UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Avaliação do papel da ácido graxo-sintase no desenvolvimento do câncer de colo de útero e proposta de formulação farmacêutica nanoestruturada para o novo alvo terapêutico.

JÉSSICA NASCIMENTO

PORTO ALEGRE, 2019

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Avaliação do papel da ácido graxo-sintase no desenvolvimento do câncer de colo de útero e proposta de formulação farmacêutica nanoestruturada para o novo alvo terapêutico.

Dissertação apresentada por Jéssica Nascimento para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas

Orientador: Prof. Dr. Ruy Carlos Ruver Beck

PORTO ALEGRE, 2019

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas, em nível de Mestrado Acadêmico da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e aprovada em 15 de março de 2019, pela Banca Examinadora constituída por:

Profa. Dr. Fátima Theresinha Costa Rodrigues Guma
Universidade Federal do Rio Grande do Sul

Profa. Dr. Léticia Cruz
Universidade Federal de Santa Maria

Prof. Dr. Helder Ferreira Teixeira
Universidade Federal do Rio Grande do Sul

CIP - Catalogação na Publicação

Nascimento, Jéssica
Avaliação do papel da ácido graxo-sintase no desenvolvimento do câncer de colo de útero e proposta de formulação farmacêutica nanoestruturada para o novo alvo terapêutico. / Jéssica Nascimento. -- 2019.
101 f.
Orientador: Ruy Carlos Ruver Beck.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Porto Alegre, BR-RS, 2019.

1. Câncer de Colo de Útero. 2. FASN. 3. Nanocápsulas. 4. Orlistate. I. Beck, Ruy Carlos Ruver, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Este trabalho foi desenvolvido no Laboratório de Análises Bioquímicas e Citológicas (LABC) do Departamento de Análises Clínicas e no Laboratório de Pesquisa do Departamento de Produção e Controle de Medicamentos da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul, na cidade de Porto Alegre, com financiamento do Programa de Pós-Graduação em Ciências Farmacêuticas (PPGCF/UFRGS), do Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), do Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE/HCPA), da Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) e da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

À minha família,

pelo amor, respeito, força e incentivo a todas as minhas escolhas.

AGRADECIMENTOS

Ao prof. Dr. Ruy Carlos Ruver Beck e prof. Dr. Diogo André Pilger, pela orientação, por todo empenho, disponibilidade e, acima de tudo, exigência. Por acreditarem em mim e me incentivarem.

Aos meus queridos colegas de Laboratório – Aline, Camila, Débora, Letícia, Júlia, Paola e Samuel. Agradeço todo o apoio, amizade e por tornarem meu caminho até aqui mais alegre.

A profa. Dra. Lúcia Maria Kliemann pelas importantes contribuições para o desenvolvimento deste trabalho.

A profa. Dra. Andréia Buffon, por ter apoiado minha ideia inicial mesmo sendo um assunto totalmente diferente de sua linha de pesquisa.

Ao Programa de Pós-Graduação em Ciências Farmacêuticas pela oportunidade.

A minha mãe e meu pai, agradeço pelo amor, educação e por nunca terem deixado me faltar nada.

À minha vó que sempre rezou por mim.

Aos meus irmãos que sempre me incentivaram a alcançar caminhos cada vez mais distantes.

Ao Giovani, pelo amor, paciência e compreensão.

RESUMO

A síntese *de novo* dos ácidos graxos ocorre no citoplasma das células e é realizada pela enzima ácido graxo-sintase (FASN) a partir de reações de condensação entre acetil-CoA e malonil-CoA. Estudos tem demonstrado que muitos tumores expressam FASN e que sua inibição resulta em atividade antitumoral. O Orlistate é um fármaco originalmente desenvolvido para o tratamento da obesidade, no entanto, tem sido demonstrada sua atividade antitumoral por ser um inibidor irreversível da FASN. O câncer de colo de útero representa um grande problema de saúde pública. É o quarto tipo mais comum de câncer que afeta as mulheres em todo o mundo. Até o momento, nenhum estudo correlacionou a expressão da FASN com o câncer do colo do útero e a possibilidade de utilizá-la como um novo alvo terapêutico. Considerando a relação da FASN com o câncer, a expressão da enzima em linhagens de câncer de colo de útero (HeLa, SiHa, C-33A, e ME-180) e em amostras de pacientes com lesões pré-malignas e carcinoma foi determinada, bem como os efeitos da sua inibição pelo Orlistate. Como esse fármaco apresenta baixa solubilidade em água e limitada biodisponibilidade oral, foi proposto, ainda, o desenvolvimento de nanocápsulas de poli(ε-caprolactona) contendo Orlistate para posterior avaliação de seus efeitos na linhagem HeLa. Todas as linhagens de câncer de colo de útero e amostras de pacientes com lesões apresentam expressão da FASN, sendo mais elevada em estágios mais avançados. O tratamento das linhagens de câncer do colo do útero com Orlistate resultou na diminuição da viabilidade celular de maneira tempo-dependente e desencadeou apoptose. Ainda, o tratamento com Orlistate causou parada do ciclo celular e autofagia em todas as linhagens avaliadas. A nanoformulação de Orlistate apresentou uma eficiência de encapsulação de quase 100% e um efeito antiproliferativo maior quando comparada ao orlistate não-encapsulado. Sendo assim, analisados de maneira global, todos os resultados indicam a importância da FASN na carcinogênese cervical e que sua inibição pode ser proposta como uma estratégia terapêutica promissora para o câncer de colo de útero.

Palavras chaves: Câncer de Colo de Útero, FASN, nanocápsulas, Orlistate.

ABSTRACT

Evaluation of fatty acid synthase role in the cervical cancer development and proposal for nanostructured pharmaceutical formulation for the new therapeutic target.

The *de novo* synthesis of fatty acids occurs in the cells cytoplasm and it is performed by the metabolic enzyme fatty acid synthase (FASN) through the condensation reaction between acetyl-CoA and malonyl-CoA. Studies have shown that many tumors express FASN and that their inhibition, results in antitumor activity. Orlistat is a drug originally developed for the treatment of obesity. However, its antitumor properties have been shown by acting as an irreversible inhibitor of the FASN. Cervical cancer represents a major public health problem. It is the fourth most common cancer type that affects women worldwide. Until now, no study related FASN expression and cervical cancer and the possibility of using it as a new therapeutic target. Considering the relationship between FASