adesão ao epitélio superficial podendo estender-se em profundidade para o tecido adiposo, muscular ou ósseo, além de feixes nervosos e vasos sanguíneos. Há regularmente uma exacerbada resposta inflamatória celular ou imune invadindo o tumor, e necrose pode estar presente. A neoplasia pode induzir uma densa fibrose (desmoplasia) e a neoformação vascular. As células neoplásicas apresentam abundante citoplasma eosinofílico com núcleos volumosos e hipercromáticos e uma relação núcleo-citoplasma aumentada. Graus variados de pleomorfismo celular e nuclear são observados. E pérolas de ceratina (focos arredondados de camadas concêntricas de células queratinizadas) podem ser produzidas no interior do epitélio neoplásico (NEVILLE; DAMM; ALLEN; CHI, 2016).

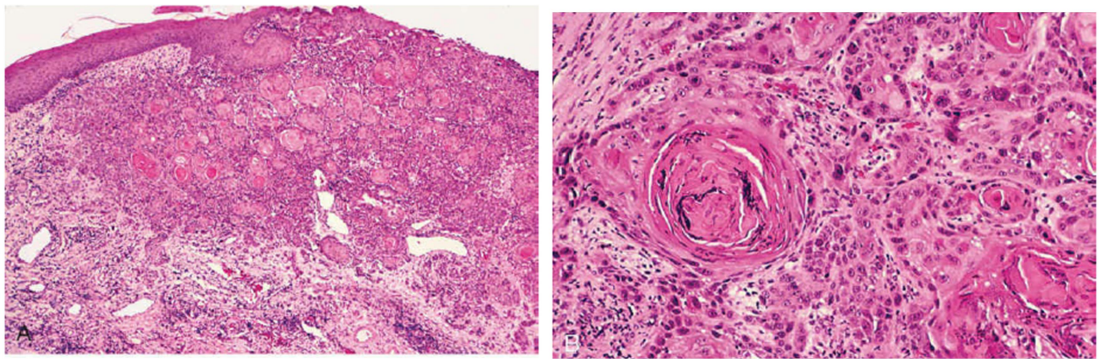

Figura 2: Carcinoma Espinocelular oral. A - Ilhas de células epiteliais malignas invadindo o tecido conjuntivo. **B -** Células epiteliais displásicas com a formação de pérolas de queratina (NEVILLE; DAMM; ALLEN; CHI, 2016).

1.1.3 Fatores de risco

O CEC oral é multifatorial e desta forma, diferentes fatores de risco tem sido tem sido relacionados com a iniciação desses tumores (Figura 3). A mucosa bucal é suscetível a uma série de alterações induzidas por agentes físicos, químicos e biológicos, sendo considerados como principais fatores de risco tabagismo, etilismo e radiação solar. Trabalhos mais recentes têm concentrado evidencias de que o papiloma vírus humano é um fator etiológico importante para alguns subtipos de tumores de cabeça e pescoço(CHI; DAY; NEVILLE, 2015; NEVILLE; DAMM; ALLEN; CHI, 2016). Em nível molecular, a carcinogênese envolve o acúmulo de mutações ou alterações epigenéticas de proto-oncogenes e genes supressores de tumores, no qual esses podem ter sua expressão ativada ou suprimida. Assim, promovendo uma série de eventos que são característicos dos tumores, como: divisão celular descontrolada, resistência à morte celular, e capacidade de invasão e metástase (HANAHAN; WEINBERG, 2011).

Dentre os fatores químicos da carcinogênese do CEC oral, o fumo é um importante fator de risco, associado a 75% dos casos e eleva o risco de desenvolvimento de câncer em 6 vezes quando comparado com pessoas não fumantes (MARKOPOULOS, 2012). A Agência Internacional de pesquisa em câncer classifica o tabaco como o grupo número 1 em carcinógenos para a cavidade bucal e a faringe, que está relacionado com a quantidade e o tempo de consumo (CHI; DAY; NEVILLE,

2015). Os produtos químicos componentes do cigarro sofrem alterações coordenadas por enzimas oxidativas e o produto final torna-se capaz de se ligar ao DNA originando mutações (HEMA; SMITHA; SHEETHAL; MIRNALINI, 2017; LEE; TANEJA; VASSALLO, 2012; RIVERA, 2015). Outro elemento importante é álcool que possui ação local promovendo o aumento da permeabilidade da mucosa bucal, dissolvendo componentes lipídicos do epitélio, interferindo na síntese e reparo do DNA. Além disso, sua ação sistêmica afeta a capacidade do fígado de lidar com compostos tóxicos ou potencialmente carcinogênicos, e seu uso crônico está associado a um comprometimento da imunidade inata e adquirida, resultando em maior suscetibilidade a infecções e neoplasias (REIDY; MCHUGH; STASSEN, 2011; RIVERA, 2015). O álcool e fumo possuem um efeito sinérgico, e a combinação de ambos produz um risco aumentado em 15 vezes para o desenvolvimento de câncer de boca (MARKOPOULOS, 2012).

Em relação aos fatores biológicos que contribuem para a carcinogênese oral, os tipos de papilomavírus humano 16 e 18 são os mais correlacionados com o carcinoma espinocelular oral, e esta associação prediz um prognóstico favorável para tumores orofaríngeos localmente avançados (KANG; KIESS; CHUNG, 2015). Este comportamento pode ser explicado pela diferença em perfis moleculares e no comportamento clínico dos tumores HPV positivo e negativo, particularmente no que diz respeito à invasão, metástase e resposta à quimioterapia e radioterapia (DAYYANI; ETZEL; LIU; HO *et al.*, 2010; FLEMING; WOO; MOUTASIM; MELLONE *et al.*, 2019; KRUPAR; ROBOLD; GAAG; SPANIER *et al.*, 2014). As evidências mostram que o HPV contribui para a carcinogênese por meio de duas proteínas codificadas por vírus: a proteína E6 que promove a degradação do produto do gene supressor de tumor p53 e E7 que promove a degradação do produto do gene supressor de tumor pRb (proteína de retinoblastoma), causando uma desregulação do controle do ciclo celular, o que também leva a uma superexpressão do inibidor da ciclina dependente de quinase $p16^{Ink4a}$ (DALIANIS, 2014).

Entre os fatores físicos, destaca-se a radiação solar, ambas UVA e UVB podem produzir danos no DNA, tanto diretamento, como indiretamente por meio da produção de espécies reativas de oxigênio (ROS) (RODUST; STOCKFLETH; ULRICH;

LEVERKUS *et al.*, 2009). Ainda pode ocorrer a ativação de oncogenes, mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases (JNK), p38 e a inativação de genes supressors de tumor, p53 (EBERLE; FECKER; FORSCHNER; ULRICH *et al.*, 2007; NISHIGORI, 2006). O processo de carcinogênese do CEC oral é complexo e composto por múltiplas etapas que pode ser moduladas pelos fatores de risco associados ao desenvolvimento da doença.

1.1.4 Processo de tumorigênese

O desenvolvimento do câncer, processo denominado de tumorigênese, se inicia a partir de um grupo de células que sofreram mutações genéticas, principalmente nos genes que controlam a proliferação e a apoptose celular, levando a um crescimento descontrolado (HANAHAN; WEINBERG, 2000). Conforme o tumor progride, inúmeras alterações ocorrem em diferentes células presentes no local e na matriz extracelular (MEC), propiciando que a lesão se desenvolva, sendo a metástase – estabelecimento de um novo tumor à distância do tumor primário o estágio mais avançado desse evento (TSANTOULIS; KASTRINAKIS; TOURVAS; LASKARIS *et al.*, 2007). Além do descontrole na proliferação, as células tumorais reprogramam seu metabolismo, preferindo a glicólise para atender às altas demandas de ATP (ROMERO-GARCIA; LOPEZ-GONZALEZ; BÁEZ-VIVEROS; AGUILAR-CAZARES *et al.*, 2011). Outra característica do microambiente tumoral é a acidose, que resulta do aumento local da produção de ácido lático pelas células tumorais direcionando a reprogramação metabólica das células tumorais e serve como uma pressão seletiva adicional (KOUKOURAKIS; GIATROMANOLAKI; WINTER; LEEK *et al.*, 2009). A associação de diferentes capacidades adquiridas pelas células tumorais suporta a invasão tecidual e a manutenção do crescimento do tumor.

A cavidade bucal é revestida por epitélio estratificado queratinizado e não queratinizado e a proliferação celular é restrita à camada basal. As células progenitoras do epitélio da região de cabeça e pescoço equilibram proliferação e diferenciação durante a homeostase, no entanto, quando há um acúmulo sequencial de mutações genéticas ocorre a ruptura dessa homeostase e o início do processo neoplásico (LEEMANS; BRAAKHUIS; BRAKENHOFF, 2011; SÁNCHEZ-DANÉS; BLANPAIN,

2018). As mutações podem ser do tipo somáticas (Som mut), de amplicação (Amp) ou de deleção (Del) em genes que controlam o ciclo celular, os receptores de tirosina quinase (RTK), vias de sinalização como RAS e AKT, diferenciação epitelial e remodelamento da cromatina (Comprehensive genomic characterization of head and neck squamous cell carcinomas, 2015; STRANSKY; EGLOFF; TWARD; KOSTIC *et al.*, 2011). O gene supressor de tumor P53, que induz a parada no ciclo celular e apoptose, é o mais comumente mutado (CHEN, 2016), assim como a amplificação de MYC e ciclina D1 que se liga a CDK4 e CDK6 promovendo a progressão da fase G1 para S do ciclo celular (PICKERING; ZHANG; YOO; BENGTSSON *et al.*, 2013). Além disso, os tumores de cabeça e pescoço apresentam amplificação do receptor do fator de crescimento epidérmico (EGFR) em 80 a 90% dos casos e está relacionado a um pior prognóstico e no receptor do fator de crescimento de fibroblastos (FGRR1) (PICKERING; ZHANG; YOO; BENGTSSON *et al.*, 2013; SOLOMON; YOUNG; RISCHIN, 2018). Estudos tem mostrado que EGFR pode ativar a via de sinalização PI3K/Akt/mTOR que apresenta-se desregulada em tumores de cabeça e pescoço, sendo que o gene PI3KCA apresenta uma taxa de mutação de 16%, e a inibição de AKT tem sido sugerida para contornar a resistência do tumor ao tratamento (KANG; KIESS; CHUNG, 2015; SWICK; PRABAKARAN; MILLER; JAVAID *et al.*, 2017).

O acúmulo de alterações genéticas e epigenéticas em células epiteliais são capazes induzir proliferação e rompimento da membrana basal, e então as células migram em direção ao tecido conjuntivo dando origem ao carcinoma espinocelular oral. Durante este processo as células demonstram menor expressão de E-caderina e um aumento na expressão de N-caderina, que está associado a um comportamento mais agressivo do tumor devido à modulação da migração celular pela mudança no comportamento das adesões (M; PIERANTONI; FEOLA; ESPOSITO *et al.*, 2011; MATTE; KUMAR; PLACONE; ZANELLA *et al.*, 2019). A capacidade das células tumorais em invadir o tecido conjuntivo é essencial para acessar os vasos sanguíneos e promover metástases. Estes eventos, invasão tecidual e metástase, são processos altamente heterogêneos que exigem a adaptação das células tumorais a novos ambientes (SETHI; KANG, 2011). A migração das células neste novo ambiente envolve a interação coordenada de moléculas estruturais e de sinalização que resultam na

polimerização da actina, adesão à MEC por meio de integrinas, contração do corpo celular e rompimento das adesões na parte traseira da célula (RIDLEY; SCHWARTZ; BURRIDGE; FIRTEL *et al.*, 2003). A família das Rho GTPases está envolvida em alterações na organização da actomiosina que conduzem alterações na migração celular. Por exemplo, Rac1 regula a formação dos filamentos de actina associados à formação de adesões nascentes, enquanto RhoA controla contratilidade celular e maturação das adesões (PARSONS; HORWITZ; SCHWARTZ, 2010; RIDLEY, 2011).

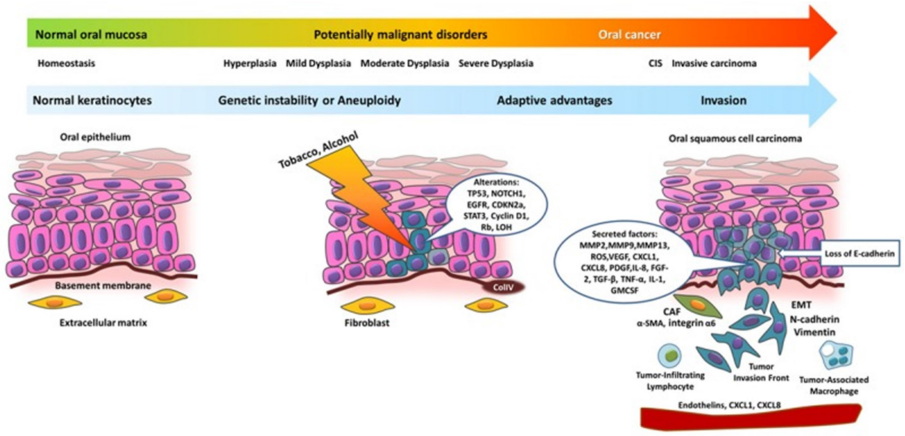

Figura 3: Modelo de progressão tumoral. O desenvolvimento do carcinoma espinocelular oral é um processo complexo composto por várias etapas. Os queratinócitos da mucosa oral normal são expostos cronicamente a fatores de risco, que podem modular a homeostase e promover instabilidade genômica (RIVERA, 2015).

1.2 Transição epitélio-mesênquima (EMT)

A mudança em direção a um estado mesenquimal faz parte de um processo conhecido como transição epitélio-mesênquima (EMT) (Figura 4), no qual há a modificação de moléculas de adesão expressas pela célula epitelial, permitindo que estas adotem um comportamento migratório e invasivo (THIERY; ACLOQUE; HUANG; NIETO, 2009). Pesquisas tem revelado que este processo não é definitivo nem estático, podendo ocorrer o caminho reverso, chamado como transição mesênquima-epitélio (MET). Este configura a perda da liberdade migratória, na qual as células adotam polarização ápico-basal e voltam a expressar proteínas que são características dos tecidos epiteliais (NIETO; HUANG; JACKSON; THIERY, 2016). A grande maioria

das vias de sinalização conhecidas que promovem a EMT (TGFβ, Wnts, Notch, EGF, HGF, FGF e HIF) convergem para a indução de repressores de E-caderina (THIERY; ACLOQUE; HUANG; NIETO, 2009). Assim, a expressão de fatores específicos de transcrição, chamados de marcadores de EMT, Snail, Zeb e Twist são os mais citados, exibindo aumento em sua expressão em linhagens de CEC oral com elevado grau de agressividade (MATTE; KUMAR; PLACONE; ZANELLA *et al.*, 2019).

Foi demonstrado a correlação de proteínas relativas à EMT no prognóstico de pacientes com CEC oral e observou-se que superexpressão de vimentina em 53% dos pacientes, redução da expressão de E-caderina e β-catenin em 84% das amostras, sendo associada a recorrência do tumor e morte dos pacientes (LIU; JIANG; ZHOU; WANG *et al.*, 2010). Adicionalmente, os fatores de transcrição Zeb2 e Snail ativam a expressão de metaloproteases de matriz (MMP) que são proteases que facilitadoras da degradação da membrana basal suportando a invasão das células (MIYOSHI; KITAJIMA; KIDO; SHIMONISHI *et al.*, 2005). O remodelamento da MEC e mudanças na interação da célula com a matriz são fundamentais para a iniciação e progressão da EMT. Essa interação é mediada por subtipos de integrinas, sendo que algumas são superexpressas e outras suprimidas, essa modulação colabora para o aumento da expressão de MMPs (LAMOUILLE; XU; DERYNCK, 2014; PAGE-MCCAW; EWALD; WERB, 2007). A plasticidade epitelial é dependente de um contexto de mudanças em vias de sinalização distintas e *crosstalk* das células com componentes extracelulares que irão influenciar a iniciação e a progressão tumoral.

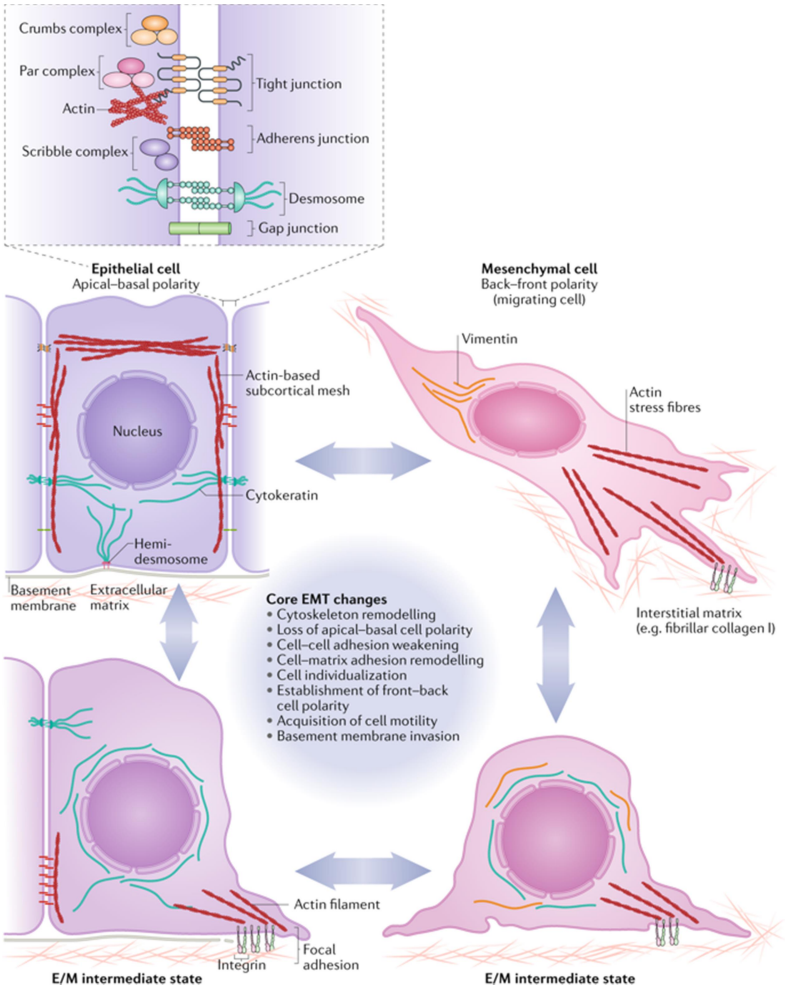

Figura 4: Processo de transição epitélio-mesênquima. As células epiteliais são conectadas umas às outras por meio de uma variedade de junções epiteliais, incluindo junções aderentes, desmossomos e junções tipo gap. As junções estão conectadas aos feixes de actina, enquanto os desmossomos estão ligados a filamentos intermediários de citoqueratina. A polaridade ápico-basal orienta a organização adequada das junções aderentes e desmossomos nas células epiteliais. As células epiteliais são conectadas à membrana basal por meio de hemidesmossomos, que contêm integrinas que permitem a ligação à membrana basal e também estão ligadas a citoqueratinas no interior da célula. Por outro lado, as células mesenquimais não contêm junções epiteliais funcionais (YANG; ANTIN; BERX; BLANPAIN *et al.*, 2020).

1.3 Invasão e metástase

A invasão tecidual é caracterizada por alterações moleculares importantes, que levam a modificação da adesão célula-célula e célula-MEC, facilitando o processo de migração celular (ETIENNE-MANNEVILLE, 2008; FRIEDL, 2004; PAINTER; ARMSTRONG; SHERRATT, 2010). A invasão tecidual e o desenvolvimento de potencial metastático de tumores malignos é a maior causa de insucessos clínicos em termos de terapia e prognóstico. A invasão tecidual pode ocorrer por meio de migração celular coletiva ou individual, caracterizada por alterações moleculares importantes, como expressão e fosforilação de proteínas (PAINTER; ARMSTRONG; SHERRATT, 2010).

A migração celular é um processo essencial para o desenvolvimento e também está envolvida em patologias importantes, como o câncer (WEBB; ZHANG; HORWITZ, 2005). Quando a migração é iniciada, ocorre a polimerização de actina na porção frontal da célula (*leading edge*), promovendo a formação de grandes projeções de membrana (lamelipódios) e o estabelecimento de novas adesões ao substrato. A adesão celular é um processo dinâmico e ocorre a partir da interação de integrinas com proteínas da MEC resultando na liberação de moléculas sinalizadoras intracelulares bem como no recrutamento de proteínas moduladoras da ligação entre integrinas e proteínas do citoesqueleto (LAUFFENBURGER; HORWITZ, 1996; RIDLEY; SCHWARTZ; BURRIDGE; FIRTEL *et al.*, 2003). Cada etapa da migração celular é regulada por GTPases de baixo peso molecular pertencente à família Rho (de Ras-homology), que desempenham um papel fundamental nesse processo. Estas proteínas ciclam entre um estado inativo (ligadas a GDP) e um estado ativo (ligadas a GTP). A ativação das Rho GTPases, principalmente RhoA (envolvida, principalmente, na formação de fibras de estresse) e Rac1 (envolvida na formação de lamelípodios e complexos focais), estão associados a uma maior capacidade de invasão e migração de diferentes tumores, e a expressão elevada dessas proteínas tem sido associada a um pior prognóstico dos pacientes (ALAN; LUNDQUIST, 2013; HANNA; EL-SIBAI, 2013; HORWITZ; WEBB, 2003).

1.4 Microambiente tumoral

A lesão tumoral não consiste somente de uma população heterogênea de células tumorais, mas também de uma série de células residentes e infiltradas no tecido, fatores secretados e proteínas da matriz extracelular, constituindo o microambiente tumoral (TME) (JOYCE; POLLARD, 2009). Dessa forma, o TME compreende componentes biológicos, químicos e físicos presentes na região do tumor (Figura 5)(GILKES; SEMENZA; WIRTZ, 2014). As células tumorais, fibroblastos associados ao tumor (CAFs), células endoteliais, pericítos, células inflamatórias, células residentes e recrutadas para o TME, fazem parte dos componentes biológicos, no entanto, os diferentes tipos celulares podem ter ações antagonistas entre atividades pró e antitumorais (HANAHAN; WEINBERG, 2011). Os macrófagos são células inerentemente plásticas, e essa adaptabilidade pode ser explorada pelo tumor para desencadear funções distintas em diferentes estágios de progressão(JOYCE; POLLARD, 2009). Dependendo do estímulo, os macrófagos podem polarizar-se no perfil M1 ou M2, onde M1 atua como pró-inflamatório e antitumoral, e M2 como anti-inflamatório e pró-tumoral(LEWIS; POLLARD, 2006). Células tumorais e macrófagos também utilizam fibras colágenas como facilitadores para percorrer rapidamente o estroma (CONDEELIS; SEGALL, 2003; INGMAN; WYCKOFF; GOUON-EVANS; CONDEELIS *et al.*, 2006). Vários estudos demonstraram que os macrófagos são importantes para o prognóstico de pacientes com diferentes tipos de câncer, e uma revisão sistemática avaliou o valor prognóstico em pacientes com CEC oral demonstrando que é mais relevante para a avaliação a presença de macrófagos polarizados do que a região do tumor em que se encontra (ALVES; DIEL; LAMERS, 2018; POLLARD, 2008).

Os fibroblastos associados ao tumor (CAFs) apresentam sinalização modificada desenvolvendo um fenótipo ativado e resistência a mecanismo de morte celular, alguns trabalhos relatam sua transformação em miofibroblastos, e passam a expressar marcadores como actina do músculo liso α (α-SMA) e proteína de ativação de fibroblastos (ZHOU; CHEN; WANG; LIN *et al.*, 2014). Estas células demonstram um papel importante no microambiente tumor devido a secreção de proteínas capazes de remodelar a matriz extracelular (ECM) (AUGSTEN, 2014; PELTANOVA; RAUDENSKA; MASARIK, 2019; SHIGA; HARA; NAGASAKI; SATO *et al.*, 2015). Foi demonstrado em

tumores de cabeça e pescoço que fibroblastos associados ao tumor regulam a capacidade de resposta à terapia, exibindo resistência a cetuximabe e a radioterapia (BERNDT; BÜTTNER; GÜHNE; GLEINIG *et al.*, 2014; HUELSKEN; HANAHAN, 2018; SCHMITZ; BINDEA; ALBU; MLECNIK *et al.*, 2015; ZHANG; HUA; JIANG; YUE *et al.*, 2019).

Os componentes químicos caracterizados por fatores de crescimento e hipóxia interagem com as células influenciando na progressão tumoral (GILKES; SEMENZA; WIRTZ, 2014). A hipóxia aumenta o recrutamento de células mesenquimais o que resulta em aumento das metástases linfáticas e vasculares, por intermédio de fator de crescimento endotelial vascular (VEGF) - que é liberado pelas células tumorais hipóxicas, e mais frequentemente pelas células endoteliais, fibroblastos e células inflamatórias (CHATURVEDI; GILKES; WONG; LUO *et al.*, 2013; KARNOUB; DASH; VO; SULLIVAN *et al.*, 2007). Induz permeabilidade microvascular, que por sua vez medeia um influxo de fibroblastos, células inflamatórias e células endoteliais no tumor primário (BROWN; GUIDI; SCHNITT; VAN DE WATER *et al.*, 1999). Além disso, VEGF tem sido está associado com fibrose devido a ativação de células do estroma e a produção de uma matriz rica em fibronectina e colágeno tipo I (KALLURI; ZEISBERG, 2006). Além desses componentes, a MEC faz parte do TME como um componente não-celular, regulando o comportamento tumoral por meio de mudanças mecânicas no estroma.

Essas capacidades adquiridas pelas células tumorais podem influenciar e ao mesmo tempo sofrer alterações por meio do recrutamento de outros tipos celulares, que irão associar-se ao tumor. Ainda, há a produção de uma série de fatores (fator indutor de hipóxia, VEGF, TGFβ) e mudanças na composição e na proporção de componentes da ECM tumoral. Este rearranjo ambiental e celular é chamado de microambiente tumoral. A progressão tumoral é profundamente influenciada pelas interações das células tumorais com o ambiente, o qual determina se o tumor primário é erradicado ou metastatiza, por exemplo. Além disso, TME também pode moldar respostas terapêuticas e resistência a terapia, justificando os esforços recente para obter uma terapia alvo que possa atingir os componentes do microambiente tumoral.

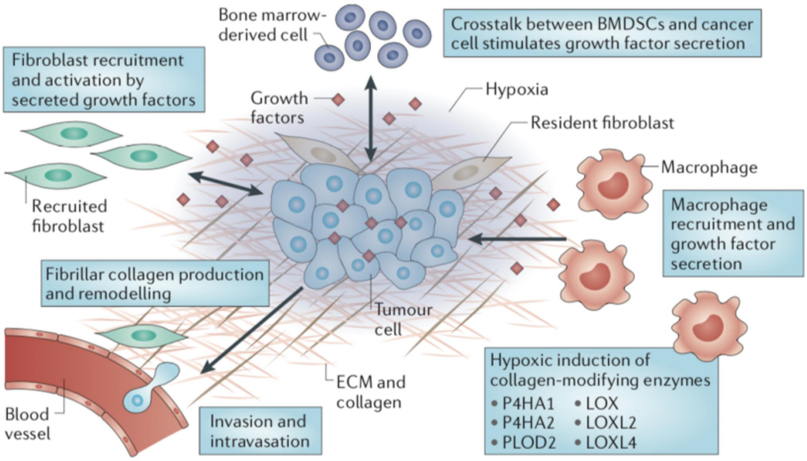

Figura 5: Microambiente tumoral (TME). A secreção de HIF pelas células tumorais promove o recrutamento de macrófagos e fibroblastos para regiões hipóxicas do tumor primário. Os macrófagos produzem fatores de crescimento, como TGFβ1 e o fator de crescimento derivado de plaquetas que ativam fibroblastos recrutados e residentes para estimular a deposição de colágeno. As células cancerígenas hipóxicas também sinalizam para as células-tronco mesenquimais, que podem participar da deposição de colágeno. O HIF regula a produção de enzimas modificadoras de colágeno, para facilitar a maturação adequada das fibras de colágeno. Juntas, essas vias de sinalização promovem a produção de uma rede de colágeno fibrilar (produzida por vários tipos de células), o que aumenta a capacidade das células cancerígenas de invadir os vasos sanguíneos(GILKES; SEMENZA; WIRTZ, 2014).

1.4.1 Matriz extracelular - Fatores físicos

As propriedades físicas do tumor se referem a rigidez, topografia, porosidade e solubilidade da matriz extracelular, refletindo as alterações do microambiente tumoral.

A MEC é formada por um complexo de proteínas e glicoproteínas que são secretados e formam uma rede organizada que envolve as células. É constituída por substância fundamental e pelo componente fibrilar, como laminina, fibronectina, vitronectina, elastina, glicoproteínas, fibras colágenas e elásticas, porém essa composição pode variar de tecido para tecido (ALBERTS, Bruce et al., p. 1035-1072). Além de propiciar a sustentação dos tecidos, a MEC desempenha um papel essencial

no desenvolvimento de processos biológicos de reparo tecidual e metástase através da regulação da proliferação, diferenciação, adesão, migração e expressão gênica celular (ROSS, Michel H.; PAWLINA, Wojciech., cap.6). A remodelação da MEC ocorre tanto durante processos fisiológicos como o processo de remodelação óssea, quanto em processos patológicos como o câncer.

As MMPs são enzimas proteolíticas que estão envolvidas na degradação da MEC. Em condições fisiológicas, desempenham funções importantes na regulação da MEC durante a embriogênese ou remodelação de tecidos (remodelação óssea). Em condições patológicas, as MMPs participam da destruição da MEC em processos inflamatórios, além do crescimento de tumores e cistos, podendo ser secretadas por células como fibroblastos, macrófagos, neutrófilos, linfócitos, queratinócitos e células tumorais. Cerca de vinte e quatro tipos de MMPs já foram identificadas em humanos, sendo classificadas de acordo com sua estrutura molecular e o substrato que degradam. As MMPs podem clivar componentes da MEC resultando no aumento do espaço para célula ou tecido, podem gerar a clivagem de produtos específicos e assim iniciar uma sinalização autócrina ou parácrina. Ou ainda possuem capacidade de regular diretamente a arquitetura do tecido epitelial através da clivagem das junções intercelulares ou da célula com a sua membrana basal (PAGE-MCCAW; EWALD; WERB, 2007; WOLF; WU; LIU; GEIGER *et al.*, 2007).

As células e a MEC interagem por meio de estímulos específicos, no qual proteínas sinalizadoras são secretadas podem alterar propriedades físicas da matriz e consequentemente essa modificação pode alterar o comportamento celular (GEIGER; YAMADA, 2011). O fenômeno pelo qual as células “sentem” e respondem a estímulos mecânicos da matriz, convertendo-os em sinais bioquímicos que provocam respostas celulares específicas é denominado: mecanotransdução (Figura 6) (WANG; TYTELL; INGBER, 2009). As integrinas são capazes de mudar de conformação quando se conectam ao ligante permitindo que a integrina emita sinais em duas direções, através da membrana plasmática de dentro para fora e de fora para dentro da célula. Existem diferentes tipos de integrinas que se ligam a componentes específicos da matriz. Então, após um estímulo mecânico (mudança de composição ou aumento da rigidez da MEC, por exemplo) as integrinas são ativadas, e se ligam a talina-1 no citoplasma da célula,

promovendo a associação de vinculina-talina e Src, com posterior ativação de quinase de adesão focal (FAK). Estas etapas culminam na contratilidade de actomiosina e remodelamento do citoesqueleto de actina, associado a esta cascata de sinalização há a geração de uma resposta específica, como aumento da fosforilação de proteínas e mudanças na transcrição gênica que culminam na alteração de comportamento da célula (JANSEN; ATHERTON; BALLESTREM, 2017; STUTCHBURY; ATHERTON; TSANG; WANG *et al.*, 2017).

O processo de mecanostransdução pode ser didaticamente dividido: 1- *Mechanosensing* que compreende o ato pelo qual a célula "sente" o estímulo mecânico, 2- *Mechanosignalling* que corresponde aos eventos de sinalização intracelular que ocorrem em resposta ao estímulo mecânico e 3- *Mechanoresponse* entendida como a resposta específica das células ao estímulo mecânico. Algumas vias de sinalização já foram descritas como vias de mecanotransdução, a via do Yap (Yes-associated protein) é a principal já descrita (Figura 7), esta via atua promovendo proliferação celular, sobrevivência e manutenção do controle da diferenciação da célula indiferenciada. E devido ao processo de mecanostrandução, o yap está localizado no citoplasma da célula é translocado para o núcleo, modificando o comportamento celular (DASGUPTA; MCCOLLUM, 2019; NARDONE; OLIVER-DE LA CRUZ; VRBSKY; MARTINI *et al.*, 2017; ZANCONATO; CORDENONSI; PICCOLO, 2016). Outro componente clássico já descrito é o fator de transcrição Twist, conhecido por promover a EMT, ele também é translocado para o núcleo de acordo com as mudanças nas propriedades físicas da MEC (BRODERS-BONDON; NGUYEN HO-BOULDOIRES; FERNANDEZ-SANCHEZ; FARGE, 2018).

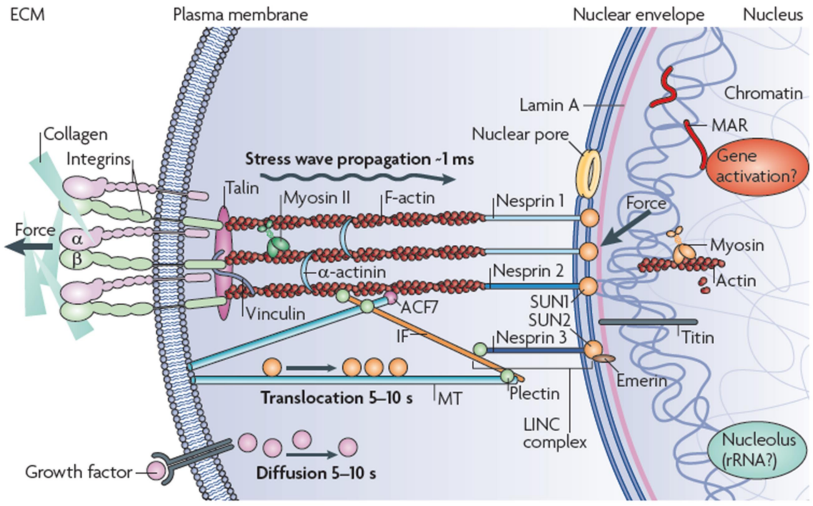

Figura 6: Mecanotransdução. As integrinas conectam as células à MEC, dessa forma: as integrinas inativas, são estimuladas, por exemplo pelo aumento da rigidez do substrato, e assim ativadas, as integrinas se ligam a talina, ativando FAK, e consequentemente há o aumento da contratilidade celular por meio do recrutamento de miosinas e remodelamento do citoesqueleto de actina. Devido à viscoelasticidade citoplasmática, a propagação de força para o núcleo pode levar as proteínas que mantem a sua posição à conectar-se à cromatina e ao DNA, e assim afetar diretamente a ativação de genes (WANG; TYTELL; INGBER, 2009).

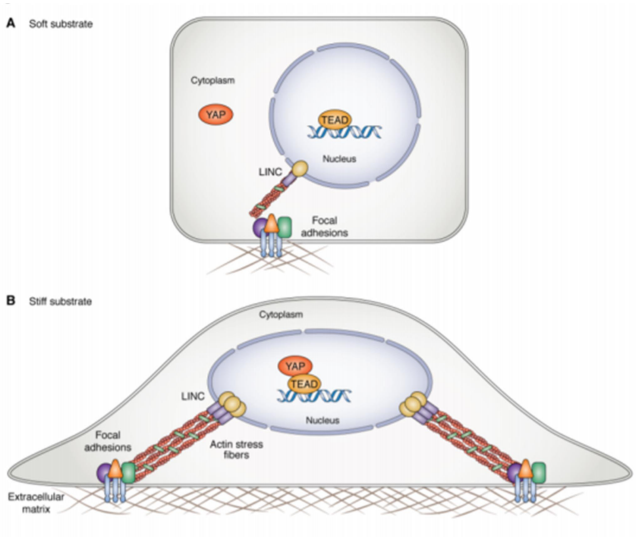

Figura 7: Localização da proteína YAP de acordo com a rigidez do substrato. (DASGUPTA; MCCOLLUM, 2019).

A MEC pode ser remodelada de acordo com a sinalização local, no entanto, essas alterações na dinâmica da MEC podem resultar em alteração da normalidade tecidual, normalidade da MEC é mantida pela atividade dos fibroblastos residentes, apresentando uma rede “solta” de colágeno, elastina e fibronectina ancorada na membrana basal. Em situações patológicas como fibroses, os fibroblastos podem se diferenciar em miofibroblastos, altamente contráteis, que depositam altos níveis de moléculas na matriz aumentando a rigidez do tecido através de ligações covalentes do colágeno com a elastina. Adicionalmente, tanto no sítio primário como no metastático a matriz é rígida contendo ligações de colágeno, altos níveis de fibronectina, tenascina c e hialuronana (BURGSTALLER; OEHRLE; GERCKENS; WHITE *et al.*, 2017; WEI; FATTET; TSAI; GUO *et al.*, 2015). A matriz é fisicamente diferente dependo da origem do tecido: neurogênico (1 kPa), miogênico (17 kPa) e osteogênico (40 kPa), a partir disso foi demonstrado que células mesenquimais indiferenciadas quando presentes em matrizes com diferentes graus de rigidez (em nível compatível com os respectivos tecidos) são capazes de se diferenciar em linhagens celulares de diferentes origens: neurogênica, miogênica e osteogênica (ENGLER; SEN; SWEENEY; DISCHER, 2006). Portanto, a matriz associada ao tumor é substancialmente remodelada levando a mudanças em sua composição e densidade, essas resultam em alterações nas propriedades mecânicas, como a modificação da rigidez da matriz (Figura 8). Os tumores são tipicamente mais rígidos que os tecidos saudáveis, e esta propriedade é utilizada para o diagnóstico clínico durante palpação tecidual, por exemplo.

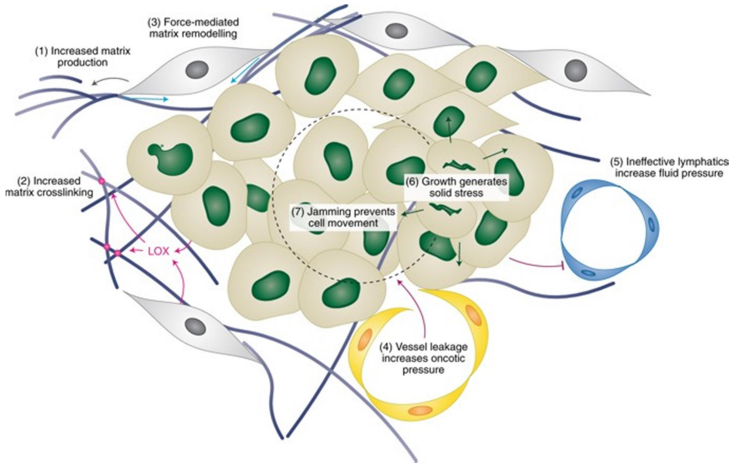

Figura 8: Alterações da mecânica tecidual. (1) aumento da deposição da matriz; (2) aumento do *crosslinking* da matriz (pontos cor de rosa); (3) força contrátil que impulsiona a compactação local e organização de fibras (setas azuis); (4) drenagem linfática defeituosa; (5) crescimento celular gerando pressão sólida (setas verdes); (6) alta densidade celular (MOHAMMADI; SAHAI, 2018).

Nesse contexto, a rigidez da MEC vem sendo amplamente estudada e foi comprovado que o aumento é capaz de promover fenótipos malignos por causar alterações na expressão gênica e comportamento celular (LEVENTAL; YU; KASS; LAKINS *et al.*, 2009; PASZEK; ZAHIR; JOHNSON; LAKINS *et al.*, 2005; PROVENZANO; INMAN; ELICEIRI; KEELY, 2009). Já foi demonstrado para tumores de mama que a rigidez altera a organização da cromatina, ou seja, na matriz rígida há mais pontos de cromatina acessível quando compara com uma matriz menos rígida (*soft*). Além disso, houve aumento da expressão de FAK fosforilada, vimentina e perda de integrina normal β4 (STOWERS; SHCHERBINA; ISRAELI; GRUBER *et al.*, 2019).

O aumento da rigidez promove proliferação e invasão das células tumorais de pulmão, e associada à macrófagos contribuem conjuntamente para um fenótipo invasivo modulando a expressão dos principais marcadores relacionados à EMT (ALONSO-NOCELO; RAIMONDO; VINING; LOPEZ-LOPEZ *et al.*, 2018). Sabemos que TWIST é um mecanomediador regulado pelo aumento da rigidez, e pesquisadores mostraram que o aumento da proteína G3BP2 impede a translocação desse fator de transcrição. Além disso, o mesmo trabalho demonstrou que a baixa expressão de

G3BP2 e o aumento da organização de colágeno em tumores de mama predizem pior prognóstico (WEI; FATTET; TSAI; GUO *et al.*, 2015).

O aumento da rigidez da matriz revela resultados similares para diferentes tipos de tumor, promovendo maior organização do citoesqueleto de actina, aumento da expressão de paxilina, FAK fosforilada, fatores de transcrição relacionados com EMT (TILGHMAN; COWAN; MIH; KORYAKINA *et al.*, 2010). Adicionalmente, células de CEC oral podem ser mecanicamente condicionadas por uma matriz rígida e isso promove a EMT, aumenta a velocidade de migração e a organização das fibras colágenas reflete estágios avançados do tumor (MATTE; KUMAR; PLACONE; ZANELLA *et al.*, 2019).

Associado a rigidez da matriz outro conceito foi aplicado ao estudo das células tumorais, a memória mecânica de células migratórias, expandindo o conceito básico de mecanotransdução que analisa o contexto de microambiente imediato. Nasrollahi e colaboradores demonstraram que a rigidez da ECM a qual a células foi exposta no passado pode influenciar a migração futura quando esta é transferida para outro grau de rigidez. Ainda nesse contexto, células com depleção da atividade de YAP demonstram incapacidade de armazenar memória mecânica, mas continuam sensíveis a rigidez da matriz por meio de adesões focais (NASROLLAHI; WALTER; LOZA; SCHIMIZZI *et al.*, 2017). Novos horizontes no tratamento de tumores estão sendo apresentados nos estudos recentes, e a partir desses conceitos novas abordagens para a modulação do comportamento tumoral e adicionalmente elucidação da relação entre a rigidez e a recidiva do tumor são necessárias.

1.5 Modulação do comportamento tumoral

1.5.1 Terapias antitumorais

Na última década, foram notáveis os avanços na terapia antitumoral, devido ao desenvolvimento de abordagens de precisão em medicina e imunoterapia, além de melhorias contínuas nas opções e estratégias terapêuticas disponíveis. No entanto, a capacidade persistente de resistência ao tratamento continua sendo um grande desafio que limita a eficácia de todas as terapias. A plasticidade celular, cada vez mais

reconhecida no processo de resistência à drogas, pode ser dependente e motivadora da heterogeneidade intratumoural (QUINTANAL-VILLALONGA; CHAN; YU; PE'ER *et al.*, 2020). O que aumenta a diversidade de células tumorais implicando na retenção de progenitores pluripotentes, e sua persistência pode permitir o repovoamento em tumores resistentes com um diversificado repertório de fenótipos celulares (CALBO; VAN MONTFORT; PROOST; VAN DRUNEN *et al.*, 2011; LIM; IBASETA; FISCHER; CANCILLA *et al.*, 2017). Surpreendentemente, a maior parte das drogas antitumorais aprovadas na Europa entre 2009 e 2013 entraram no mercado sem evidencia de benefício na sobrevida ou qualidade de vida dos pacientes (DAVIS; NACI; GURPINAR; POPLAVSKA *et al.*, 2017).

O modo de tratamento para CEC oral depende de vários fatores, como estadiamento clínico da doença, sítio primário e estado geral de saúde do paciente. O foco terapêutico das neoplasias malignas de cabeça e pescoço é o tratamento cirúrgico e/ou radioterapia e/ou quimioterapia. Tratamentos cirúrgicos são, normalmente, bastante invasivos, nos quais ocorre grande perda de tecido, levando, na maioria dos casos, a uma elevada morbidade e diminuição da qualidade de vida do paciente. Já a radioterapia e a quimioterapia são procedimentos que possuem muitos efeitos adversos ao paciente, uma vez que não conseguem diferenciar o tecido maligno do tecido sadio (HOSKIN; RAMAMOORTHY, 2008). Drogas antiproliferativas, pró-apoptóticas, antiangiongênicas, como a Cisplatina, uma das terapias mais utilizadas no tratamento de tumores de cabeça e pescoço, são empregadas na tentativa de atingir sucesso clínico, com avanço no prognóstico do paciente (DASARI; TCHOUNWOU, 2014; PENDLETON; GRANDIS, 2013).

Nesse contexto, terapias alvo-específicas para componentes do TME vêm sendo propostas na tentativa de conter a progressão tumoral e reduzir à resistência a terapia. Para a progressão tumoral vasos sanguíneos e células endoteliais são recrutados para fornecer aporte de oxigênio e nutrientes, assim terapias anti-angiogênicas, como anticorpo específico para VEGF e bevacizumab tem sido testados (JOYCE, 2005). Ainda drogas que inibem o turnover da matriz extracelular associada ao tumor (Suramin/ Volociximab) tem sido testadas a fim de impedir o remodelamento dessa

matriz e bloquear a função de proteínas associadas (FGF, PDGF, IGF-1, TGF-α e TGF-β) ou integrinas (anti-α5β1) (JIN; VARNER, 2004).

Em contrapartida, ainda é amplamente realizado o tratamento tradicional no qual o objetivo principal é atuar sobre as características básicas adquiridas pelo tumor. Nesse contexto, a proliferação e a resistência à morte celular são uma parte fundamental da progressão tumoral devido a atividade alterada de proteínas relacionadas ao ciclo celular, sendo este o alvo terapêutico mais comumente utilizado (FEITELSON; ARZUMANYAN; KULATHINAL; BLAIN *et al.*, 2015). A cisplatina é o quimioterápico mais utilizado em tumores de cabeça e pescoço, e sua ação se dá pela interferência nos mecanismos de reparo do DNA, causando danos e subsequentemente induzindo apoptose (DASARI; TCHOUNWOU, 2014). Outra droga muito utilizada é o Paclitaxel, o composto natural com função antitumoral mais bem sucedido, que promove a montagem dos microtúbulos e evita a sua dissociação, bloqueando a progressão do ciclo celular (WEAVER, 2014; ZHU; CHEN, 2019). Nesse sentido, substâncias como os sais imidazólicos estão demonstrando resultados promissores por meio da indução de apoptose, parada do ciclo celular e redução de proliferação celular *in vitro* e *in vivo* (GOPALAN; KE; ZHANG; KNG *et al.*, 2011; GOPALAN; NARAYANAN; KE; LU *et al.*, 2014; STROMYER; SOUTHERLAND; SATYAL; SIKDER *et al.*, 2020).

O uso combinado de agentes quimioterápicos tem demonstrado resultado superior em comparação a terapia isolada. Por exemplo, inibidores de histona desacetilase (HDAC) e inibidores de DNA methyltransferase (DNMT) em doses baixas pode reverter a resistência a agentes citotóxicos, em parte, induzindo a remoção de alterações epigenéticas adquiridas que direcionam o fenótipo de resistência (CANDELARIA; GALLARDO-RINCÓN; ARCE; CETINA *et al.*, 2007; ZHANG; ZHENG; WANG; LU *et al.*, 2014). Para CEC oral, a adição de uma terapia alvo, como inibidor de EGFR, demonstrou melhorar a sobrevida dos pacientes em comparação a radioterapia primária isolada(BONNER; HARARI; GIRALT; AZARNIA *et al.*, 2006). Em contraste, ensaios clínicos randomizados, realizados em pacientes com tumores de cabeça e pescoço, combinando inibidores de EGFR com outros quimioterápicos não mostraram melhora na sobrevida dos pacientes (*overall survival*), assim como não exibiram efeito

superior em relação a sobrevida livre de progressão (PFS *progression-free survival*) ou resposta completa (CR *complete response*) (COHEN; LICITRA; BURTNESS; FAYETTE *et al.*, 2017; DE PAUW; LARDON; VAN DEN BOSSCHE; BAYSAL *et al.*, 2018; HARTMANN; NECKEL; SEHER; MUTZBAUER *et al.*, 2016; SPECENIER; VERMORKEN, 2016). Nesse sentido, as pesquisas tem buscado substâncias que apresentem poucos efeitos colaterais e baixa toxicidade, e isso levou a crescente pesquisa por substâncias de origem natural que apresentassem atividade antitumoral. Além disso, a possibilidade de sintetizar substâncias análogas as naturais, também se mostrou promissora.

1.5.1.1 Compostos naturais

Produtos naturais até os dias atuais desempenham um papel relevante na terapia antitumoral com substancial número de agentes antineoplásicos utilizados na clínica derivando de produtos naturais de várias fontes (plantas, animais e microorganismos)(NOBILI; LIPPI; WITORT; DONNINI *et al.*, 2009). A descoberta e a triagem de drogas antitumorais em larga escala promovidos pelo *National Cancer Institute* (NCI) têm desempenhado um importante papel no desenvolvimento de compostos naturais anticâncer. Nos últimos anos, a descoberta de medicamentos baseados em produtos naturais está aumentando com base em novas tecnologias, como a síntese combinatória e triagem de alta performance. Esses medicamentos são caracterizados por uma variedade de mecanismos de ação, incluindo interação com microtúbulos, inibição das topoisomerases I ou II, alquilação do DNA e interferência na transdução(NOBILI; LIPPI; WITORT; DONNINI *et al.*, 2009). Resultados *in vitro* e *in vivo* sugerem que doses baixas de Curcumina podem influenciar várias etapas envolvidas na tumorigênese, incluindo migração celular, sugerindo um possível uso na terapia antitumoral (DE CAMPOS; MATTE; DIEL; JESUS *et al.*, 2017). Nutracêutico, termo surgiu combinando “nutrição” e “farmacêutico” e foi definido como um alimento que proporcionava benefícios médicos ou à saúde, é um produto isolado ou purificado de origem alimentar que é vendido em forma medicinal (FOSTER; ARNASON; BRIGGS, 2005). Polifenóis, considerados nutracêuticos, são compostos orgânicos classificados em dois grupos principais flavonoides e ácidos fenólicos e demonstram

atividade antioxidante e efeito em diferentes tipos de tumor (AMORATI; VALGIMIGLI, 2012; CROZIER; JAGANATH; CLIFFORD, 2009).

O flavoperidol é um tipo de flavonoide que demonstra efeito inibidor de kinase dependente de ciclina, induz apoptose por meio da ativação de caspase-3 (FLINN; BYRD; BARTLETT; KIPPS *et al.*, 2005; GRENDYS; BLESSING; BURGER; HOFFMAN, 2005). Esta substância já encontra-se em fase de ensaios clínicos, demonstrando bons resultados quando utilizado como agente único no tratamento de leucemia, exibindo alta atividade clinica(BYRD; LIN; DALTON; WU *et al.*, 2007; LIN; RUPPERT; JOHNSON; FISCHER *et al.*, 2009). No entanto, quando foi testado como um agente único para tumores sólidos, demonstrou mínima atividade ou não nenhum efeito, mas quando combinado com outros quimioterápicos, o flavopiridol demonstra atividade, apesar de sua toxicidade significativa (BIBLE; PEETHAMBARAM; OBERG; MAPLES *et al.*, 2012; CARVAJAL; TSE; SHAH; LEFKOWITZ *et al.*, 2009; GRENDYS; BLESSING; BURGER; HOFFMAN, 2005).Já a isoflavona, outro tipo de flavonoide, mostrou induzir apoptose em células de tumor de pâncreas e melhorou a atividade antitumoral de drogas padrão-ouro já utilizadas na terapia antineoplásica (EL-RAYES; PHILIP; SARKAR; SHIELDS *et al.*, 2011). Além disso, demonstrou eficácia em ensaios clínicos randomizados de fase I/II como agente antimetastático para câncer de próstata (ABBASZADEH; KEIKHAEI; MOTTAGHI, 2019; PAVESE; KRISHNA; BERGAN, 2014).

1.5.1.2 Compostos sintéticos

O anel de imidazol é universal na natureza e desempenha um papel crucial em muitas estruturas e funções dentro do corpo humano. A funcionalidade do anel de imidazol é possível através da sua capacidade de aderir a metais como ligante e também de formar ligações de hidrogênio com drogas e proteínas (RIDUAN S.N., ZHANG Y., 2013). O anel imidazólico é o equivalente catiônico do imidazol, sendo que os sais imidazólicos possuem a capacidade de interagir com sistemas biológicos (ANDERSON; LONG, 2010). Esses compostos são líquidos iônicos e possuem ponto de fusão a 100 °C.

De acordo com a literatura, os líquidos iônicos são divididos em três gerações de acordo com a suas aplicações. Assim, a primeira geração foi baseada em suas

propriedades físicas como ponto de fusão, densidade, viscosidade, estabilidade térmica, condutividade e hidrofobicidade. A segunda geração é caracterizada pela possibilidade de agrupar as propriedades físicas e químicas dos líquidos ionicos (reatividade química, coordenação e solvatação). Mais recentemente, a terceira geração utilizada na indústria farmacêutica visa a produção de líquidos iônicos com atividade biológica (DIAS; COSTA-RODRIGUES; FERNANDES; FERRAZ *et al.*, 2017; FERRAZ; BRANCO; PRUDÊNCIO; NORONHA *et al.*, 2011).

As propriedades físicas e químicas únicas destes compostos, especialmente a não volatilidade, a estabilidade térmica e química, são atraentes em aplicações farmacêuticas como antifúngico, antibacteriano e anestésico local(HOUGH; SMIGLAK; RODRIGUEZ; SWATLOSKI *et al.*, 2007). Os sais imidazólicos são produzidos a partir da alquilação do imidazol, formando um par de um ânion e um cátion, o que confere uma forma ajustável a esses compostos, possibilitando o delineamento das propriedades químicas, físicas e biológicas, que tornam esses compostos muito visados pela indústria farmacêutica (HOUGH et al., 2007). Essa característica permite incorporar elementos, para aperfeiçoar a sua utilização (STOIMENOVSKI; MACFARLANE; BICA; ROGERS, 2010). Os sais imidazólicos N-substituídos vem sendo testados como antifúngicos, apresentando-se promissores contra leveduras e demonstrando maior atividade e baixa toxicidade em relação aos agentes antifúngicos atualmente disponíveis no mercado. Associado à isso, não apresentaram efeito genotóxico para a estrutura da membrana celular ou danos no DNA em células de leucócitos humanos (BERGAMO; DONATO; DALLA LANA; DONATO *et al.*, 2015; SCHREKKER; DONATO; FUENTEFRIA; BERGAMO *et al.*, 2013). As propriedades antifúngicas desta classe de compostos já foram comprovadas e, recentemente, tem-se estudado as propriedades antitumorais destes. Gopalan e colaboradores (2011) demonstraram que dois tipos de sais imidazólicos livres de metal tiveram efeito antitumoral em modelo *in vivo* e *in vitro*, inibindo o crescimento de hepatocarcinoma por meio da supressão de Cdk4 e da modulação do microambiente estromal. A proliferação celular também foi afetada, além de induzir à apoptose independente de p53 (GOPALAN; KE; ZHANG; KNG *et al.*, 2011; GOPALAN; NARAYANAN; KE; LU *et al.*, 2014). A ação dos sais imidazólicos ainda é demonstrada em outras linhagens tumorais

como: carcinoma hepático mielóide (SMMC-7721) e câncer de pulmão (NCI-H460) através da indução de apoptose (WRIGHT; DEBLOCK; WAGERS; DUAH *et al.*, 2015; XU; WANG; YU; CHEN *et al.*, 2014). Devido à possibilidade de modulação da atividade biológica dessa classe de compostos por meio da modificação dos componentes de sua molécula, essas substâncias vêm demonstrando um efeito promissor em diferentes tipos de tumores.

2. HIPÓTESE

Comportamento agressivo do carcinoma espinocelular oral é influenciado por elementos físicos do microambiente tumoral e por compostos químicos seletivos.

3. OBJETIVOS

3.1 Objetivo geral:

Avaliação dos efeitos de fatores físicos e químicos na modulação do comportamento de células tumorais.

3.1.1 Objetivo Específico 1:

Analisar os mecanismos envolvidos na modulação do comportamento celular induzido pela rigidez do microambiente tumoral.

3.1.2 Objetivo Específico 2:

Estabelecer uma relação estrutura-atividade de diferentes formulações de Sais Imidazólicos no comportamento de células de carcinoma oral e queratinócitos.

3.1.3 Objetivo Específico 3:

Avaliar o desfecho de pacientes após o tratamento com polifenóis como terapia antitumoral por meio de ensaios clínicos randomizados (fase II e III).

A presente tese será apresentada na forma de três artigos científicos, abordando as estratégias experimentais e os resultados referentes a cada um dos objetivos específicos.

4.1 Artigo científico 1:

Artigo científico será submetido ao periódico *Journal of Cell Science* (ISSN 1477-9137, Fator de Impacto: 4.517).

As células tumorais são capazes de sentir o ambiente ao seu redor, processar e transformar essa informação em sinais bioquímicos que irão alterar o comportamento das próprias células, mas também poderão modificar o microambiente associado ao tumor. Nesse contexto, as características físicas da matriz extracelular vem sendo avaliadas na tentativa de elucidar seu papel na progressão e manutenção do tumor. A rigidez da matriz é um dos aspectos físicos mais estudados e já foi demonstrado a associação da rigidez em diferentes tipos de tumor com genótipo e fenótipo alterados das células e também pior prognóstico para o paciente. Já foi demonstrado para CEC oral que um alto grau de rigidez resulta em aumento da velocidade de migração celular, e uma maior organização de fibras colágenas prediz um pior prognóstico para esses pacientes. A partir destes dados prévios, buscamos responder algumas questões que ainda não estão claras como quais as vias de sinalização envolvidas nesse processo e como se dão as etapas de aquisição, consolidação e recuperação da memória mecânica induzida pelo substrato rígido. Este trabalho foi conduzido no laboratório do professor Adam Engler, na Universidade da Califórnia – San Diego, durante o período do meu estágio sanduíche, sendo este um dos principais centros de pesquisa em mecanobiologia.

Cell contractility drives mechanical memory of oral squamous cell carcinoma

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Abstract

Increased tissue stiffness is observed in a variety of tumors, like in oral cancer, and the increased tissue rigidity on tumor influences its progression, by increasing epithelial-mesenchymal transition and migration. We have previously demonstrated that poorly invasive oral cancer cells acquire an aggressive phenotype and migrate faster when exposed to a prolonged time on stiff substrate throught a process that recall a mechanical memory. It is unclear how the mechanical memory signaling occurs and we hypothesized that it might be dependent on contractile proteins. We evaluated the mechanisms that drive mechanical memory in oral squamous cell carcinoma (OSCC). We observed that the non-muscle type II myosin (MII) isoform B is overexpressed in tumor adjacent epithelia and center of tumor in patient biopsies comparing to MIIA and MIIC, while the isoforms MIIA and MIIB were overexpressed in high invasive OSCC cell line. We plated OSCC cell lines for 5 days in soft (0.48kPa) and stiff (20kPA) substrate made with polyacrylamide (PA) hydrogels and observed that stiff substrate induces cell changes in cell morphology in low invasive OSCC cell line. Then, in the group soft (0.48kPa) we induced cell contractility with Lysophosphatidic acid (LPA – 10μM), while in the group stiff (20kPa) we blocked contractility with blebbistatin (10μM) for 5 days. Cells from all groups (0.48kPa, 0.48kPa+LPA, 20kPa, 20KpA+blebbistatin) were re-plated onto either soft or stiff substrate and imaged for analysis of migration performance. Low invasive cells exhibited increase in migration velocity after prolonged exposure to a stiff substrate when compared to soft exposure ($p<0.001$). Cells plated in initial stiff condition did not change migration velocity when expose to soft substrate, indicating a mechanical memory of cells. Treatment with LPA enhanced migration velocity in collective migration ($p<0.001$) when compare to the soft control condition, while blebbistatin treatment overcome “memory” induce by stiff niche in individual cell migration ($p<0.001$). For analysis in a 3D environment, we repeated the same groups for 5 days treatment and performed spheroids that were seeded in collagen gel (1.8mg/ml). We observed that cells from stiff substrate had a significant increase in spheroid area ($p<0.05$) when compare to spheroid formed to cells in soft condition. The treatment with blebbistatin in stiff condition demonstrated decrease in area ($p<0.01$) when compare to stiff control, while LPA shows a trend to increase spheroid spread in soft substrate.

Therefore ours data suggest that contractility is necessary for cells to acquire a mechanical memory according to the stiffness of the substrate.

Introduction

Oral and pharynx cancer corresponds to 354,000 new cases and 177,000 death per year worldwide [1] and oral squamous cell carcinoma (OSCC) represents 80–90% of all malignant tumors of the oral cavity [2; 3; 4] with a 5-survival rate that varies from 40 to 63% in 5 years [5; 6; 7; 8]. Clinically, OSCC presents as an ulcer with fissuring or exophytic mass associated to a raised, rolled and stiff border [9]. The stiff tumor border, also observed in other tumors, results from extracellular matrix modifications during carcinogenesis, where changes on several enzymes increases matrix stiffness and total adjacent ECM volume [10; 11; 12; 13; 14]. It was demonstrated that an increase on collagen content and organization correlates to a poor outcome for patients [15; 16; 17], probably due to changes on proliferation and invasive phenotype [15; 18; 19; 20].

Several cancer hallmarks require communication between cells and their environment in order to improve the tumor performance, including invasion and metastasis [21; 22]. The extracellular and intracellular compartments are mechanically linked by transmembrane integrins which connect the ECM with actin cytoskeleton involving adhesion, contractile and signaling proteins [23]. In summary, active integrins recruits adhesion molecules that dynamically assemble nascent adhesions which mature in focal adhesions. These focal adhesions modulate cell signaling as well as the contractile actomyosin machinery [24; 25; 26; 27]. This mechanism may also play outside-in, in which increase on cell signaling, such as RhoGTPase and AKT signaling, and/or non-muscle myosin-II activity may modulate adhesions and integrin/ECM binding [28; 29]. As a result of these dynamic interactions, it was demonstrated that increase on cell contractility enhances tumor growth, survival and invasion [18; 30; 31].

Chronic modulation on cytoskeleton machinery elicited by stiffer environment might result in broader modifications on cell behavior due to changes on intracellular biochemistry and gene expression [32; 33; 34]. Mechanotransduction is the process through cells sense and respond to mechanical stimuli by converting them to biochemical signals and promote specific cellular responses [32]. For instance, stiffer substrates induce

tumorigenic phenotype in mammary cells by promoting chromatin accessibility peaks [34] while lung tumor cells increases the expression of epithelial to mesenchymal transition (EMT)-related genes [35; 36]. Potential players for mechanotransduction involves the crosstalk of cell-ECM [37] and cell-cell adhesion [38], as well as the myosin activity [39; 40] with mechanoregulator proteins. Together, these players migth generate a mechanical memory in invading cells contributing to tune the tumor ability to adapt to a new tissue [41].

We previously showed that stiff substrate potentially induce mechanical memory in OSCC cells, since low invasive OSCC acquired higher migratory profile in stiffer environment that was maintained when cells switched to soft matrix [16]. Herein, we hypothesized that OSCC mechanical memory involves different steps (acquisition, consolidation and recovery) that may be modulate through cytoskeleton machinery and cell signaling. We demonstrated that cell contractility and AKT signaling are involved in memory acquisition in stiffer environment, while Focal Adhesion Kinase signaling is required for memory recovery in soft environment.

Acquisition of mechanical memory in 2d and 3d environment is affected by contractility

We previously showed that OSCC response to stiff substrate is mediated by an increase on adhesion properties [16]. Since adhesion maturation is mediated mainly by cell tension [23; 39; 42; 43], we hypothesized that contractility might be involved in the acquisition of mechanical memory. As an indirect evidence for the role of contractility in tumor cell behavior, we analyzed the expression of non-muscle myosin isoforms in OSCC biopsies (n=15) and cell lineages. At the tumor adjacent epithelia, NMIIB was detected mainly at the basal cell layer and the staining was stronger in cells at the invasive front of tumor (Fig 1A). In addition, NMIIA and NMIIB are overexpressed in low differentiated/low E-cadherin:N-cadherin ratio (SCC25, Inv^{H}/E:N^{L}) when compared to high differentiated/ high E-cadherin:N-cadherin ratio (Cal27, Inv^{L}/E:N^{H}) cell lineage, suggesting a higher contractility level in more aggressive tumor cells. Thus, we used Inv^{L}/E:N^{H} and artificially increased (LPA) or blocked (blebbistatin) the contractility when plated in soft (soft+LPA) or stiff (stiff+ blebbistatin) substrates. We observed that the rise

in contractility in cells plated in soft environment affected cell morphology (Fig 1C), increased the expression of the transcription factor TWIST (EMT promoter) as well as there was a trend to increase Snail1 and Zeb1 (E- cadherin suppressors) (Fig 1D) at similar levels to those observed in cells plated in stiff environment. These results suggest that there is a correlation of cell contractility and the mechanical memory induced by stiff environment.

To analyze the role of contractility in the acquisition of this mechanical memory, we plated Inv^{L}/E:N^{H} cells for 5 days in a soft or stiff niche with LPA or blebbistatin, respectively, then re-plated cells in soft or stiff condition without the drugs and performed an analysis of cell migration (Fig 2A). As previously observed, stiff niche conditioned Inv^{L}/ E:N^{H} cells into a more aggressive phenotype with a higher migration velocity and directionality, even when cells were re-plated in soft conditions, when compared to cells that stayed in a soft niche. Interestingly, addition of LPA during pre-conditioning on soft environment forced cells to migrate with higher velocity when re-plated on a soft niche. Additionally, Inv^{L}/E:N^{H} cells pre-conditioned in stiff niche with blebbistatin impaired migration velocities and directionality when cells were re-plated in soft niche without the drug. To confirm these results, we repeated the preconditioning in soft or stiff niche with LPA or blebbistatin for 5 days (Fig 2A), performed spheroids (24h) that where transferred to a collagen gel (1.8 mg/ml) and imaged for 5 days. We observed that spheroids derived of cells pre-conditioned in a soft substrate demonstrated smaller cell area when compared to stiff conditioned cells ($p<0.05$) (Fig. 2C/D). The addition of LPA to soft pre-conditioning induced an increase on spheroid spread similar to stiff pre-conditioning, while association of blebbistatin to stiff pre-conditioning severely impaired spheroid invasion ($p<0.01$). Taken together, the 2D and 3D experiments indicates that increase on cell contractility in soft environments artificially induced a stiff-like mechanical memory while the inhibition of cell contractility during stiff pre-conditioning blocked the acquisition of a mechanical memory.

Acquisition and recovery of mechanical memory respectively involves AKT and FAK signaling

To understand the signaling pathways involved in the mechanical memory induced by the stiffness of the niche we performed a phospho-kinase array. Thus, the Inv^{L}/E:N^{H} cells were cultivated for 5 days in stiff or soft substrate and after the cells were re-plated in the same substrate or switch to the another stiffness. The analysis of 43 protein target indicated that the switch from soft to stiff induced an increase on AKT (S308) phosphorylation levels, indicating this pathway as an initial step necessary to mechanical memory acquisition. When cells switched from stiff to soft, there was an increase on FAK (Y397) phosphorylation levels, indicating FAK is involved in the recovery of mechanical memory (Fig.3A).

So, was performed time lapses with cells seed in soft substrate and after 5 days we re-plate this cells in stiff substrate with Akt inhibitor for 24h. In collective migration Inv^{L}/E:N^{H} cells exposed to a secondary matrix with different concentrations to AKT inhibitor (0.5 and 5μM) demonstrated decrease in migration velocity ($p<0.001$) when compare to the control just with high dose (5μM) (Fig.3B). Furthermore, individual migration exhibited reduction in cell migration with both doses (0.5- $p<0.001$ and 5μM- $p<0.05$). Accordingly, AKT phosphorylation blocks the acquisition to a new memory.

Discussion

The bi-directional crosstalk between cell and ECM leads to change on cell behavior, thus dynamics in ECM features can modify cell mechanical properties promoting malignancy [44; 45]. Contractile forces is claim to several processes during embryogenesis and pathogenesis, modulating cells property by myosin activity [30; 46]. Increase on cell contractility impairs in cell–cell adhesions and promote tumor cell invasion and migration [18; 47].Oncogenes are promoters of EMT process and inducing tumor progression through NMII activation. Also, NMII support the collective to single-cell migration by accelerating turnover of cell–cell adhesion[48]. Expression of type II non-muscle myosin (MIIB) in non-neoplastic adjacent epithelial may indicate the potential for regional metastasis to head and neck patients [49]. As well as our results showed strong staining to NMIIB on basal cell layer at tumor adjacent epithelia and at the invasive tumor front (Fig 1A), also we observed expression of MIIA and MIIB on OSCC cell line moreover higher expression on Inv^{H}/E:N^{L} cells. Furthermore, its observed changes on

cell morphology and EMT markers expression when the Inv^{L}/E:N^{H} cells were challenge in different stiffness substrates. Thus, we chemically modulate cell contractility and evaluate it influence in cell behavior on soft or stiff substrate. Stiff substrate promotes malignant features in tumor cells and increasing in cell contractility on soft matrix trend to enhance EMT markers to similar values to stiff. Accordingly the results contractility is require to oral squamous cell carcinoma through myosin expression and modulating it we observed change in cell behavior that influence tumor aggressiveness.

The cells are able to sense and respond to mechanical stimuli converting it into a biochemical signals that follow to a specific cellular response[50; 51]. Thus, this process: mechanotransduction requires to regulation of cytoskeleton tension that provides mechanical support to the cells and controls motility, shape and tension homeostasis. The cell-ECM interaction starts after extracellular changes are perceived by integrin clusters whose morphological changes or distribution recruit FAK [24; 52; 53; 54]. And them, proteins such as talin, vinculin and paxillin transfer the mechanical cues from integrins to the actin component of the cytoskeleton[55]. The isoforms of Myosin II as described before, MIIA is responsible for generating traction force in order to stabilize FAs in a Rho/ROCK-dependent mechanism and rapid cytoskeleton remodeling, and MIIB stabilizer actin fiber with no motor function, involved in maintenance of cell polarity [56]. The contractility promote cell migration through myosin II-based contractility that generates force necessary for migration and disassembly of cell adhesions at the rear of the cell, besides that contributes to cell polarity by positioning the nucleus, suppressing the formation of lateral membrane protrusions and establishment of a leading edge during cell migration and tumor cell invasion [57; 58; 59; 60; 61; 62]. Stiffness has been demonstrate to influence phenotypic and genotypic cellular responses enhancing the malignant profile, so the papers try to explain the mechanical memory that mean the persistent influence of cellular mechanosensitivity on cell migration[15; 34; 51]. Cancer cells and stem cells demonstrate have mechanical memory in which cells save mechanical information from primary physical substrate and it going to influence cell process[41; 63]. Cell contractility increase is able to promote cell migration on stiff substrate, however the contractility impairment on soft substrate showed block the mechanical memory

acquisition. Our data corroborate that stiffness improve oral cancer cell migration speed[16] in 2D and 3D, also demonstrate that contractility is require during this process.

In order to investigate the signaling pathways are involved in memory acquisition and recovery we performed phospho-kinase array, for this Inv^{Low}/E:N^{High} was plated on stiff and soft substrate for five days. And then it was re-plate in the same substrate or switch to another one for one hour and started the assay. We observed high levels of AKT (Thr 308) when we switch cells from soft to stiff substrate, to prove this we keep the cell during five days in soft substrate and then switch to stiff with 24h treated with AKT inhibitor (0.5 and 5μM). Thus, we observed decrease on collective and individual cell migration with both doses (5μM collective and 0.5 and 5μM individual, $p<0.05$). AKT plays important role in cancer initiation and progression through regulating biological processes such as cell growth and survival, thereby AKT also PI3K appears to be activated and is responsible to cancer cell survival by hypoxia in different tumor cell types[64; 65; 66], but the mechanism by which these are activated is not well understood. However, opposite roles of AKT signaling has been demonstrated to different tumor cell types about cell migration, invasion and metastasis. On lung cancer AKT appeared to negatively regulate migration and invasion, and its inhibition promoted metastasis by with KRAS or EGFR mutations[67]. It was demonstrate that AKT activation was observed in OSCC cells where the TRPV4 (Ca2+-permeable nonselective cation channel) expression level was high, in which tumor stromal stiffness stimulate this receptor resulting in increasing of AKT signaling[68; 69; 70]. In result to increase in stiffness matrix the evidences demonstrate cooperation between distinct pathways, in which integrin-induced AKT activation by focal adhesion kinase (FAK), also contributes to enhance cytoplasmic β-catenin (support its translocation into the nucleus) by inhibition of GSK3β[71; 72] .

Cell migration plays a important role in normal and diseases phenomena, also is a multi step process, that involve many molecules such as FAK that have a critical function on adhesive complexes formation, cell shape, adhesion and motility[57; 73; 74]. As a result to increase on matrix stiffness FAK is highly phosphorylated and is able to regulate RhoGTPases that going to regulated cytoskeleton organization[74; 75; 76]. The phosphorylation of FAK at Y397 has also been shown to be higher on stiff substrates

than on soft, cells without FAK demonstrated loss of response to durotaxis[55; 74; 77; 78]. The FAK inhibition demonstrated that focal adhesion was promptly formed and exhibited the same composition of FA from normal cells with normal phosphorylation levels, but the maturation did not affected. Also, FAK and paxillin phosphorylation do not contribute to the generation of traction forces required to sense ECM stiffness and to pull the cell body forward during migration. Accordingly, FAK and paxillin phosphorylation is essential to driven cellular processes as lamellipodial protrusions, cell migration and spreading by Rac-1[24; 79]. Therefore ours data suggest that contractility is necessary for cells to acquire a mechanical memory according to the stiffness of the substrate.

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Conflict of interests

The authors declare that there is no conflict of interests.

MATERIALS AND METHODS

Human Biopsies

The experimental design and the informed consent procedures were approved by the Ethical Committee of Federal University of Rio Grande do Sul - Brazil and of Hospital de Clínicas de Porto Alegre (HCPA) - Brazil (GPPG nº 11-0289; GPPG nº 14-0019) and all patients in this study provided written informed consent. Patients (n=19) with oral lesions were interviewed and submitted to surgery. OSCC diagnosis was confirmed histopathologically by a pathologist and fragments from regions corresponding to the center of the tumor and the carcinoma edge tissue, named as tumor adjacent epithelia (TAE) were collected. Clinical information is described in supplementary material (S. Table 1).

Cell culture and reagents

OSCC cell lines were a kind gift from Akihiro Sakai, University of California San Diego (UCSD). Cal27 cells were cultivated in DMEM high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gemni Bio) and 1% penicillin/streptomycin (Gibco); SCC25 cells were maintained in DMEM/F12 with 15 mM HEPES (Teknova) supplemented with 10% FBS, 1% penicillin/streptomycin, and 400 ng/ml of hydrocortisone (Sigma). Cells were maintained in incubator at 37°C with 5% CO_2. The cells were treated with Blebistatin (10μM, Cayman Chemical), Lysophosphatidic acid (LPA) (10μM, Enzo Life Sciences), Perifosine, AKT inhibitor (0.5 and 5 μM, InvivoGen).

Real-time PCR

RNA was extracted from cells using Trizol and total RNA quantity and quality was checked using absorbance (A280/A260). cDNA was generated by adding random hexamer primers and Super Script III reverse transcriptase (Thermo) to 2 μg of RNA. Quantitative PCR was performed (45 cycles, 95°C for 15 s followed by 60°C for 1 min) using a 7900HT Fast Real-Time PCR System (Thermo) with the primer sets described below and SYBR Green Supermix (Bio-Rad Laboratories). A fibronectin standard was used to analyze absolute RNA expression. Experiments were performed in biological and technical triplicates using the CFX Connect (Bio-Rad) realtime PCR detection system. Human primer sequences were used as follows: Twist1 (5′-TGCATGCATTCTCAAGAGGT-3′, 5′-CTATGGTTTTGCAGGCCAGT-3′), Snail1 (5′-CTAGCGAGTGGTTCTTCTG-3′, 5′-CTGCTGGAAGGTAAACCTCTG-3′), Snail2 (5′-ATGAGGAATCTGGCTGCTGT-3′, 5′-CAGGAGAAAATGCCTTTGGA-3′), Zeb1 (5′-GCCAATAAGCAAACGATTCTG-3′, 5′-CTTGTCTTTCATCCTGATTTCC-3′), Zeb2 (5′-CAGTCCAGACCAGTATTCCT-3′, 5′-GCAATTCTCCCTGAAATCCT-3′), GAPDH (5′-TCGACAGTCAGCCGCATCTTC-3′, 5′-ACCAAATCCGTTGACTCCGAC-3′).

Fabrication of polyacrylamide hydrogels

Polyacrylamide hydrogels (PAAGs) were made on No. 12 mm and 25 mm glass coverslips that had been methacrylated by first oxidizing the surface through UV/ozone exposure (BioForce Nanosciences) followed by functionalization with 20 mM 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich, cat # 440159) in ethanol. A polymer

solution containing either 3%/0.06% acrylamide/bis-acrylamide (Fisher) for 0.48 kPa hydrogels or 8%/0.264% for 20 kPa hydrogels, 1% v/v of 10% ammonium persulfate (Fisher), and 0.1% v/v of N,N,N′,N′-tetramethylethylenediamine (VWR) was prepared. 15µl for 12 mm coverslips and 30 µl for 25 mm coverslips of hydrogel solution was sandwiched between a functionalized coverslip and a dichlorodimethylsilane-treated glass slide and polymerized for 15 min. Hydrogels were incubated in 0.2 mg/ml sulfo-SANPAH (Fisher, cat #22589) in sterile 50 mM HEPES pH 8.5, activated with UV light (wavelength 350 nm, intensity 4 mW/cm2) for 10 min, washed three times in HEPES, and then incubated in 150 µg/ml collagen solution (Corning) overnight at 37°C.

Migration assay

OSCC cells were plated on either 0.48 or 20 kPa PAAGs for 12h and were then imaged with a Nikon Eclipse Ti-S microscope equipped with a motorized temperature- and CO_2-controlled stage. Cells were imaged at 10x in brightfield at multiple positions every 15 min for 24 h. For long-term conditioning in a soft or stiff niche, cells were cultivated for 5 days in either soft or stiff PAAGs, then trypsinized and plated onto soft and stiff hydrogel (with or without respective treatment) to analyze migration as mentioned. For analysis of migration parameters, the nucleus of each migratory cell was used as a reference point to track each cell with the 'Manual Tracking' plugin on ImageJ. Migration was considered to be in single-cell mode when a cell did not touch any other cell during its migration movement. Migration was considered to be in collective-cell mode when cells migrated in a group of two or more cells.

Phospho-kinase array

Protein phosphorylation was examined with the Proteome Human Phospho-Kinase Array Kit (Proteome Profiler Array, R&D Systems, Abingdon, Oxford, UK), which is a membrane based sandwich immunoassay. The assay was performed according to the manufacturers' instructions. Briefly, total cell extracts (Inv^{L}/E:N^{H}) were tripsinazed after 5 days in soft (0.48kPa) or stiff (20kPa) substrate and re-plated in the same substrate or switch to other stiffness for 1h in 6-well plates. Cells plated in the same substrate after 5 days were used as control. The cell extracts containing 300 µg of total protein were

incubated with the Human Phospho-Kinase Array. The proteins present in a lysate sample were captured by discrete antibodies printed in duplicate across the nitrocellulose membranes. The array was washed 3× with 1X Wash Buffer for 10 minutes on a rocking platform shaker to remove unbound proteins. Washing was followed by incubation with a cocktail of biotinylated detection antibodies (monoclonal anti-human of phosphorylated Akt (S473), Akt (T308), AMPK alpha1 (T174), AMPK alpha2 (T172), beta-Catenin, Chk-2 (T68), c-Jun (S63), CREB (S133), EGF R (Y1086), eNOS (S1177), ERK1/2 (T202/Y204, T185/Y187), FAK (Y397), Fgr (Y412), Fyn (Y420), GSK-3 alpha/beta (S21/S9), Hck (Y411), HSP27 (S78/S82), HSP60, JNK pan (T183/Y185 T221/Y223), Lck (Y394), Lyn (Y397), MSK1/2 (S376/S360), p27 (T198), p38 alpha (T180/Y182), p53 (S15), p53 (S392), p53 (S46), p70 S6 Kinase (T421/S424), PDGF R beta (Y751), PLC gamma-1 (Y783), PRAS40 (T246), Pyk2 (Y402), RSK1/2/3 (S380/S386/S377), Src (Y419), STAT2 (Y689), STAT3 (S727), STAT3 (Y705), STAT5a (Y694), STAT5a/b (Y694/Y699), STAT5b (Y699), STAT6 (Y641), TOR (S2448), WNK-1 (T60), Yes (Y426) and subsequent application of streptavidin-HRP conjugate. The signals were detected with Chemi Reagent Mix (R&D Systems, Abingdon, Oxford, UK). Developed signals were analyzed using ImageJ analysis software.

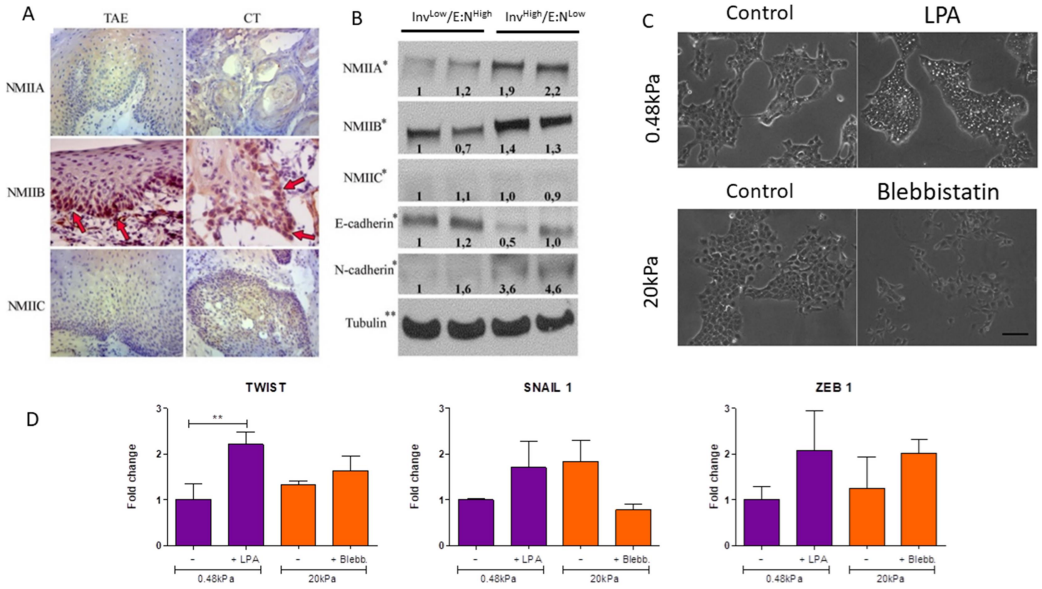

Figure 1 – Contractility modifies cell behavior in oral squamous carcinoma and trends to affect EMT markers. (A) Immunohistochemistry staining of NMIIA, NMIIB and NMIIC at adjacent epithelia (TAE) and center of the tumor (CT). (B) Representative western blottings for NMIIA, NMIIB, NMIIC, E-cadherin, N-cadherin and tubulin in low (Cal27) or high (SCC25) invasive OSCC cell lines. * Densitometry values were normalized to the loading control and then compared to the first band (arbitrary value of 1). ** Densitometry values were compared to the first band (arbitrary value of 1). (C) Brightfield microscopy demonstrating cell morphology for InvL /E:NH cells after being cultured on soft or stiff substrates for five days. (D) Fold change to mRNA expression for the indicated transcription factors, normalized to GAPDH and to soft control (0.48kPa), is plotted for the indicated cells cultured on soft or stiff substrates, *p<0.05 One-way ANOVA, Tukey'post-test, n=3 samples for each condition from triplicate experiments.

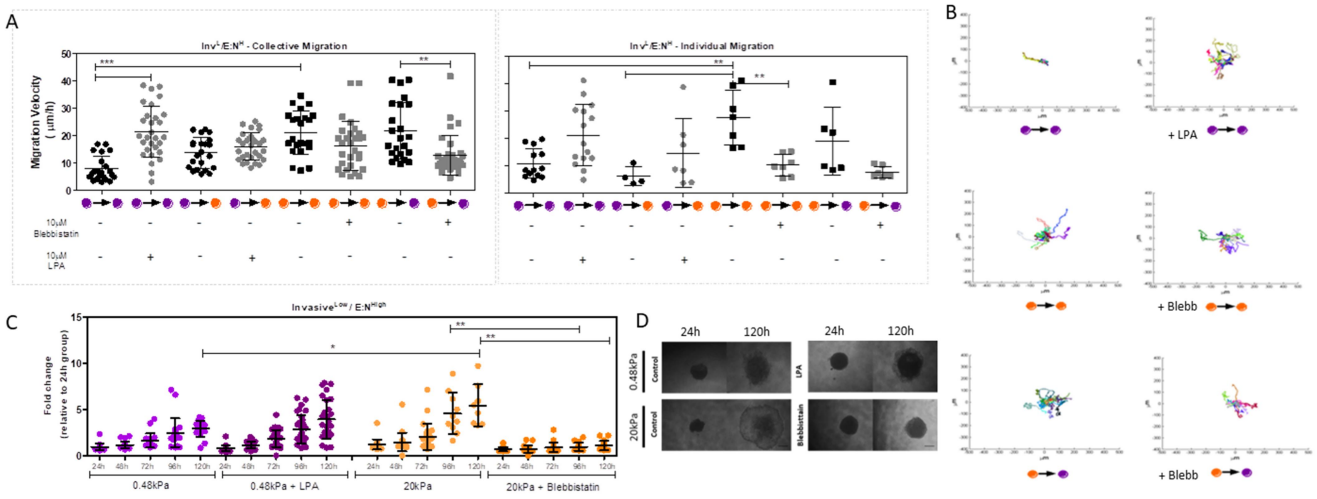

Figure 2 – Acquisition of mechanical memory in 2d and 3d environment is affected by contractility. (A) Collective (left) and individual (right) migration velocity was analyzed for $Inv^{L}/E:N^{H}$ cells on stiffness combinations as indicated (soft=purple; stiff=orange) plus treatment with LPA (10μM) or blebbistatin (10μM). All data were analyzed using Two-way ANOVA with Tukey's multiple comparisons test with *P<0.05, **P<0.01, n=22, 23, 29, 32, 22, 23, 30 and 29 cells for collective migration and n=13, 06,

15, 07, 04, 07 and 08 cells for individual migration. (B) Rose plots of collective cell migration on the respective substrate (soft=purple; stiff=orange) plus treatment with LPA (10μM) or blebbistatin (10μM). Each line represent a trajectory of one cell. (C) Fold change to OSCC spheroids pre-conditioning in soft and stiff substrate (control and treated with LPA/blebbisttain) normalized to 24h sphere. (D) Brightfield microscopy of OSCC-derived spheroids incubated in control conditions (soft or stiff) or with drugs that increase (LPA) or blocks (blebbistatin) contractility.

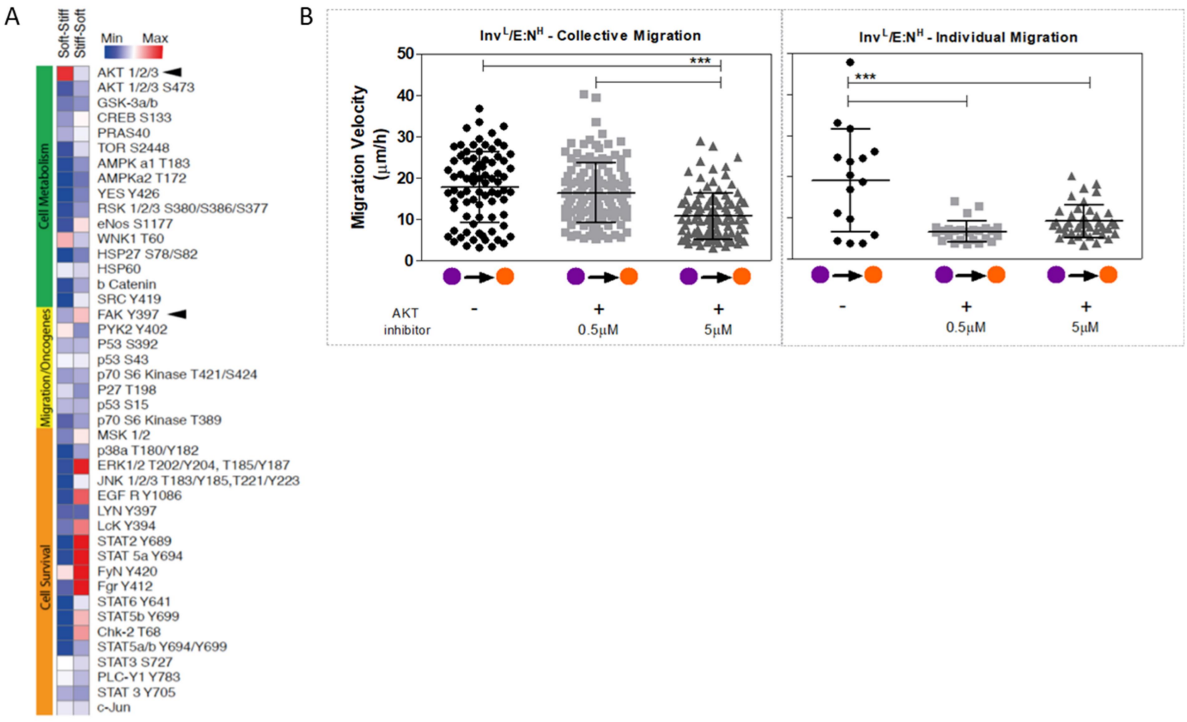

Figure 3 – Mechanical memory recall is affected by AKT and FAK signaling. (A) Heat map of phosphorylation level of 43 signaling proteins. Cells were plate in soft or stiff substrate for 5 days and re-plated on the same substrate (soft to soft; stiff to stiff) or onto inverse substrate (soft to stiff, stiff to soft) per 1 h and normalize to primary substrate; red: up-regulation and blue: down-regulation. (B) Collective (left) and individual (right) migration velocity was analyzed for $Inv^{L}/E:N^{H}$ cells on stiffness combinations as indicated (soft=purple; stiff=orange) plus treatment with AKT inhibitor (0.5 or 5μM). All data were analyzed using Two-way ANOVA with Tukey's multiple

comparisons test ***p<0.001. n=80, 124 and 105 cells for collective migration, and n=16, 26 and 42 cells for individual migration from triplicate experiments, n=4 independent assays.

Supplementary information

		Number of cases
Gender	Female	5
	Male	14
Localization	Border of tongue	5
	More the one localization	4
	Floor of the month	2
	Others	7
Age	51 – 80 years (mean 65,5)	
Clinical staging	Staging I	4
	Staging II	3
	Staging III	1
	Staging IVa	5
	Staging IVb	1

Supplementary images 1: Clinical information.

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4.2 Artigo científico 2:

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Este artigo surgiu de uma colaboração entre o Laboratório de Migração Celular (LAMOC) e o laboratório de Processos Tecnológicos e Catálise (TECNOCAT), no qual foi realizado a síntese das substâncias, e então, estas foram testadas na Faculdade de Odontologia. Atualmente, há um grande número de pesquisas *in vitro* realizando a testagem de um número substancial de novas drogas para o tratamento de tumores, no entanto, uma pequena proporção dessas substâncias avançam para testes clínicos. Isto se dá pela utilização de modelos de estudo *in vitro* que não reproduzem ou mimetizam a complexidade do tumor *in vivo*, dessa forma, nós buscamos utilizar ensaio 2D e 3D durante o *screening* das substâncias para avaliar seu desempenho em diferentes condições de substrato. Além disso, o efeito citotóxico dos antineoplásicos é bastante elevado, nesse contexto, os Sais Imidazólicos estão sendo apresentados como seletivos para células tumorais quando comparado a células normais. Sendo possível melhorar sua atividade biológica por meio de modificações (cátions, ânions, número de carbonos) na molécula dessas substâncias. Na literatura é possível encontrar evidências consistentes da atividade antitumoral dessa classe de compostos em diferentes tipos de tumores. Então, esta pesquisa demonstra a atividade antitumoral de diferentes substâncias de Sais Imidazólicos modificadas sobre o comportamento de células de carcinoma espinocelular oral.

Anti-tumor effects of Imidazolium Salts on Oral Squamous Cell Carcinoma

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Highlights

- Imidazolium salt-oral squamous cell carcinoma structure-activity relationship;
- N-alkyl chain-length dependent anti-tumor effect of imidazolium salts;
- Impaired OSCC proliferation and anti-tumor effect similar to cisplatin;
- 1-n-Hexadecyl-3-methylimidazolium chloride is more effective in tumor than in keratinocyte cell death.

ABSTRACT

Imidazolium salts (IS), ionic derivatives of the neutral imidazoles, have adjustable properties through structural modifications in their cations and anions, turning this class promising for the development of biologically active compounds. Herein, the anti-tumor effects of the IS 1-*n*-butyl-3-methylimidazolium chloride (**C_4MImCl**), 1-*n*-decyl-3-methylimidazolium chloride (**C_{10}MImCl**), 1-*n*-hexadecyl-3-methylimidazolium chloride (**C_{16}MImCl**), 1-*n*-hexadecyl-2,3-dimethylimidazolium chloride (**$C_{16}M_2$ImCl**), 1-*n*-octadecyl-3-methylimidazolium chloride (**C_{18}MImCl**), 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate (**C_{16}MImMeS**), and 1-*n*-hexadecyl-2,3-dimethylimidazolium methanesulfonate (**$C_{16}M_2$ImMeS**) on oral squamous cell carcinoma (OSCC) was studied. OSCC cells (CAL27) were incubated with increasing IS doses and then submitted to proliferation (2D), cell death (2D) and spheroid assay (3D). The IS anti-tumor effect was both dependent on its *N*-alkyl chain length and anion, where **C_{16}MImCl** showed an optimum combination for inhibition on cell proliferation and cell-cell adhesion, outperforming the methylated **$C_{16}M_2$ImCl** derivative and, most importantly, the gold standard cisplatin. In addition, **C_{16}MImCl** had little effect on keratinocytes and more pronounced effects on more aggressive tumor cells. This compound showed an increased spreading area of the tumor sphere, and an enhanced number of apoptotic and necrotic cells in the tumor cell line, demonstrating only a small rise in the healthy cells. These data indicate the promising effect of **C_{16}MImCl** on OSCC, being selective for cancer cells.

KEYWORDS: Ionic liquids, oral cancer, apoptosis, spheroids

Introduction

There was a worldwide estimation of 18.1 million new cancer cases in 2018, 9.5 million cancer deaths, and 43.8 million people living with cancer within 5 years of diagnosis [1]. Surprisingly, most of the cancer drugs that were approved in Europe between 2009 and 2013 entered the market without evidence of benefit on survival or quality of life. Also, when there were survival gains over existing treatment options or placebo, they were often marginal [5]. Actually, the current therapies have several side effects because these are not selective for tumor cells. This balance between the anti-tumor effect and side effects could be improved by structural modifications in substance classes with known anti-tumor activity.

Ionic liquids are salts that are in the liquid state at 100 °C, which have tunable physicochemical and biological properties by adjusting the structures of their ions [6-8]. Imidazolium-based ionic liquids (from now one referred to as imidazolium salts (IS)) are cationic derivatives of neutral imidazoles, and a broad variety of biological activities have been reported for this class of compounds [9, 10], including antifungal [11, 12] and fungal antibiofilm [13].

The anti-tumor activity of some IS has already been demonstrated in some types of cancer, where certain compounds induced apoptosis in the non-small-cell lung carcinoma line by caspase-3, and PARP cleavage [15]. Still on lung cancer, some compounds displayed high anti-cancer activity, comparable to cisplatin, in cell lines [16]. Gopalan *et al*. presented both *in vitro* and *in vivo* data for two IS that inhibited the growth of HCC by suppressing the expression of survivin and cyclin-dependent kinases, induced the apoptosis independent of p53, and suppressed the HCC cell proliferation by arresting cells at G1 phase [17, 18].

In this study, we explored the anti-tumor potential of seven structurally complementary IS in oral squamous cell carcinoma (OSCC). The *N*-alkyl chain-length of the imidazolium cation played a crucial role in their anti-tumor activity in both 2D and 3D cell cultures. **C_{16}MImCl** was identified as the most promising anti-tumoral agent, outperforming the reference compounds cisplatin, **C_{16}Im**, the neutral equivalent of **C_{16}MImCl** and **C_{16}MImMeS** and **C_{16}PyrCl**, the pyridinium equivalent of **C_{16}MImCl** that is commercially applied in medicines [20].

Methodology

Imidazolium salts and reference compounds

NMR spectra were recorded on a Bruker (400 MHz) equipment at ambient temperature. The chemical shifts are given in parts per million (ppm) and referenced to the residual solvent signal ($CDCl_3$ = 7.26 (^1H), 77.16 (^{13}C); DMSO-d6 = 2.50 (^1H)). Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), in the mid infrared range (4000–500 cm^{-1}), was performed on an ALPHA-P compact Bruker FTIR spectrometer. High-resolution mass spectrometry spectra were recorded on an electrospray ionization (ESI) Q-Tof Micro^{TM} equipment (Micromass, Manchester, UK) in the positive mode.

1-*n*-Butyl-3-methylimidazolium chloride (**C_4MImCl**; Sigma-Aldrich), 1-*n*-decyl-3-methylimidazolium chloride (**C_{10}MImCl**; Sigma-Aldrich), **C_{16}MImCl** (CJC CHINA JIE CHEMICAL), 1-*n*-octadecyl-3-methylimidazolium chloride (**C_{18}MImCl**; CJC CHINA JIE CHEMICAL), **C_{16}PyrCl** (Sigma-Aldrich), and cisplatin (Blau Farmacêutica S.A.) were purchased and used as received. *n*-Hexadecyl methanesulfonate (**C_{16}MeS**) [21],**C_{16}MImMeS** [22], and **C_{16}Im** [23] were synthesized according to previously reported procedures. **C_{16}MeS** was used as source for the synthesis of **C_{16}MImMeS**. A modified literature procedure was used for the synthesis of 1-*n*-hexadecyl-2,3-dimethylimidazolium chloride (**$C_{16}M_2$ImCl**) and 1-*n*-hexadecyl-2,3-dimethylimidazolium methanesulfonate **$C_{16}M_2$ImMeS** [24], which are yet unpublished IS.

$C_{16}M_2$ImCl: The mixture of *n*-hexadecyl chloride (21.0 mmol, 1.05 equiv.), 1,2-dimethylimidazole (20.0 mmol, 1.00 equiv.) and acetonitrile (3 mL) was stirred and heated at 78 °C for 42 h. Next, the crude product was washed 5X with a 1:1 mixture of acetonitrile/diethyl ether. Finally, the solvent was removed under reduced pressure.

$C_{16}M_2$ImMeS: The mixture of **C_{16}MeS** (21.2 mmol, 1.00 equiv.), 1,2-dimethylimidazole (21.5 mmol, 1.01 equiv.) and acetonitrile (15 mL) was stirred and heated at 80 °C for 48 h. The crude product was recrystallized from hot acetonitrile to give crystals, which were filtered off and dried, under vacuum.

Reagents and cell culture

HaCaT (human aneuploid immortal keratinocyte – BCRJ® CRL – 0341 ™), CAL27 (OSCC – ATCC® CRL-2095 ™), SCC-9 (OSCC – ATCC® CRL-1628 ™) and SCC-25 (OSCC – ATCC® CRL-1628 ™) were cultivated on Dulbecco's modified Eagle's medium high glucose (DMEM – Gibco, Thermo Fischer Scientific, Massachusetts, USA) (HACAT, CAL27 and SCC-9) or DMEM/DMEM F12 medium (SCC-25). All media were supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin (Gibco, Thermo Fischer Scientific, Massachusetts, USA). For the SCC-25 cell line, hydrocortisone (400 ng/mL) was added.

Cell proliferation assay

Cells (CAL27) were cultured in 96-well plates for 12 hours. IS and reference compounds were added to the medium at 0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml. After 24 hours, cell proliferation was analyzed by using the CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) according to the instructions of the manufacturer. The DNA amount was measured on a fluorometer (Spectra Max Gemini XPS) with excitation wavelength of 485 nm and emission wavelength of 530 nm.

Spheroid assay

Cells (HaCaT, CAL27, SCC-9 and SCC-25) were plated in non-adherent agarose-coated 96-well plates ($1x10^4$ cells/well) to form spheroids [25]. After 24 h of spheroid formation, IS and reference compounds (0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml) were added. Then, after 24, 48 and 72 hours of treatment, pictures from the spheroids were taken with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AxioVision Software (Zeiss, Göttingen, Germany).

Cell death assay

Cells (HaCaT and CAL27) were plated in 12-well plates ($1x10^4$ cells/well) for 24 h, followed by the addition of an IS or a reference compound (0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml). After 24 h, the cells were stained with Trypan Blue and dead and/or alive cells

were counted in a hemacytometer (Gibco, Thermo Fischer Scientific, Massachusetts, USA).

Cumulative Population Double (CPD)

For this assay, the HaCaT, CAL27, SCC-9 and SCC-25 cell lines were plated in 12-well plates at (1 x 10^4 cells/well), and treated with 0.1 µg/ml of IS or culture medium (it was used as positive control) for 28 days. The culture medium was used as positive control. After every 7 days, the cells were stained with Trypan blue and counted in a hemacytometer (Gibco, Thermo Fischer Scientific, Massachusetts, USA), and the cell lines were re-plated (1 x 10^4 cells/well) in new wells. The adhesion period of cells to the new plate (4 h) was followed by the addition of an IS, a reference compound or medium. The population double (PD) value was calculated by the formula: $PD = \frac{\log_n [final\ cell\ number] - [initial\ cell\ number]}{\log_n [2]}$. Then, the cumulative population double (CPD) was calculated through the equation: [26] $CPD_{7day} = PD$ $CPD_{14days} = PD1°week + PD\ 2°week$, $CPD_{21days} = PD1°week + PD\ 2°week + PD3°week$, $CPD_{28days} = PD1°week + PD\ 2°week + PD3°week + PD4°week$.

Flow Cytometry

Cell apoptosis and necrosis were analyzed by flow cytometry (BD FACSVerse™) using the software BD Facsuite. Forward scatter and sideward scatter patterns were established representing the size and granularity of the cells, respectively. Cellular debris with low forward scatter/sideward scatter characteristics were excluded. Evaluation of apoptosis was performed using the FITC annexin V and PE 7AAD. CAL27 and HaCaT were treated with 2 and 5 µg/ml for C_{16}MImCl and Cisplatin for 24 h, after which the medium was collected and attached cells were trypsinized. Cells from the medium and from the trypsinization process were mixed and centrifuged (5 min, 2000 rpm). The pellet was washed with phosphate-buffered saline, and the cells were centrifuged again. Supernatant was removed, and cells were incubated with 100 µL of 1× Annexin buffer containing 2.5 µL of FITC annexin V (BD Biosciences, Franklin Lakes, Nova Jersey, USA) and 2.5 µL of PE 7AAD (BD Biosciences, Franklin Lakes, Nova

Jersey, USA). After incubation at room temperature for 15 min in the dark, cells were analyzed. FITC and PE were detected using green (FL1) and red fluorescence (FL-3) detectors, respectively. Flow cytometry results were expressed as percentages of viable (Annexin $V^-/7AAD^-$), early apoptotic ($Annexin^+/7AAD^-$) and necrotic or late apoptotic ($Annexin^+/7AAD^+$). At least 10,000 counts were counted for each sample.

Cytotoxicity sulforhodamine B (SRB) colorimetric assay

Cells (CAL27 and HaCaT) were plated in triplicate at a concentration of 6 x 10^3 in 96-well plates, and, after adhesion to the plate (4 h), cells were treated with different concentrations of IS or Cisplatin (0.1, 0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml). After 24 h, cells were fixed with a 10% trichloroacetic acid solution (50% TCA), incubated at 4° C for 1 h, and subsequently washed (6 times) under running water and dried at room temperature. Sulforhodamine B 4% (SRB, Sigma-Aldrich, St. Louis, USA) in 1% acetic acid (Sigma-Aldrich, St. Louis, USA) was added to stain the cells and the plate was incubated for 30 min at room temperature. The wells were washed (4 times) with 1% acetic acid to remove unbound excess dye and allowed to dry completely at room temperature. Trizma (Trizma base, Sigma-Aldrich, St. Louis, USA) solution was added to the wells and the plate incubated for 1 h to allow complete solubilization of the dye. At the end of this process the microplates were read at 560 nm.

Statistical analysis

Statistical analysis was performed with Microsoft Office Excel (Microsoft Co, Redmond, Washington, USA) and GraphPad version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as number, percentage, mean, and standard deviation. To compare means between groups, we applied analysis of variance (ANOVA) followed by Tukey's or Bonferroni post-test. The level of statistical significance was 5% ($p < 0.05$).

Results

Different Imidazolium Salt formulation impairs tumor cell behavior in 2D and 3D cell cultures

In order to analyze the potential anti-tumor effect of the IS and the reference compounds (Figure 1), the CAL27 OSCC cell line was incubated for 24 h with increasing doses (0.5, 1.0, 2.5, 5.0, 10 and 20 µg/ml) of these substances in the 2D proliferation assay (Figure 2A). This cell line was established from tissue taken prior to treatment, and it has showed resistance to treatment with CDP (cis-platinum) (ATCC) [29]. Initially, the effect of the IS *N*-alkyl chain-length was evaluated (Figure 2A). Although the IS with the shortest chain (**C_4MImCl**) did not affect cell proliferation, IS with longer chains (**C_{10}MImCl**, **C_{16}MImCl** and **C_{18}MImCl**) reduced the cell proliferation by ~50% at 2.5 µg/ml ($p<0.05$), which was more effective than cisplatin at 20 µg/ml. Conventional 2D culture are not capable of mimicking the environment of clinical tumors, such as limited drug penetration, differences in hypoxia, proliferation status and cell contact effect [30, 31], which might impair drug screening outcomes [32, 33], we also tested the effectiveness of the compounds in a 3D environment using spheroids of CAL27. Now, **C_{16}MImCl** was the only compound to induce loss of cell cohesion at 20 µg/ml, which did not occur when spheroids were treated with cisplatin (Figure 2B). These results indicate that the C_{16} compound is a potential candidate to modify tumor cell behavior in both 2D and 3D organization.

Structural modifications in C_{16}MImCl affect cell behavior

Since **C_{16}MImCl** caused alterations in the CAL27 tumor cell line, the *n*-hexadecyl side chain was fixed to test the effect of other structural changes in its ions (Figure 3). The role of the imidazolium group was investigated through comparisons with the reference compounds **C_{16}PyrCl** (pyridinium group) and **C_{16}Im** (neutral imidazole). **C_{16}PyrCl** showed effects only at high doses (10 and 20 µg/ml; $p<0.05$). Although **C_{16}Im** affected the cell proliferation (0.5 µg/ml; ~30%; $p<0.05$), this compound did not affect cell cohesion when compared to **C_{16}MImCl**. Substitution of the C2-H with a methyl group (**$C_{16}M_2$ImCl**) decreased the effect on both cell proliferation and cell adhesion. The methanesulfonate equivalents of **C_{16}MImCl** (**C_{16}MImMeS**) and **$C_{16}M_2$ImCl** (**$C_{16}M_2$ImCl**) impaired cell proliferation and cell cohesion, but at higher doses. These results indicate

that despite all modifications, the **C_{16}MImCl** demonstrated a higher efficiency on both cell-cell adhesion and cell proliferation when compared with other formulations.

C_{16}MImCl affects different oral tumor cell types

Since the tumor shows a heterogeneity of tumor cells phenotypes, we analyzed the effect of **C_{16}MImCl** in spheres derived from different cell lines: HaCaT (keratinocyte), CAL27 (high differentiated/low invasive OSCC), SCC-9 and SCC-25 (low differentiated/high invasive OSCC). First we analyzed the effects of C_{16}MImCl on cell-cell adhesions and it was observed that keratinocytes are less responsive to the compound (Figure 4B and C). The low differentiated OSCC (CAL27) exhibit loss cohesion with 10 and 20 µg/ml showing increased in spreading area (Figure 4B and C). Both lineages of high aggressive OSCC (SCC-9 and SCC-25) showed weak cell-cell cohesion in external area at tumor spheroids under control conditions (Figure 4B), which was increased when cells were treated with 20 µg/ml for 72 hours when compare to the control (Figure 4B).

In order to address a potential resistance in long-term treatment we assessed the effects of Imidazolium Salt **(C_{16}MImCl)** on cell proliferation during four weeks (Figure 4A). Keratinocyte (HaCaT), highly differentiated (CAL27) and low differentiated (SCC-25, SCC-9) OSCC cell lines were plated ($1x10^4$ cells), treated for seven days with 0.1µg/ml of Imidazolium Salt **(C_{16}MImCl)**, tripsinized, counted and then plated again ($1x10^4$ cells) for another week of treatment until the treatment completed 28 days. By the end, it was observed a reduction in cell proliferation of the treated group when compared to control groups as follow HaCaT (24.9%, $p<0.001$), CAL27 (26.22% – $p<0.001$), SCC-9 (20.26% – $p<0.05$) and SCC-25 (60.11% –$p<0.001$). Taken together, these results indicate that **C_{16}MImCl** Imidazolium Salt impairs cell adhesions on more aggressive cells and it had lower effect on keratinocytes.

C_{16}MImCl leads to cell death

Since **C_{16}MImCl** affected the proliferation and adhesion of different cell types, we also investigated what would be the cytotoxic effect of the compound. The dose-response curves (the half maximal inhibitory concentration (IC50) were established for

both the HaCaT (2.047 and 4.336 µg/ml) and CAL27 (2.449 and 4.066 µg/ml) cell lines after treatment with IS and CDP for 24 hours (Figure 5, A), respectively. From that data we set up the doses to assess the cell death by flow cytometry by keratinocyte (HaCaT) and highly differentiated OSCC (CAL27) cell line. Thus, we analyzed the effects of IS and gold standard (CDP) in apoptosis (Annexin V) and necrosis (7AAD) of both cells lines (HaCaT and CAL27) (Fig. 5B/C). Our results demonstrated that **C_{16}MImCl** decreased the number of viable tumor cells (59.15%, $p<0.01$) at similar values than CPD (47.12%, $p<0.001$) (Fig. 5C). Also, normal epithelial cells exhibited a small reduction in cell viability after treatment with IS (62.34%, $p<0.05$) and CPD (65.76%, $p>0.05$). Together, our data demonstrate that IS had similar effects in cell death when compared to the gold standard chemotherapy – CDP, but it was more effective to control cell proliferation and cell-cell adhesion.

Discussion

Initially, **C_{16}MImMeS**, **$C_{16}M_2$ImMeS**, and **$C_{16}M_2$ImCl** were synthesized, which along with the commercially available **C_4MImCl**, **C_{10}MImCl**, **C_{16}MImCl**, and **C_{18}MImCl**, constituted the group of seven IS tested (Figure 1). The spectral data of **C_{16}MImMeS** [22], **C_{16}MeS** [21], and **C16Im** [23] are in agreement with those previously reported. For the new IS **$C_{16}M_2$ImMeS** and **$C_{16}M_2$ImCl**, the NMR, FTIR and MS data support their structures and purity.

OSCC is the most common oral cancer and corresponds to the eighteenth most incident type of cancer [1]. Surgery is the first choice for OSCC treatment but the resection margins may result in increased esthetic and functional morbidities. Also, adjuvant radiotherapy or chemotherapy is used for specific indications [34-36], but late diagnosis and the failure of the therapies leads to a poor prognosis and survival rate that varies from 40 to 63% in 5 years [36-39]. Despite extensive research in cancer and the discovery of new drugs, the gold standard for treatment of head and neck cancer is still cisplatin-based concurrent radiochemotherapy (RCT). The results of meta-analysis showed a modest survival benefit for induction chemotherapy (ICT) compared to RCT alone, which was not statistically significant [40, 41]. Thus, this tumor type has a poor prognosis and the gold standard therapy used for treatment is not resolutive. Herein we

demonstrated that IS compounds might be considered as a potential substance for OSCC treatment due to induction of cell death similar to cisplatin but with an inhibition of cell proliferation and impairment of cell-cell adhesion that outperformed cisplatin.

Due to the complexity of cancer, more selective therapies are sought to control the signaling pathways used by tumor cells to progress and survive. In fact, IS might be a potential antitumor drug due to its selective effect on fungal growth [12]. *In vivo* assays already demonstrate the pharmacokinetic properties of IS in albino Wistar rats and the uptake of ^{131}I-ITFSI reached maximum level in the spleen, liver, and blood at 60 minutes, large intestine and heart at 30 minutes, and ovary at 120 minutes [14]. Also, IS showed almost no effect in leukocytes [12]. *In vitro*, it was observed that intracellular uptake of the radiolabeled anion compound is higher in the colon adenocarcinoma tumor cell line (CaCo-2) than human epithelial kidney (HEK-293) cell line [14]. Herein we demonstrated that IS might also be applied for OSCC treatment with low effect in normal keratinocyte when compared to the gold standard chemotherapeutic drug, cisplatin. This corroborates the hypothesis that known multitarget substances could be used for cancer treatment with selective action in tumor cells.

Tumor progression requires escape from proliferative suppression [2] and most of the chemotherapeutic drugs such as cisplatin are known to impair cell proliferation. The IS **C_{16}MImCl** reduced the proliferation rate in OSCC cells (CAL27) around 50%, with better efficacy than cisplatin. This effect was related to the *N*-alkyl chain-length of the imidazolium cation, showing an optimal anti-proliferative effect for the *n*-hexadecyl group in **C_{16}MImCl**. Such chain-length dependence was also observed in other studies against bacteria [13], fungi [12], A potential mechanism might be related to the hydrophobicity in the interior of plasma membrane [45, 46], since we also observed anti-proliferative effects when the Cl^- anion was changed to MeS^- in the imidazolium ring. In general, IS with pronounced amphiphilic character interact strongly with biological substances and structures, including lipid-membranes, which plays an important role in their mechanism of action [47]. Also, the **C_{16}MImCl** modifies the ergosterol ability to regenerate membrane by reducing the amount of available sterol in the fungal cell [12, 48], resulting in the depletion of intracellular constituents that are necessary for fungal growth and survival. In mammalian cells, IS have been related to changes on cell cycle

[10] and inhibition of nuclear translocation of Cdk4 [17]. As such, it is possible that all these mechanism are favored by the imidazolium ring, since the structurally related compounds with the pyridinium **(C_{16}PyrCl)** and the neutral imidazole **(C_{16}Im)** rings only affected cell proliferation at high doses. Interestingly, the effect of **C_{16}MImCl** on cell proliferation was more pronounced in the highly aggressive OSCC cell line [49, 50] and the use of an assay that resembles the extensive cancer clinical treatment [26] showed that OSCC cells remained responsive to the compound even during a continuous 4 weeks-treatment. Therefore, the short and long-term exposure with the imidazolium compound demonstrated effectiveness to control tumor proliferation on OSCC and these data indicates a potential use as antineoplastic drug.

The tumor environment is a current challenge in cancer treatment. The abnormal OSCC tumor cell proliferation changes tissue architecture resulting in tumor islands in the invaded tissue [51, 52]. It would be desired in an adjuvant antitumor drug to be able to dissociate these islands in order to increase the bioavailability of chemotherapeutic drugs [53]. Herein, we used a 3D approach for drug screening that generates spheroids resembling OSCC tumor islands [54, 55]. Most of the tested *N*-*n*-hexadecyl-substituted IS (Figure 3) affected cell-cell adhesion, similar to observed by Schrekker *et al.*, 2016 on antibiofilm activity in fungi. However, the best association on anti-proliferative effect and dissociation of tumor cell-cell adhesion was achieved with chloride-based IS. This was also observed when we tested OSCC cells with different epithelial to mesenchyme levels [49, 50], since **C_{16}MImCl** also showed these effects in more aggressive OSCC cells.

Another cancer hallmark is the ability to evade the mechanisms of cell death [2]. We observed that the IC50 of **C_{16}MImCl** is lower in tumor (2.29µg/ml) and normal (2.04µg/ml) cells than CPD (4.066 µg/ml/4.336 µg/ml). Also, it was able to induce cell death comparable to cisplatin in cancer cells. It was already demonstrated that IS suppresses the expression and activity of surviving and at high dosages induces apoptosis [10, 17, 56] independently of p53 [18]. Lung cancer cells treated with IS undergo an apoptotic cell death pathway and mitochondria could be the cellular target due to potential disruption of the mitochondrial membrane [16]. Others studies demonstrate that imidazolium salt (TPP1) with a triphenylphosphonium substituent

caused apoptotic cell death in bladder cancer cells, even when exposed for a short time [57]. The compound diosgenin–imidazolium salt a30 show low IC50 values for five human tumor cell lines and, for human hepatocarcinoma (SMMC-7721), it was 54-fold more sensitive than cisplatin to induce the G0/G1 cell cycle arrest and apoptosis [58]. Therefore, similar to our results, different formulations of IS demonstrate structure-activity relationships and may be potential anti-tumor drugs.

Herein we demonstrated in 2D and 3D assays that variations in the IS ring might have different anti-tumor affects, but the formulation **C_{16}MImCl** outperformed other IS compounds in order to simultaneously inhibit cell proliferation, impair cell-cell adhesion and induce cell death in OSCC cell lines when compared to cisplatin. Besides the need for more studies on the anti-tumoral effect of IS, these results contribute to the development of new complimentary treatments of OSCC, with the potential use of IS as an adjuvant compound.

Author's Contribution

ML Lamers, HS Schrekker and PS Campos designed research. PS Campos, LD Menti, L Pazutti, NA Bortoli, LA Ferreira and JL van Wyk performed research. All authors were involved in manuscript preparation.

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Conflict of interests

The authors declare that there is no conflict of interests.

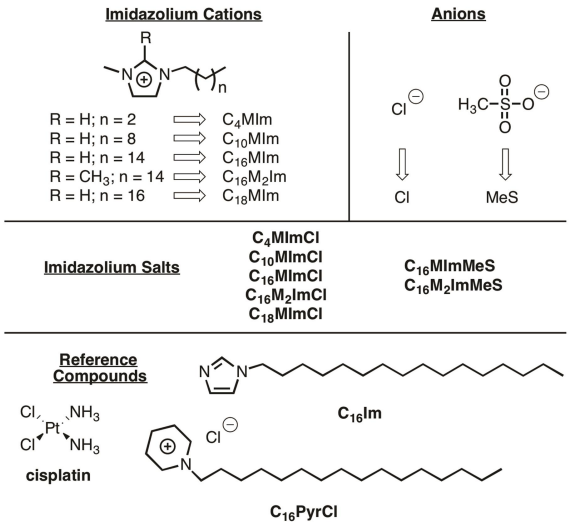

Figure 1 - Imidazolium salts and reference compounds used in this study.

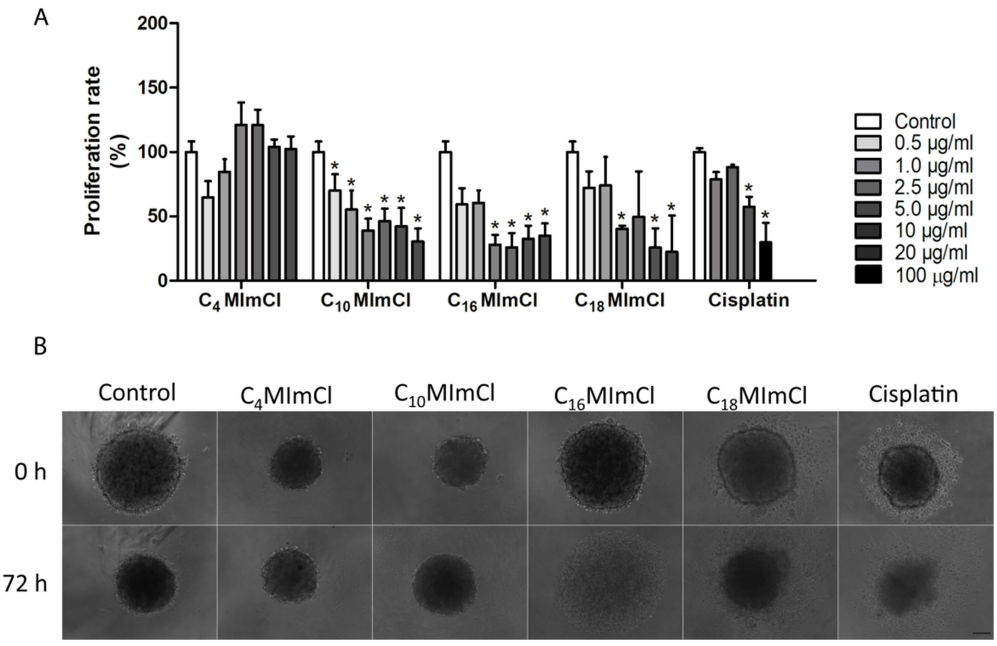

Figure 2 - Effect of the *N*-alkyl chain-length in IS on tumor cells in 2D and 3D cell cultures. (A) Cell viability showed the effect of different formulation of IS in OSCC. (B) Representative images of CAL27 spheroids after 72 h of treatment with IS, with higher spheroid spreading with C_{16}MImCl treatment. One-way ANOVA, Bonferroni's multiple comparison test, $p<0.05$, n = 3.

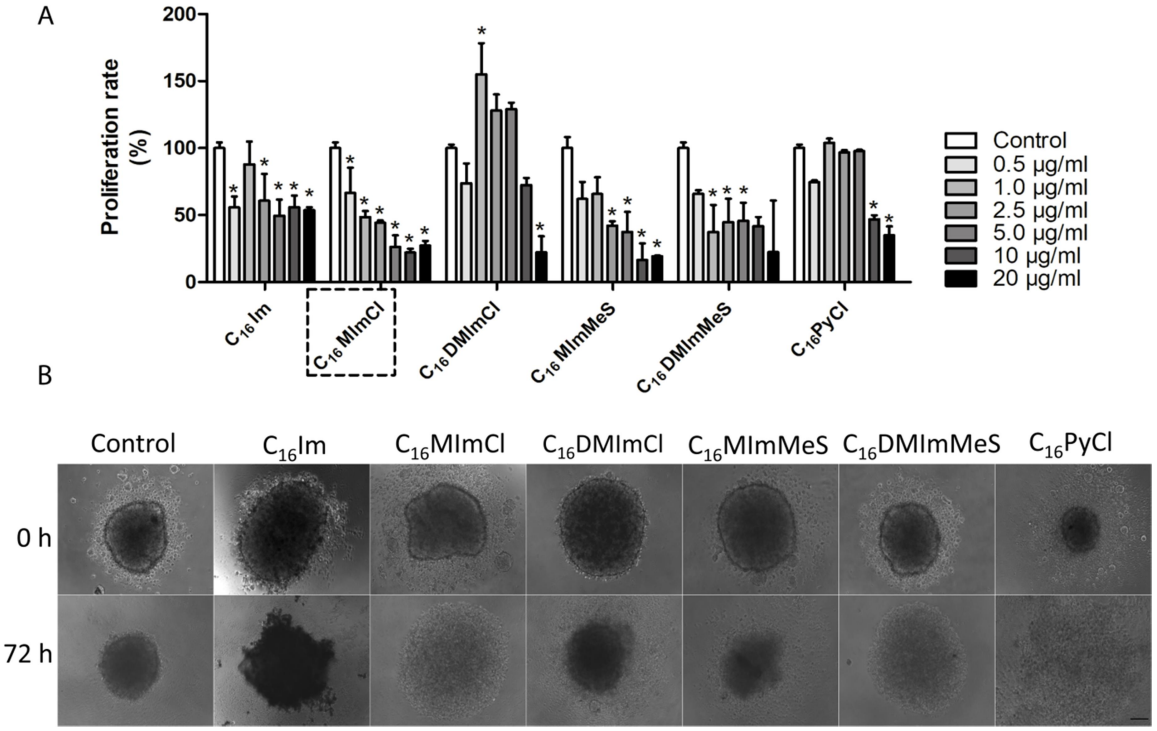

Figure 3 - Effect of structural modifications in both ions of *N-n*-hexadecyl-substituted IS on tumor cells in 2D and 3D. (A) Cell viability assay showed the result of chain modification, and it demonstrated changeable advantage. (B) Representative images of CAL27 spheroids after 72h of treatment with different compounds. One-way ANOVA, Bonferroni's multiple comparison test, $p<0.05$, $n = 3$.

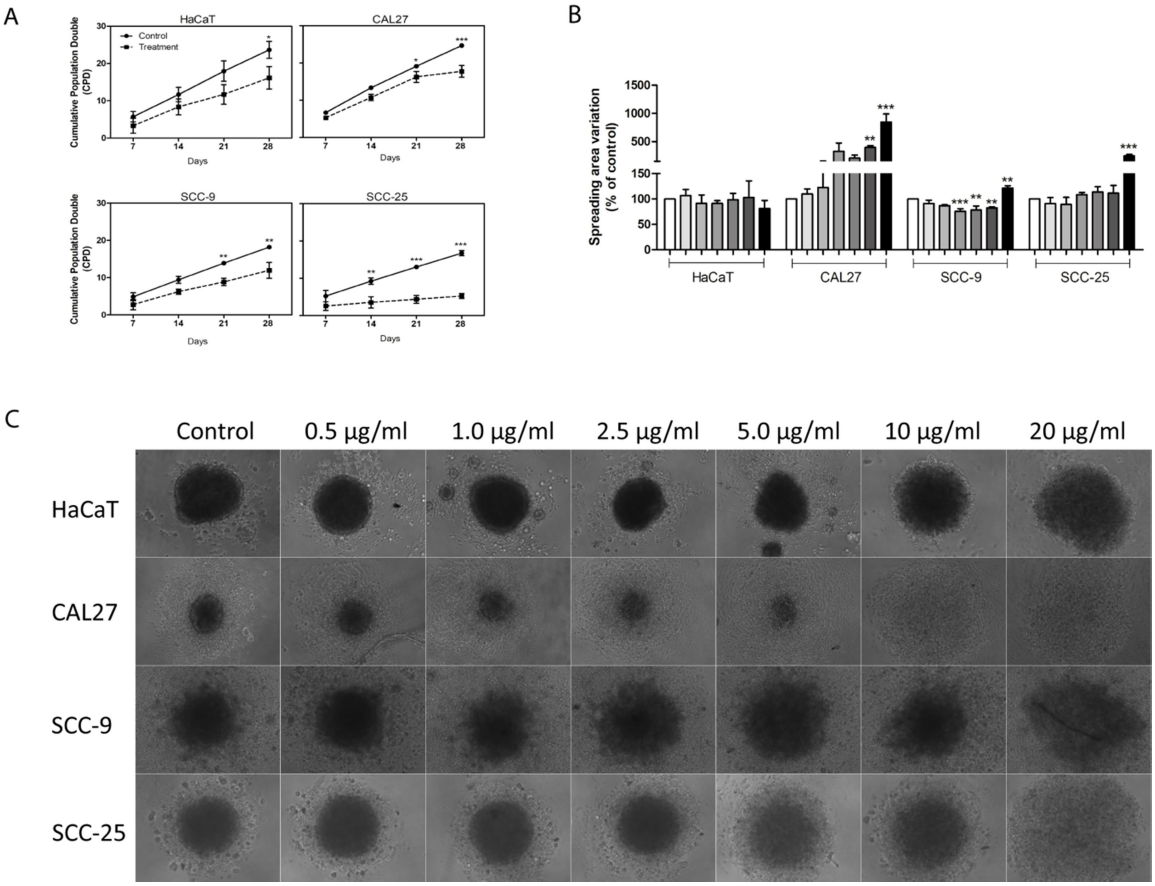

Figure 4 - C_{16}MImCl modifies cell adhesion in oral tumor spheroids. (A) Keratinocyte (HaCaT), highly differentiated (CAL27) and low differentiated (SCC-25, SCC-9) OSCC cell lines were treated for 28 days with 0.1 µg/ml of Imidazolium Salt (C_{16}MImCl). Two-way ANOVA, Bonferroni posttest, *p<0.05, **p<0.01, ***p<0.001. (B) Graphs represent the quantification of spheroid spreading area. One-way ANOVA, Tukey's multiple comparison test, *p<0.05. (C) Representative images of spheroids of HaCaT, CAL27, SCC-9 and SCC-25 after 72h of treatment with C_{16}MImCl, n = 3.

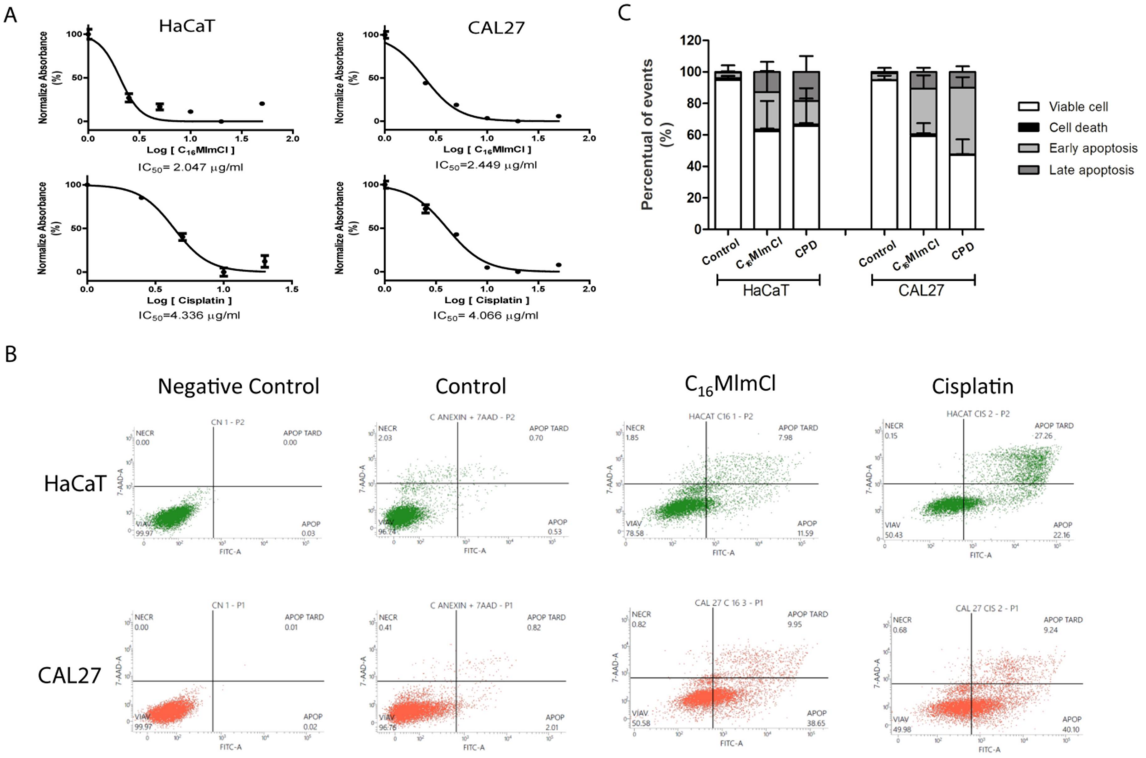

Figure 5 – Imidazolium salt leads to cell death in short term treatment. (A) Dose-response curves, IC50 values for each cell line treated with C_{16}MImCl or CPD it was measure by SRB viability assay. (B) To analyze apoptosis by flow cytometry after 24h for treatment, cells were selected from gate R1. (C) C_{16}MImCl showed decrease tumor viable cells at similar values to exhibited by cisplatin.

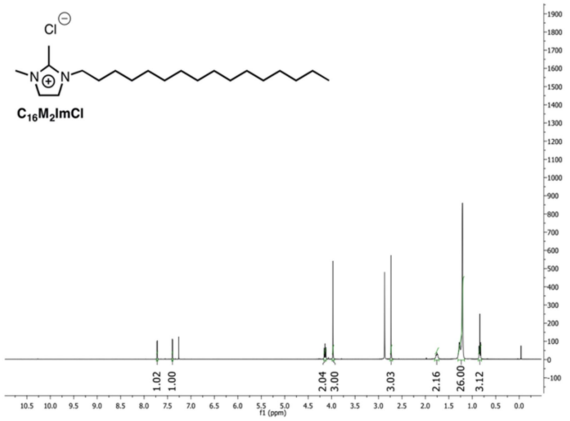

Figure S1. ^{1}H NMR spectrum of **$C_{16}M_2$ImCl** (400 MHz, $CDCl_3$).

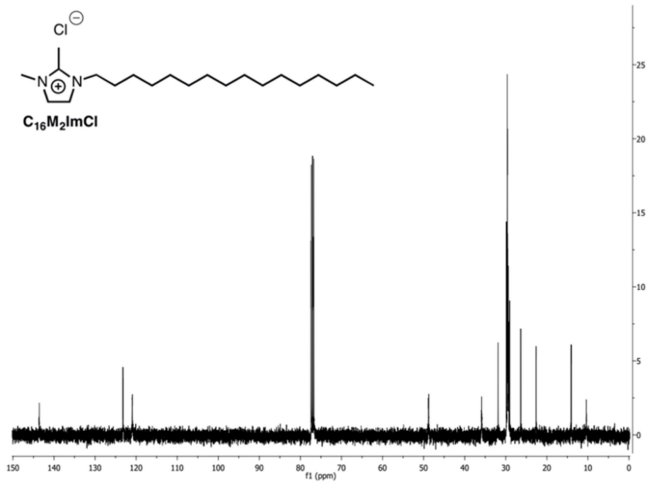

Figure S2. ^{13}C NMR spectrum of **$C_{16}M_2ImCl$** (101 MHz, $CDCl_3$).

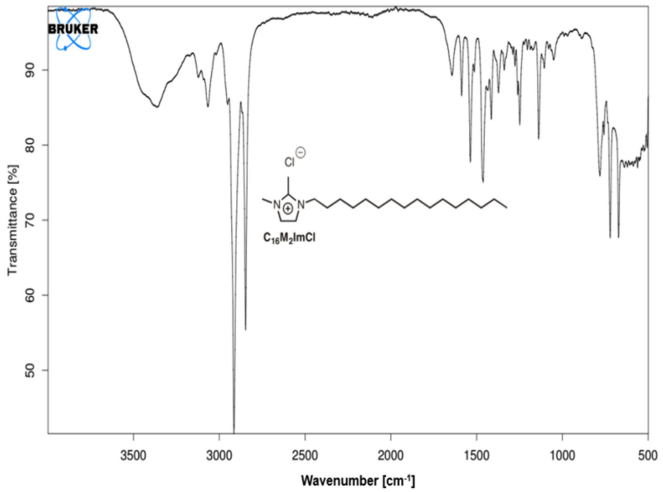

Figure S3. FTIR spectrum of **$C_{16}M_2ImCl$**.

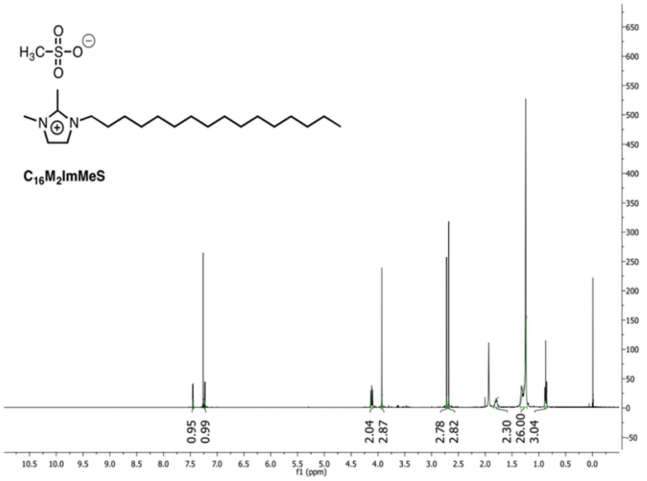

Figure S4. ^{1}H NMR spectrum of **$C_{16}M_2$ImMeS** (400 MHz, $CDCl_3$).

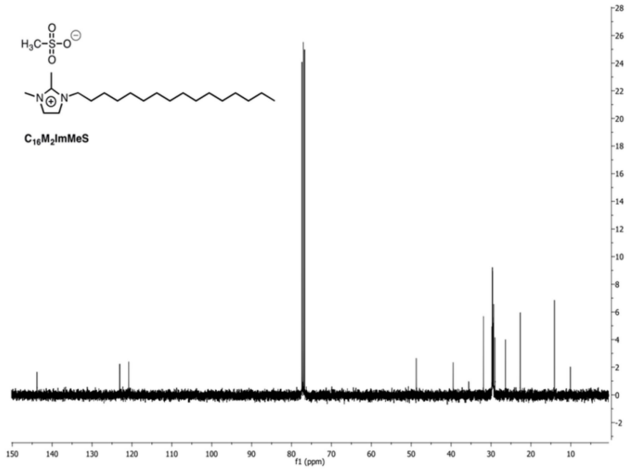

Figure S5. ^{13}C NMR spectrum of **$C_{16}M_2$ImMeS** (101 MHz, $CDCl_3$).

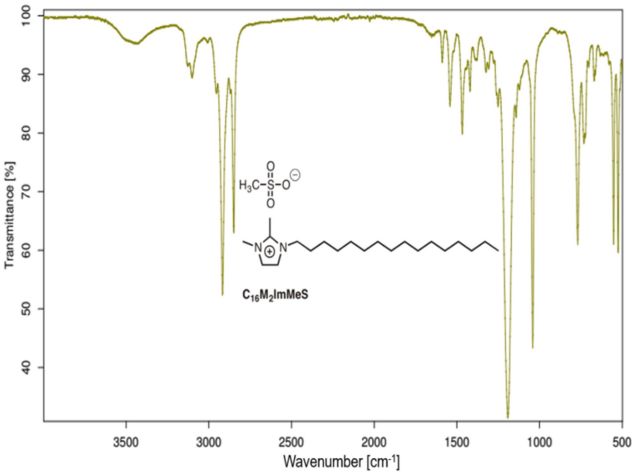

Figure S6. FTIR spectrum of **$C_{16}M_2ImMeS$**.

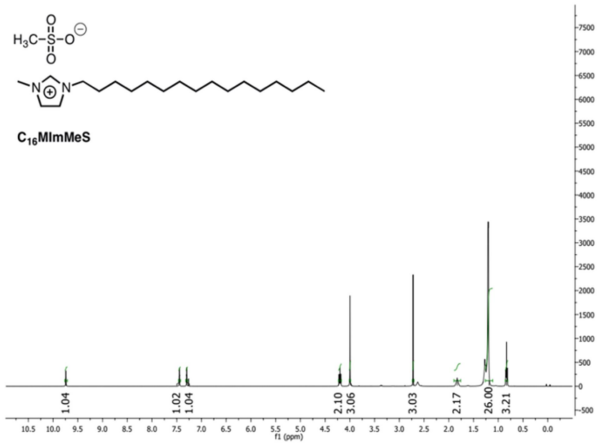

Figure S7. ^{1}H NMR spectrum of **$C_{16}MImMeS$** (400 MHz, $CDCl_3$).

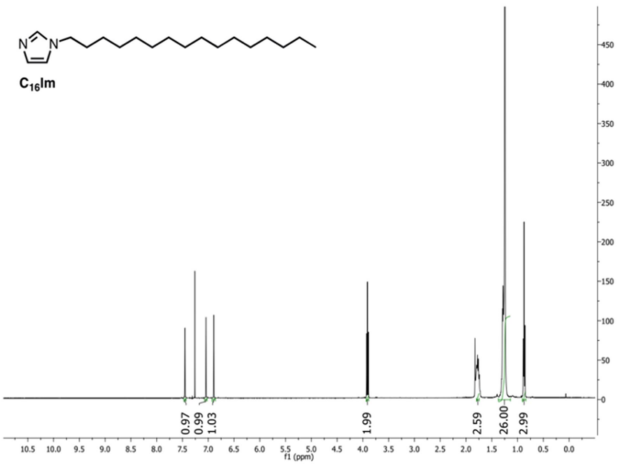

Figure S8. ^{1}H NMR spectrum of **C_{16}Im** (400 MHz, $CDCl_3$).

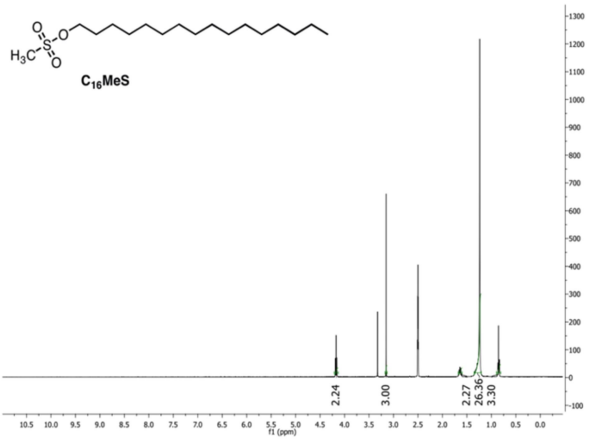

Figure S9. ^{1}H NMR spectrum of *n*-hexadecyl methanesulfonate (400 MHz, DMSO-d6).

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4.3 Artigo científico 3:

Artigo científico publicado no periódico *Phytotherapy Research* (ISSN 1099-1573, Fator de Impacto: 3.766, DOI: 10.1002/ptr.6551).

Este trabalho se deu como continuação da minha pesquisa de iniciação científica que demonstrou o efeito da Curcumina na modulação de caraterísticas fenotípicas de células de carcinoma espinocelular oral, publicada no periódico Phytotherapy Research em 2017. Assim como a Curcumina os polifenóis, em sua maioria, são compostos naturais, que tem sido estudado pelo seu potencial antineoplásico, apresentando seletividade e toxicidade para células tumorais e não para células normais. Nesta revisão sistemática foi avaliado a eficácia dos flavonóides na quimioterapia de pacientes com diferentes tipos de tumor, sólido e não sólido.

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REVIEW

WILEY

Flavonoids as anticancer therapies: A systematic review of clinical trials

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Flavonoids have been proposed as potential chemotherapeutic agents because they are toxic against cancer cells but not harmful to healthy cells. This systematic review analyzed flavonoid effectiveness in human cancer chemotherapy. Overall, 22 phase II and 1 phase III clinical trials (PubMed, Scopus, and Web of Science) that used flavonoids as a single agent or combined with other therapeutics against hematopoietic/lymphoid or solid cancer published by January 2019 were selected for analysis. Flavopiridol was the most commonly used flavonoid (at a dose of 50-mg/m^2 IV) for all tumor types. Aside from the relatively low rate of complete response (CR) or partial response (PR) with any administration protocol, flavonoids showed higher positive outcomes for hematopoietic and lymphoid tissues (140 patients with CR and 88 with PR among 615 patients in 11 trials) than for solid tumors (4 patients with CR and 21 with PR among 525 patients in 12 trials). However, because of the high variety in administration schedule, more studies are needed to further understand how flavonoids can promote positive outcomes for cancer patients.

KEYWORDS

chemotherapy, flavopiridol, hematopoietic tumors, solid tumor, polyphenols

1 | INTRODUCTION

Polyphenols are considered organic compounds with a chemical structure consisting of multiple phenol units. They are classified into two main classes, flavonoids and phenolic acids, and appear to have antioxidant activity (Crozieral et al., 2009; Amorati & Valgimigli, 2012). Flavonoids are polyphenolic compounds comprising 15 carbons linked to two benzene rings and have been demonstrated to possess anticancer properties because of their ability to downregulate mutant p53 protein, arrest cell cycle, and inhibit Ras protein expression (Lamson & Brignall, 2000; Brusselmans et al., 2003; Brusselmans et al., 2005; Mishra et al., 2013). Phenolic acids are derived from plants and have antioxidant activity as well as apoptotic effects on cancer cells (Galati et al., 2002).

Polyphenols have demonstrated positive effects against oral cancer, leukemia, and liver, prostate, breast, and skin cancer, and these compounds have been shown to act on multiple targets in several cellular pathways (Lin et al., 2006; Fu et al., 2007; Khan et al., 2011; Lin et al., 2012; Karthikeyan et al., 2013; Liu et al., 2014; Tenta et al., 2017; Koval et al., 2018; Sheng, et al., 2018; Wang et al., 2018). Some evidence suggest that polyphenols have antitumor suppressor potential because they inhibit proliferation and suppress protein kinase C and AP-1-dependent transcriptional activity (Kuntz et al., 1999; Park, 2015). In addition, polyphenols may neutralize free radicals during carcinogenesis (Agullo et al., 1996; Dong et al., 1997; Barthelman et al., 1998; Kuntz, 1999; Amorati & Valgimigli, 2012). In fact, men with prostate cancer that received green tea polyphenols showed a significant reduction in prostate-specific antigen, hepatocyte growth factor, and vascular endothelial growth factor serum levels prior to prostatectomy (McLarty et al., 2009; Wang et al., 2015).

The aim of this systematic review was to assess Phases II and III clinical trials in which patients were treated with polyphenols as anticancer therapy. Among the 23 selected studies, intravenous flavopiridol at 50 mg/m^2 was the most frequent flavonoid administered and was used primarily combined with a chemotherapeutic agent for treatment of solid, hematopoietic, or lymphoid tumors. Promising results were observed for polyphenols applied combined with other agents for

hematopoietic and lymphoid tumor treatment, but trials with a higher methodological quality and better standardization of administration protocols are still necessary to draw conclusions about the clinical benefits of polyphenol administration for cancer therapy.

2 | METHODS

2.1 | Search strategy

Searching was performed in the PUBMED, SCOPUS, and Web of Science databases with the following terms: "cancer" OR "neoplasm" OR "tumor" AND "drug and therapies" OR "chemotherapy" OR "therapy and drug" AND "humans" AND "clinical trial" AND "flavonoids" OR "polyphenols" (the last access was realized on January 2019). Furthermore, the references of the selected papers were searched. Two independent authors (A. B. and P. S. C.) reviewed the articles and the studies that generated disagreement between reviewers, and these papers were reevaluated so that a consensus could be reached. PROSPERO registration: CRD42018090987.

2.2 | Inclusion and exclusion criteria

Clinical trials (Phases II and III) that evaluated the use of flavonoids or polyphenols as chemotherapy for cancer patients were included in this study. in vitro and animal model studies, reviews, and Phase I trial studies were excluded.

2.3 | Data extraction

Using a standardized instrument, two authors independently extracted the following data: first author, country, year, sample size, age, gender, tumor type, clinical trial phase, drug type, administration route, dose, therapeutic scheme, median follow-up time, and main results.

2.4 | Methodological quality assessment

The JADAD score (Jadad et al., 1996) evaluation system was applied for methodological quality assessment of the 23 selected clinical trials. In the JADAD scale, studies are scored according to the presence of three key methodological features of clinical trials: randomization, blinding, and withdrawal and dropout of all patients. For an overall score of 0–5, a score of 1 is obtained for each one of the points described. A further point is added when the method of randomization and/or blinding is given and is appropriate, and a point is deducted when the method is inappropriate. Studies with a final JADAD score $\geq$3 points are considered to represent high-quality methodological reporting (Yang et al., 2015).

3 | RESULTS

A flowchart illustrating the progressive study selection stages is shown in Figure 1. The initial database searches identified 371 papers: 157 from PubMed, 36 from Scopus, and 178 from Web of Science. In the first analysis step, 41 papers were excluded because they were indexed in two or more databases and thus were considered duplicates. Next, the remaining 330 papers were screened, and 07 were found to be written in a language other than English, 111 were not clinical trial studies (43 were in vitro studies, 18 were animal model studies, and 40 analyzed other aspects, such as ulcerative colitis and hormone metabolism, or prevention of mucositis and cardiovascular disease). Additionally, 12 papers described studies in healthy patients in order to prevent hormonal diseases or dermatitis, and 118 were review papers, resulting in a total of 248 manuscripts that were excluded. The remaining 82 papers were then submitted for abstract analysis, and consequently, 59 were excluded because they did not fit the inclusion criteria of a Phase II or Phase III Clinical Trial using a flavonoid against cancer: 38 were Phase I clinical trials, 15 analyzed flavonoid pharmacokinetics and pharmacodynamics, 2 applied flavonoid via food administration, 1 used a plant extract, 2 used flavonoids for chemoprevention, and 1 was a Phase II maintenance therapy study, which was not our focus. After these selections, the 23 eligible papers were submitted for full-text analyses.

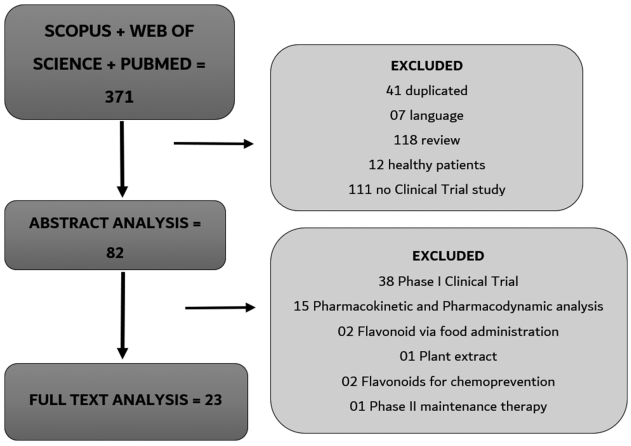

FIGURE 1 Flowchart of studies selection strategy

All the 23 selected papers were designed as Phase II (n = 22) or Phase III (n = 1) Clinical Trial studies for flavonoids as a chemotherapeutic drug for cancer treatment, and all cancer types were considered (Table 1). The publication dates of the 23 eligible papers ranged from 1990 to 2015. For all included papers, the median sample size was 49.5 patients and the median age of the patients was 59.3 years old among a total of 1,140 patients. The majority of enrolled patients were male. Cancers of hematopoietic and lymphoid tissues, such as acute myelogenous leukemia, chronic lymphocytic leukemia, and mantle cell lymphoma represented 48% (11 trials) of the studies, whereas solid tumors, including ovarian, fallopian tube, peritoneal, pancreatic, and prostate tumors; endometrial carcinoma; renal cell carcinoma; melanoma; colorectal cancer; and breast, colon, and head and neck carcinoma represented the remaining 52% (12 trials).

The majority of the clinical trials (16) used the flavopiridol flavonoid, with 10 manuscripts related to hematopoietic and lymphoid tissue tumors and six using flavopiridol against solid tumors. The flavone

TABLE 1 Demographic features, flavonoids, and tumor types from selected studies

Author, Year	Country	Multicenter/One center	Tumor	Flavonoid	Sample Size (*n*)	Age Median (Range)	Gender (%)
Hematopoietic and lymphoid tissues tumors							
Zeidner et al. (2015)	USA	Multicenter	Acute myeloid leukemia	Flavopiridol	165	44.5 (19–70)	Male 56% Female 44%
Shanafelt et al. (2013)	USA	One center	Chronic lymphocytic leukemia	Polyphenon E	42	60 (41–78)	Male 71% Female 29%
Karp et al. (2012)	USA	Multicenter	Acute myelogenous leukemia	Flavopiridol	78	49 (20–78)	Male 58% Female 42%
Karp et al. (2010)	USA	Multicenter	Acute myelogenous leukemia	Flavopiridol	45	61 (22–72)	Male 55% Female 45%
Lin et al. (2009)	USA	One center	Chronic lymphocytic leukemia	Flavopiridol	64	60.5 (31–82)	Male 76% Female 34%
Karp et al. (2007)	USA	One center	Acute myelogenous leukemia	Flavopiridol	62	58 (23–73)	Male 47% Female 53%
Byrd et al. (2007)	USA	One center	Chronic lymphocytic leukemia	Flavopiridol	42	61 (44–84)	Male 65% Female 35%
Byrd et al. (2005)	USA	Multicenter	Chronic lymphocytic leukemia	Flavopiridol	51	59 (37–81)	Male 70% Female 30%
Flinn et al. (2005)	USA	Multicenter	Chronic lymphocytic leukemia	Flavopiridol	26	63 (43–74)	Male 77% Female 33%
Kouroukis et al. (2003)	Canada	Multicenter	Mantle-cell lymphoma	Flavopiridol	30	68 (41–82)	Male 83% Female 17%
Lin et al. (2002)	USA	Multicenter	Mantle-cell lymphoma	Flavopiridol	11	62.5 (49–76)	Male 10% Female 90%
Solid tumors							
Fotopolou et al. (2014)	UK	Multicenter	Ovarian cancer	Phenoxodiol	142	58 (39–82)	Female 100%
Bible et al. (2012)	USA	Multicenter	Ovarian cancer, peritoneal cancer	Flavopiridol	45	59 (29–79)	Female 100%
Kelly et al. (2011)	USA	One center	Ovarian, fallopian tube, peritoneal cancers	Phenoxodiol	32	56.5 (43–70)	Female 100%
El Rayes et al. (2011)	USA	One center	Pancreatic cancer	Isoflavones	20	57.9 (39–75)	Male 60% Female 40%
Carvajal et al. (2009)	USA	One center	Pancreatic cancer	Flavopiridol	10	64 (49–81)	Male 40% Female 60%
Vaishampayan et al. (2007)	USA	One center	Prostate cancer	Isoflavone	71	76 (50–91)	Male 100%
Grendys et al. (2005)	USA	Multicenter	Endometrial carcinoma	Flavopiridol	23	57.5 (39–76)	Female 100%
Van Veldhuizen et al. (2005)	USA	Multicenter	Renal cell carcinoma	Flavopiridol	38	60.3 (38.6–76.9)	Male 65% Female 35%
Burdette-Radoux et al. (2004)	Canada	Multicenter	Melanoma	Flavopiridol	17	53 (29–77)	Male 71% Female 29%
Aklilu et al. (2003)	USA	Multicenter	Colorectal cancer	Flavopiridol	20	61 (42–80)	Male 55% Female 45%
Thatcher et al. (1990)	UK	Multicenter	Melanoma	Flavone Acetic Acid	34	58.5 (26–67)	Male 56% Female 44%
Kaye et al. (1990)	UK	Multicenter	Breast, colon, melanoma, head and neck carcinoma	Flavone Acetic Acid	73	56 (36–76)	—

Note: (—) not measured by authors.

acetic acid (FAA) was used in two clinical trials, one for patients with solid tumors, such as melanoma (Thatcher et al., 1990), and the other for treatment of breast, colon, and head and neck carcinoma and endometrial carcinoma (Kaye et al., 1990). Additionally, two trials used phenoxodiol for ovarian cancer patients (Kelly et al. 2011, Fotopoulou et al., 2014), two used isoflavone for prostate cancer (Vaishampayan et al., 2007) and pancreatic cancer patients (El-Rayes et al., 2011), and one used polyphenon E (Shanafelt et al., 2013) for treatment of leukemia, associated or not with the first line chemotherapy drug schema (Table 1).

3.1 | Response assessment of hematopoietic and lymphoid tissue tumors to flavonoids

The clinical trials that treated tumors of the hematopoietic and lymphoid tissues used the "1996 NCI Working Group Response Criteria" and "WHO Criteria—World Health Organization Criteria, 1981" when analyzing patient responses to treatment. Flavopiridol and polyphenon E were the flavonoids used for these types of cancer, and a total of 615 eligible patients were enrolled in these trials. Among the 11 trials, 10 studies used flavopiridol at different doses (50, 60, 75, 80, 100, 120, and 140 mg/m^2) via continuous intravenous or bolus infusion for acute myelogenous leukemia (Karp et al., 2005; Karp et al., 2010; Karp et al., 2012; Zeidner et al., 2015), chronic lymphocytic leukemia (Byrd et al., 2005; Byrd et al., 2007; Lin et al., 2009), and mantle cell lymphoma (Lin et al., 2002; Kouroukis et al., 2003) and just one clinical trial for chronic leukemia used polyphenon E at 1,000 or 2,000 mg through oral administration (Shanafelt et al., 2013).

The majority of study groups that examined these types of tumors (10 of 11) were from the USA, and one (Kouroukis et al., 2003) was from Canada. Additionally, 7 of the 11 clinical trials were multi-institution studies that involved two or more groups in the trial, and 4 studies were performed with patients from only one institution. The median follow-up time among the clinical trials for hematopoietic tissues was 20.5 months, with the shortest follow-up time being 8 months (Lin et al., 2009) and the longest follow-up time being 32 months (Shanafelt al., 2013).

Some clinical trials excluded patients who received prior chemotherapy (Karp.,et 2010, Shanafelt., et al. 2013), but the majority of clinical trials for hematopoietic tumors accepted this group of patients, requesting a pause of approximately 3 or 4 weeks since the last treatment (Lin et al., 2002).

Flavonoids were used as single agent chemotherapy for hematopoietic and lymphoid tissues in the following clinical trials: Shanafelt et al. (2013) used polyphenon E, whereas Lin et al. (2009), Byrd et al. (2007), Byrd et al. (2005), Flinn et al. (2005), Kouroukis et al. (2003), and Lin et al. (2002) used Flavopiridol. Zeidner et al. (2015) combined flavopiridol with cytarabine and mitoxantrone and compared flavopiridol with daunorubicin. Karp et al. (2012, 2010, 2007) combined flavopiridol with cytosine, arabinoside, and mitoxantrone with the intention of increasing the antitumoral activity of the gold standard treatment established (Table 2).

For objective responses to treatment, among the total 615 patients with hematopoietic or lymphoid tissue cancer treated with flavonoids, 140 patients achieved complete response (CR), and 88 patients achieved partial response (PR; Table 2). All the patients with a CR were from clinical trials that used flavopiridol schema combined with a first-line drug schema therapy for treatment of acute myeloid leukemia (4 trials, 139 patients with CR out of 350 patients) or as single agent for treatment of chronic lymphocytic leukemia (1 trial, 1 patient with CR out of 64 patients). PRs were observed in patients with chronic lymphocytic leukemia treated with poliphenon E as a single agent (1 trial, 29 patients with a PR out of 42 patients) or flavopiridol as a single agent (6 trials, 53 patients with a PR out of 183 patients), as well as in patients with mantle cell lymphoma treated with flavopiridol as a single agent (2 trials, 3 patients with a PR out of 40 patients; Table 2).

3.2 | Response assessment of solid tumors to flavonoids

The majority of clinical trials for solid tumors evaluated response and disease progression using "RECIST Criteria—Response Evaluation on Solid tumors." From the 12 clinical trials, 6 used Flavopiridol, 2 used FAA, 2 used phenoxodiol, and 2 used isoflavone (Table 3). Flavopiridol was administered for ovarian and peritoneal cancers (Bible et al., 2012), pancreatic cancer (Carvajal et al., 2009), endometrial cancer (Grendys, Blessing et al. 2005), renal cancer (Van Veldhuizen et al., 2005), melanoma (Burdette-Radoux et al., 2004), and colorectal cancer (Aklilu et al., 2003) at doses of 50 to 100 mg/m^2 via intravenous continuous or bolus infusion. FAA was administered via intravenous infusion (4.8 mg/m^2) for melanoma (Kaye et al., 1990) and breast, colon, and head and neck carcinoma patients (Thatcher et al. 1990). Phenoxodiol was employed in patients with ovarian cancer and given at a dose of 400 to 600 mg (Fotopolou et al.,2014) or intravenously (3 mg/kg) for ovarian and peritoneal cancer (Kelly et al., 2011), whereas isoflavone was used for pancreatic and prostate cancer and administered orally at a dose of 531 and 40 mg, respectively (El-Rayes et al., 2011; Table 3).

The studies examining solid tumors were primarily from the USA (eight), three groups were from the United Kingdom, and one group was from Canada. Of the 12 clinical trials for solid tumors, eight involved patients from two or more cancer centers, whereas four clinical trials were performed in one institution only (Table 1). Five clinical trials excluded patients with solid tumors that had undergone prior chemotherapy (Thatcher, et al., 1990, El-Rayes et al., 2011). The other seven papers (Kaye et al., 1990, Grendys et al., 2005; Vaishampayan et al., 2007; Carvajal et al., 2009; Kelly et al., 2011; Bible et al., 2012; Fotopolou et al., 2014) accepted these patients but requested a pause of approximately 4 weeks after the last chemotherapy treatment was carried out. A total of 521 eligible patients qualified for these trials, and the median overall survival time was 9.2 months. The median follow-up time among the trials for solid tumors was 6 months (Table 3).

Regarding the objective response assessment for solid tumor patients, four patients achieved CR. One of these patients used FAA at 4.8 mg^2 combined with recombinant interleukin-2 (rIL-2) for

TABLE 2 Drug schema and endpoints data and main results for selected hematopoietic and lymphoid tissues tumors clinical trials

Author (year)	Tumor	Flavonoid/doses	Associated drug	Prior ther.	Therapeutic schema	Sample size (*n*)	Primary endpoints (CR, PR, SD, and PD)	Secondary endpoints (DFS, EFS, TFS, and PFS)	Overall survival (OS)	Follow-up (mo)
Zeidner et al (2015)	AML	Flavopiridol 50 mg/m^2	Cyt Mit Daun	Yes	ARM A: flavopiridol 50-mg/m^2 IV, 1–3 d, Cyt 2-mg/m^2 CI IV, 6-8 d, Mit 40-mg/m^2 IV, 9 d. ARM B: Cyt 100-mg/m^2/d CI IV, 1–7 d, Daun 90-mg/m^2 IV, 1–3 d.	165	ARM A: 71 CR ARM B: 25 CR	EFS ARM A: 9.7 mo ARM B: 3.4 mo	ARM A: 17.5 moARM B: 22.2 mo	18
Shanafelt et al (2013)	CLL	Polyphenon E (Poly E) 1,000 and 2, 000 mg	No	No	Poly E 1,000 mg orally 2×/day, 7 days. Till 2,000 mg.	42	0 CR 29 PR	79% TFS of 24 mo	—	32
Karp et al (2012)	AML	Flavopiridol 50 and 30 mg/m^2	Mit Ara-C	No	ARM A: flavopiridol 50 mg/m^2, 1-3 d. ARM B: hybrid flavopiridol 30-min IVB, 30-mg/m^2 + 4-hr CI, 40 mg/m^2 (70 mg/m^2) 1–3 d. Ara-C 2 mg/m^2 72-hr CI (667 mg/m^2/24 hr), 6 d. Mit, 40-mg/m^2 IVB 60–120 min, 9 d.	78	Arm A: 2 CR Arm B: 4 CR	DFS Arm A: 13.6 mo DFS Arm B: 12 mo	OS > 12 mo Arm A: 66% Arm B: 70%	18.5
Karp et al (2010)	AML	Flavopiridol 50 mg/m^2	Ara-C Mit	No	Flavopiridol 50 mg/m^2 CI, 1 hr, 3 d. Ara-C 2 mg/m^2 72-hr CI (667 mg/m^2/24 hr), 6 d. Mit 40-mg/m^2 CIV, 9 d, 12 hr after Ara. second cycle 30 ± 7 d.	45	30 CR 0 PR	DFS 6.7 mo	7.4 mo	22
Lin et al (2009)	CLL	Flavopiridol 30 mg/m^2 + 30 mg/m^2 and 50 mg/m^2	No	Yes	Flavopiridol 30 mg/m^2, 30-min IVB + 30 mg/m^2 4-hr CI VI. Dose escalation to 30-mg/m^2 IVB and 50-mg/m^2 CIVI.	64	1 CR 30 PR	PFS 8.6 mo	—	8
Karp et al (2007)	AML	Flavopiridol 50 mg/m^2	Ara-C, Mit	Yes	Flavopiridol 50 mg/m^2 1-hr CI. Day 1 + 2 gm/m^2/72-hr Ara-C day 6, 40 mg/m^2 Mit, day 9.	62	32 CR 3 PR	2-year DFS of 50%.	—	30
Byrd et al (2007)	CLL	Flavopiridol 60 and 80 mg/m^2	No	Yes	Cohorts 1 and 2: 50% flavopiridol dose 30-min IVB + 50% dose, 4-hr CIV 4 w + 2 w off. Total dose: 60 mg/m^2 Cohort 2: 80 mg/m^2 (40 mg/m^2 30-min IVB + 40 mg/m^2 4 h CI).	42	0 CR 19 PR	PFS 13 mo	—	28

(Continues)

TABLE 2 (Continued)

Author (year)	Tumor	Flavonoid/doses	Associated drug	Prior ther.	Therapeutic schema	Sample size (*n*)	Primary endpoints (CR, PR, SD, and PD)	Secondary endpoints (DFS, EFS, TFS, and PFS)	Overall survival (OS)	Follow-up (mo)
					Cohort 3: flavopiridol as cohort 1, 4-w + 30-mg/m^2 30-min IVB + 50 mg/m^2 4-hr CI. Total dose of 80 mg/m^2					
Byrd et al (2005)	CLL	Flavopiridol 50 mg/m^2	No	Yes	Group 1: flavopiridol 50-mg/m^2/d CI 72 hr, 2 w. Group 2: flavopiridol 50 mg/m^2, 1-hr IVB, 3 d, 3 w.	51	Group 1 0 CR 0 PR 23 SD 24 PD. Group 2: 4 PR 19 SD 13 PD	PFS Group 1: 2 mo PFS group 2: 3 mo	Group 1: 27 mo Group 2: 24 mo	28
Flinn et al (2005)	CLL	Flavopiridol 80, 100, 120, and 140 mg/m^2	No	Yes	Cohort 1: flavopiridol 80 mg/m^2 24-hr CI, 2 w. Cohort 2: flavopiridol 80 mg/m^2 24-hr CI, 1 w + dose escalated by 20 mg/m^2, 2 w. Maximal 140 mg/m^2	26	0 CR 0 PR	—	—	—
Kouroukis et al (2003)	MCL	Flavopiridol 50 mg/m^2	No	Yes	Flavopiridol 50-mg/m^2/day IVB, 3 d, 21 d.	30	0 CR 3 PR 20 SD 5 PD	3.3 mo	—	Till death or relapse
Lin et al (2002)	MCL	Flavopiridol 50, 60, 75 mg/m^2	No	Yes	72 h CI, 14 d.	10	0 PR 0 CR 3 SD 7 PD	PFS: 70% PD 2 mo	—	—

Note: (—) not measured by authors.

Abbreviations: AML, Acute myeloid leukemia; Ara-C, cytosine arabinoside; CI, continuous intravenous infusion; CLL, chronic lymphocytic leukemia; CR, complete response; Cyt, cytosine; Daun, danorubicin; DFS, disease-free survival; EFS, event-free survival; IVB, intravenous bolus infusion; IV, intravenous; MCL, mantle cell lymphoma; Mit, mitoxantrone; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TFS, treatment-free survival.

TABLE 3 Drug schema and endpoints data and main results for selected solid tumors clinical trials

Author year	Tumor	Flavonoid doses	Associated drug	Prior ther.	Therapeutic schema	Sample size (*n*)	Primary endpointsCR, PR, SD, and PD	Secondary endpoints TTP/ DFS/PFS	Overall survival (median OS)	Follow up(mo)
Fotopolou, et al (2014)	Ovarian cancer	Phenoxodiol (PDX) 400 mg a 600 mg	Car	Yes	Group I: PXD + Ca Group II: placebo + Car PDX and placebo = daily; Car weekly 1-hr IVB. PDX orally, 400 mg, 8-hr CI. Body weight > 100 kg 50% to 600 mg, 30 min before eating. 28-d cycle.	142	0 CR 0 PR 66 PD	Group I: 3.7 mo Group II: 5 mo	Group I: 9.5 mo Group II: 11.4 mo	2
Bible et al (2012)	Ovarian cancer and peritoneal cancer	Flavopiridol 100 mg/ m^2	Cis	Yes	21-day cycle, Cis 60-mg/m^2 IV 1d, + flavopiridol 100 mg/m^2 24-hr CIV.	45	Group 1 1 CR 6 PR Group 2 0 CR 2 PR 10 SD	Median TTP 4.3 mo	16.1 mo	Last TP
Kelly et al (2011)	Ovarian, Fallopian Tube, Peritoneal cancers	Phenoxodiol 3 mg/kg	Cis, Pac	Yes	Group 1: Cis 40-mg/m^2 IV day 2 + phenoxodiol 3 mg/kg, days 1 and 2. Group 2 Pac: 80-mg/m^2 IV day 2 + phenoxodiol 3 mg/kg, days 1 and 2.	32	Group 1: 0 CR 3 PR 10 SD 4 PD Group 2: 1 CR 2 PR 8 SD 4 PD	Median TTP 6 mo	—	6
El Rayes et al (2011)	Pancreatic cancer	Isoflavones 531 mg	Erlo, Gemc	No	Gemc 1,000-mg/m^2 IV days 1, 8, and 15. Erlo 150 mg, daily orally; Isoflavone 531 mg (177 mg, 3 tablets), 2× d, day 7 to the end. Cycles repeated every 28 d.	20	0 CR 1 PR 6 SD	Median PFS: 2 mo	5.2 mo	—
Carvajal et al (2009)	Pancreatic cancer	Flavopiridol 80 mg/m^2	Doc	Yes	Doc 35-mg/m^2 30-min IVB, 4 to 6 hr later flavopiridol 80 mg/m^2 60-min CI days 1, 8, and 15 of each 28 days cycle.	10	0 CR 0 PR 6 PD 3 SD	Median TTP: 2 mo	4.2 mo	—
Vaishampayan et al (2007)	Prostate cancer	Isoflavone 40 mg	Lyc	Yes	Lyc 15 mg orally 2× d, or Lyc 15 mg + Isoflavone (Solgen R) 40 mg orally 2 d, maximum 6 mo.	71	0 CR 0 PR 57 SD 18 PD	50% in 6 mo	—	6
Grendys et al (2005)	Endometrial carcinoma	Flavopiridol 50 mg/m^2	No	Yes	Flavopiridol 50 mg/m^2/d, IVB all days.	23	0 CR 0 PR		—	—

(Continues)

TABLE 3 (Continued)

Author year	Tumor	Flavonoid doses	Associated drug	Prior ther.	Therapeutic schema	Sample size (*n*)	Primary endpointsCR, PR, SD, and PD	Secondary endpoints TTP/ DFS/PFS	Overall survival (median OS)	Follow up(mo)
							6 SD 15 PD	Median PFS: 3.2 mo		
Van Veldhuizen et al (2005)	Renal cell carcinoma	Flavopiridol 50 mg/m^2	No	No	Flavopiridol 50-mg/m^2 IVB, 3 d, for 3 w.	34	1 CR 3 PR 14 SD	—	9 mo	—
Burdette-Radoux et al (2004)	Melanoma	Flavopiridol 50 mg/m^2	No	No	Flavopiridol 50-mg/m^2 1-hr IVB daily × 3 d, for 3 w.	17	0 CR 0 PR 7 SD 9 PD	Median TTP: 2.8 mo	—	5
Aklilu et al (2003)	Colorectal cancer	Flavopiridol 50 mg/m^2	No	No	Flavopiridol 50-mg/m^2 IV, 72-hr CI, every 14 d.	20	0 CR 0 PR 05 SD	Median TTP: 2 mo	65 w	—
Thatcher et al (1990)	Melanoma	FAA 4.8 mg^2	rIL-2	No	Five doses of rIL-2. rIL-2 dose: 11 × 10^6 Cetus units m^{-2} Dose 1: via femoral artery catheter + dose 4-hr later IV. Other doses IV on alternate days; the day before rIL-2, FAA (4.8 mg^2) as a 6-hr CIV. Total 3 courses/21-d intervals.	34	1 CR 4 PR 10 SD 19 PD	—	4 mo	9
Kaye et al (1990)	Breast, colon, melanoma, head and neck carcinoma	FAA 4.8 mg/m^2	No	Yes	Once-weekly, FAA 4.8 mg/m^2 1-hr IVB. Alkalization before and after FAA.	73	0 CR O PR 63 PD 10 SD	Median TTP: 3.2 mo	—	—

Note: (—) not measured by authors.

Abbreviations: Car, carboplatin; CI, continuous intravenous infusion; Cis, cisplatin; CR, complete remission; DFS, disease-free survival; Doc, docetaxel; Erlo, erlotinib; FAA, flavone acetic acid; Gemc, gemcitabine; IV, intravenous; IVB, intravenous bolus infusion; Lyc, lycopene; Pac, paclitaxel; PD, progressive disease; PFS, progression-free survival; PR, partial remission; rIL-2, recombinant interleukin-2; SD, stable disease; TFS, treatment-free survival; TTP, time to progression.

melanoma and had a median overall survival time of 4 months (Thatcher et al., 1990). Another CR patient used flavopiridol as a single agent at a dose of 50 mg/m^2 in a bolus infusion for renal cell carcinoma and showed a median overall survival time of 9 months (Van Veldhuizen et al., 2005). The other two CR patients were ovarian cancer patients, one from Bible (2012) who used flavopiridol combined with cisplatin and the other from Kelly (2011) who combined phenoxodiol with paclitaxel. A total of 21 patients achieved PR when treating solid tumors with flavonoids. Eight PR patients were treated for ovarian and peritoneal cancer with flavopiridol at 100 mg/m^2 combined with cisplatin and presented a median time to progression of 4.3 months (Bible et al., 2012). Five PR patients used phenoxodiol (3 mg/kg) combined with cisplatin or paclitaxel for ovarian, fallopian tube, or peritoneal cancer and showed a median time to progression of 6 months (Kelly, 2011). Four patients with melanoma achieved PR when treated with FAA (4.8 mg^2) associated with Interleukin-2 recombinant protein (rIL-2) and experienced a median survival time of 4 months (Thatcher et al., 1990). One patient with pancreatic cancer used isoflavones (531 mg) combined with erlotinib and gemcitabine and showed PR and a median progression-free survival of 2 months (El-Rayes et al., 2011). When flavopiridol at 50 mg/m^2 was used as a single agent to treat renal cell carcinoma (Van Veldhuizen et al., 2005), three patients achieved PR and presented a median survival time of 9 months (Table 3).

When flavopiridol was used at 100 mg/m^2 for ovarian and peritoneal cancer (Bible et al., 2012), patients had the best overall survival time of 16 months; however, the worst overall survival time (4 months) was observed for melanoma patients treated with isoflavone (Tatcher et al.,1990). For analysis of tumor recurrence, the longest time to progression was 16.1 months and was observed for Bible (2012) patients who combined flavopiridol with cisplatin to treat ovarian and peritoneal cancers. The shortest time to progression was 2 months in a clinical trial that used flavopiridol at 50 mg/m^2 to treat colorectal cancer patients (Aklilu et al., 2003) and in a trial that treated pancreatic cancer with flavopiridol combined with docetaxel (Carvajal et al., 2009; Table 3).

3.3 | Methodological quality assessment

To analyze the methodological quality of the clinical trials, we employed the JADAD score (Jadad, 1996), where a final score $\geq$3 points was considered to indicate a high quality of methodological reporting (Yang et al., 2015). For the hematopoietic and lymphoid tissue trials (Table 4), two manuscripts it had a score of 3 (Zeidner et al., 2015, Karp et al., 2012) and nine had a score of 1 (Shanafelt et al., 2013, Karp et al., 2010, Lin et al., 2009, Karp et al., 2007, Byrd et al., 2007, Byrd et al., 2005, Flinn et al., 2005, Kouroukis et al., 2003, Lin et al., 2002). For clinical trials on solid tumors, one manuscript had a score of 5 (Fotopolou et al., 2014), two had a score of 3 (Kelly et al., 2011; Vaishampayan et al., 2007), and nine had a score of 1 (Bible et al., 2012; El Rayes et al., 2011; Carvajal et al., 2009; Grendys et al., 2005; Van Veldhuizen et al., 2005; Burdette-Radoux et al., 2004; Aklilu et al., 2003; Thatcher et al., 1990; Kaye et al., 1990).

4 | DISCUSSION

The myriad outputs from cellular associations are a big challenge in cancer treatment, and thus, searching for novel therapies to improve patient survival is imperative (Hanahan & Weinberg, 2011). Flavonoids are described as phenol compounds with a phenyl benzopyran structure and a carbon skeleton joined to a chroman ring (Durazzo et al., 2019, Pereira, 2009). Flavonoids are derived from the aromatic amino acids tyrosine and phenylalanine, and their backbone structure can link to certain hydroxyl groups (OH; Zhang et al., 2016). The pattern of the primary molecular structure of flavonoids, as well as the substitution of chemical groups in the flavonoid structure, is correlated with their human biological activity and bioavailability (Durazzo et al., 2019). In addition, flavonoids are the most abundant polyphenols in our diet according to Abbaszadeh et al. (2019). At the same time, human epidemiological studies have demonstrated the potential impacts of dietary polyphenols on prevention of many cancer types (Pandey et al., 2009, Li et al., 2015, Wu et al., 2016), and the overall results from basic science research mostly agree with the potential role of flavonoids in the human diet as protective compounds (Grosso et al., 2007). Herein, to analyze the efficacy of flavonoids in cancer therapy, we examined only Phases II and III clinical trials. According to the Food and Drug Administration, Phase II clinical trials are conducted to measure the efficacy of a drug against a disease, and Phase III trials are conducted to verify that the potential drugs are better than the gold standard treatment agent. Therefore, our search resulted in identification of 22 Phase II and 1 Phase III clinical trial studies related to flavonoids used against solid (12 trials) and hematopoietic/lymphoid tumors (11 trials). The high variety in drug administration form, patient collection data, and randomization, as well as the variety of tumors types addressed, made it difficult to perform a meta-analysis evaluation. In total, three clinical trials found modest biologic effects or no clinical activity of flavonoids for patients with chronic leukemia (Flinn., 2005; Byrd., 2005; Shanafelt., 2013), but with the astonishing advances in pharmacology and nanotechnology (Bhise et al., 2017, Davatgaran T. et al., 2017), it is possible that new formulations that increase the availability of flavonoids might improve the success rate for patients with hematopoietic or lymphoid tissue tumors.

4.1 | Flavopiridol

The majority of papers selected in the present review were studies that used the flavonoid flavopiridol against cancer. Flavopiridol is a class of synthetic flavonoid and considered a broad cyclin-dependent kinase inhibitor that induces apoptosis in vitro (Grendys, 2005) through caspase 3 activation (Flinn et al., 2005). The antitumor activity is p53 independent and decreases the expression of mcl-1 in vitro (Byrd et al., 2007).

Indeed, when flavopiridol was used as a single agent for chronic lymphocytic leukemia, high clinical activity was observed (Lin et al., 2009; Byrd et al., 2007). On the other hand, some authors have reported diminished clinical activity due to increased binding of

flavopiridol to human serum proteins, which suggests that the schedule dependence of flavonoids is very important in improving their clinical efficacy (Byrd., 2005; Flinn et al., 2005). Most of the clinical trials for hematopoietic and lymphoid tissues utilized flavopiridol at doses from 30 to 100 mg/m^2. Additionally, the use of flavopiridol as an adjuvant with stablished chemotherapy drugs appeared to have better clinical effect (Zeidner et al., 2015) over the use of flavopiridol as a single agent against chronic or acute leukemia (Lin et al., 2009).

TABLE 4 JADAD scale for quality assessment of selected clinical trials

Author (year)	Randomization (0/1)	Sequence of randomization (0/1)	Blinding (0/1)	Method of blinding (0/1)	Withdrawals/dropouts (0/1)	Inappropriate randomization (0/−1)	Inappropriate blinding(0/−1)	JADAD final score
Zeidner et al. (2015)	1	1	0	0	1	0	0	3
Shanafelt et al. (2013)	0	0	0	0	1	0	0	1
Karp et al. (2012)	1	1	0	0	1	0	0	3
Karp et al. (2010)	0	0	0	0	1	0	0	1
Lin et al. (2009)	0	0	0	0	1	0	0	1
Karp et al. (2007)	0	0	0	0	1	0	0	1
Byrd et al. (2007)	0	0	0	0	1	0	0	1
Byrd et al. (2005)	0	0	0	0	1	0	0	1
Flinn et al. (2005)	0	0	0	0	1	0	0	1
Kouroukis et al. (2003)	0	0	0	0	1	0	0	1
Lin et al. (2002)	0	0	0	0	1	0	0	1
Fotopolou et al. (2014)	1	1	1	1	1	0	0	5
Bible et al. (2012)	0	0	0	0	1	0	0	1
Kelly et al. (2011)	1	1	0	0	1	0	0	3
El Rayes et al. (2011)	0	0	0	0	1	0	0	1
Carvajal et al. (2009)	0	0	0	0	1	0	0	1
Vaishampayan et al. (2007)	1	1	0	0	1	0	0	3
Grendys et al. (2005)	0	0	0	0	1	0	0	1
Van Veldhuizen et al. (2005)	0	0	0	0	1	0	0	1
Burdette-Radoux et al. (2004)	0	0	0	0	1	0	0	1
Aklilu et al. (2003)	0	0	0	0	1	0	0	1
Thatcher et al. (1990)	0	0	0	0	1	0	0	1
Kaye et al. (1990)	0	0	0	0	1	0	0	1

Flavopiridol demonstrated real effectiveness against acute myelogenous leukemia when combined with a first-line chemotherapy, such as cytosine, arabinoside, and/or mitoxantrone (Karp et al.,2007; Karp et al., 2010; Karp et al., 2012; Zeidner et al., 2015). On the other hand, despite its biological activity, flavopiridol has failed to demonstrate clinical activity against certain cancers, such as mantle cell lymphoma, in some clinical studies (Koukoris et al., 2003; Lin et al., 2002). Nevertheless, this cancer is aggressive and resistant to treatment, and thus, these results are in line with those of Grosso et al. (2017). However, further observational studies and experimental randomized trials are necessary to confirm these results.

When flavopiridol was used as a single agent for solid tumors, it appeared to have minimal or no clinical activity according to the authors Aklilu et al. (2003), Burdette-Radoux et al. (2004), Grendys et al. (2005), and Van Veldhuinet al. (2005). However, when it was combined with gold standard chemotherapy drugs, flavopiridol seemed to be feasible despite its significant toxicity (Carvajal et al., 2009). Moreover, flavopiridol could have some activity against ovarian cancer (Bible et al., 2012).

4.2 | FAA

FAA is a synthetic flavonoid that clearly has antitumor activity in vitro, possibly by acting as a biological response modifier. Several mechanisms of action are therefore likely, because of the high reactivity of the flavonoid structure (Kaye et al., 1990). Among the selected papers analyzed, Thatcher et al. (1990) combined FAA with rIL-2 for melanoma patients, and Kaye et al. (1990) used FAA as a single agent for breast, colon, and head and neck carcinoma and melanoma cancer patients. In addition, although disease stabilization was seen in Kaye et al. (1990), only one patient showed CR in Thatcher et al. (1990), indicating no significant clinical beneficial effects for this type of synthetic flavonoid against these types of tumors. In contrast, according to Rothwell et al. (2017), in a recent review, the evidence for the effectiveness of polyphenols against breast cancer metastasis suggests that polyphenols might be used as chemotherapeutic agents that reduce metastasis progression of that cancer. Nevertheless, further studies are needed, especially human trials. At the same time, a reduction in the risk for breast cancer was found to be associated with flavone intake, even though these optimistic outcomes have only been reported in case control studies (Rothwell et al., 2017).

4.3 | Isoflavones

Isoflavones are biologically active compounds with estrogenic properties, and genistein is one of the main components (Durazzo et al., 2019). Genistein is found in soybeans and modulates the expression of genes involved in a variety of cellular functions, such as apoptosis, proliferation, and angiogenesis. Isoflavone induces apoptosis in pancreatic cancer cells and enhanced the antitumor activity of gold standard drugs in experimental pancreatic cancer cells (El Rayes et al., 2011). In addition, epidemiological studies indicated that soybean-based diets may decrease cancer risk (Dong e al., 2011; Yan et al., 2009). Furthermore, Phase I/II clinical trials showed the potential efficacy of this isoflavone as an antimetastatic agent against prostate cancer (Pavese et al., 2014; Abbaszadeh et al., 2019).

Based on preclinical and clinical data, El Rayes et al. (2011) and Vaishampayan et al. (2007) analyzed isoflavone treatment for pancreatic and prostate cancer patients respectively, but the outcomes were disappointing. Isoflavone did not improve the clinical activity of the gold standard treatments for these cancers, and CR was not observed in either of these clinical trials. The possible mechanism proposed by El Rayes et al. (2007) to explain the activity of soy isoflavone in prostate cancer involves estrogen-like effects, prevention of oxidative DNA damage, reduction in cancer cell proliferation and modulation of steroid-metabolizing enzymes, but further studies are needed according to the author.

4.4 | Phenoxodiol

Phenoxodiol (PDX) is a laboratory-modified version of the naturally occurring plant isoflavone and has shown synergistic interaction with cisplatin and carboplatin, combined or not with gold standard drugs, in Phase II studies, with more than 55% of patients showing good tolerance for the cancer treatment (Kelly et al., 2011).

Phenoxodiol is an isoflavone derivative with proapoptotic properties. Tumor cells exposed to phenoxodiol exhibit distress almost immediately, followed by a cascade of biochemical effects (Kamsteeg et al., 2003). Phenoxodiol also reverses chemoresistance to cisplatin, carboplatin, paclitaxel, docetaxel, and gemcitabine in human ovarian cancer cells (Sapi et al., 2003; Kelly et al., 2011), which is in line with Durazzo et al. (2019), whose review showed evidence that isoflavone intake exerts a positive effect on cancer chemoprevention. He also demonstrated a trend similar to that observed for prostate cancer risk reduction; however, final conclusions could not be drawn given the size and duration of the clinical trials.

In relation to endometrial cancer, isoflavone intake has been associated with a reduction in cancer risk for both Asian and non-Asian populations in two separate meta-analyses (Rothwell et al., 2017). For PDX against ovarian cancer, the results confirmed the outcomes found in our review by Kelly et al. (2011) and Fotopoulou et al. (2014). Regarding the lack of phenoxodiol efficacy, although it was safe, there was no evidence of clinical activity, and indeed, there were patients with disease progression during the both clinical trials.

Previous Phase I/II safety and pharmacokinetic studies with oral PXD suggest that the drug is immediately conjugated to an inactive metabolite and that inactivation is reversed within tumors, providing a tumor targeting strategy (Gamble et al., 2006; Kelly et al., 2011). Actually, only one of the studies reviewed here was a Phase III clinical trial and reported no evidence of clinical activity of phenoxodiol plus carboplatin to overcome drug resistance in patients with ovarian carcinoma (Fotopoulou et al., 2014). It is important to be aware that the low success rate against solid tumors might be due to the heterogeneity of intratumor cell phenotypes in the tissues (Gerlinger et al., 2012).

4.5 | Polyphenon E (Catechin)

The name catechin is derived from the term catechu, the extract of acacia catechu L. Catechin belongs to a subgroup of monomeric flavanols. Catechins are a major polyphenol in tea and seem to be related to its health benefits and association with low cancer incidence (Durazzo et al., 2019).

In the same way, tea polyphenols exert multitargeted effects on malignant cells. Epigallocatechin gallate, the major catechin in tea, induces apoptotic cell death in animal models of human cancer cells (Nakazato et al., 2005; Lee et al., 2004). Subsequent case reports in patients with cancer suggest that these preclinical findings may have clinical relevance (Shanafelt et al., 2006). Based on this series of observations and the favorable toxicity profile of green tea extracts in human testing, Shanafelt et al. (2013) conducted a Phase II trial of daily, oral Polyphenon E, which is a standardized, pharmaceutical catechin preparation. Patients with chronic lymphocytic leukemia were treated, but only modest clinical effects were observed, and the study was not powered to demonstrated significance according to the author (Shanafelt et al., 2013). Therefore, more studies are likely to be conducted with this type of cancer patient treated with polyphenon E.

5 | CONCLUSIONS

- The results should be analyzed and compared carefully, because it was observed that a high variety of polyphenols, such as phenoxodiol, isoflavones, FAA, polyphenon E, and flavopiridol, were used for the treatment of various tumor types. Additionally, the variety in dosage and administration form made it difficult to perform a proper comparison.
- Although some studies received a low final JADAD score, these results should not invalidate the studies or the clinical outcomes of the trials.
- Nevertheless, it is important to note that future trials should follow rigorous clinical trial methodological protocols, as well as the guidelines stablished by NCI and or WHO criteria for hematopoietic and lymphoid tumors and by RECIST criteria for solid tumors, in order to obtain more comparable results among the clinical trial studies.
- To avoid potential pitfalls in clinical trials, as well as to improve the clinical outcomes, it is still suggested to test different drug combination models, develop more accurate predictive/prognostic markers, select appropriate controls, and reduce patient heterogeneity and selection bias.
- Evidence points to the use of flavopiridol associated with first line chemotherapy for the treatment of hematopoietic and lymphoid tissues, especially acute leukemia. However, schedule optimization and a better understanding of flavonoid action are still required to achieve better outcomes in clinical tumor treatments.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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5. CONSIDERAÇÕES FINAIS

A MEC é altamente dinâmica, assim é constantemente depositada, remodelada e degradada, alterações na dinâmica basal podem alterar sua composição e consequentemente o comportamento celular. O processo de mecanotransdução é a forma pela qual as células respondem a essas alterações da matriz e culminam em mudanças na expressão gênica. A interferência bidirecional entre a célula e a MEC induz modificações no comportamento celular, assim, a dinâmica de alterações da MEC podem promover características mais agressivas. Forças contráteis são requisitadas em múltiplos processos fisiológicos e patológicos, assim o aumento da contratilidade celular prejudica as adesões célula-célula e promove a invasão e migração de células tumorais. Então, a modulação química da contratilidade foi capaz de influenciar no comportamento celular em substrato pouco rígido e rígido. O aumento da rigidez da matriz está relacionado à transformação maligna, devido ao aumento da proliferação celular e promoção do processo de EMT, migração e invasão em células epiteliais. O substrato rígido promove fenótipo e genótipos mais agressivos em células tumorais e o aumento da contratilidade celular em ambientes pouco rígidos são capazes de promover comportamento semelhante a células expostas a substratos rígidos. Dessa forma, a contratilidade celular mostrou-se necessária para a modulação das células de CEC oral influenciando a agressividade do tumor.

A modulação do comportamento tumoral por meio de fatores químicos é uma prática comum, mas com alta taxa de insucesso devido a não seletividade dos quimioterápicos e drogas adjuvantes disponíveis para o tratamento ou em fase avançada de testes. Desta forma, busca-se uma substância que seja capaz de atuar sobre as células tumorais e associadas, porém que desenvolvam pouco ou nenhum efeito citotóxico sobre as células saudáveis. Assim, os sais imidazólicos são substâncias capazes de serem “tunadas”, ou seja, sua estrutura química pode ser alterada para melhorar sua atividade biológica. Foi demonstrado relação estrutura atividade de uma das substâncias avaliadas, comprovando que o comprimento da cadeia da molécula e o ânion associado podem melhorar o desempenho das substâncias sobre diferentes processos biológicos. Então, o sal imidazólico 1-*n*-

hexadecyl-3-methylimidazolium chloride (**C_{16}MImCl**), demonstrou uma atividade superior aos demais analisados, além de exibir seletividade à células tumorais em 2D e 3D, além de demonstrar resultados semelhante ao quimioterápico padrão-ouro em alguns processos celulares, como apoptose e necrose.

Ainda em relação aos fatores químicos na modulação do comportamento tumoral, os flavonóides foram propostos, inicialmente como potenciais agentes quimioterapêuticos devido a sua toxicidade para células tumorais, mas não para células saudáveis. Essa classe de compostos demonstrou atividade antitumoral *in vitro* e *in vivo*, por meio de efeito anti-proliferativo e capacidade antioxidante. O desfecho de pacientes após o tratamento com polifenóis como terapia antitumoral, associado ou não a outro tratamento, por meio de ensaios clínicos randomizados demonstrou que o flavopiridol intravenoso foi o flavonóide mais frequente administrado e utilizado em combinação com quimioterápicos padrão para o tratamento de tumores sólidos e não sólidos. No entanto, devido à alta variedade no cronograma de administração do tratamento, é necessário elevar a qualidade das evidências científicas, por meio de protocolos padrão de administração e dosagem para que os dados de remissão total e parcial possam ser comparados entre os trabalhos.

A mecanobiologia ganhou espaço nas pesquisas nos últimos tempos, e sua participação no microambiente tumoral é fundamental. A partir das descobertas nesse campo os questionamentos sobre a aplicabilidade desse conhecimento estão sendo levantadas, e assim é importante elucidar o mecanismo no qual o tumor aumenta o stress mecânico e sua relação com comportanto mais agressivo. Além disso, buscar a padronização de modelos tumorais biomiméticos, desenvolvendo biomateriais para simular a complexidade do tumor. Assim, o conhecimento de cada componente do microambiente tumoral, celular e não-celular, e seu papel na progressão da doença ainda não são completamente elucidados, o que reflete a taxa de insucesso nos tratamentos e falha no desenvolvimento de novas drogas. Sendo, então, o maior desafio associar estes novos parâmetros descobertos recentemente no estabelecimento de biomarcadores para detecção precoce do tumor, desenvolvimento de terapias alvo e atribuição de valor prognóstico.

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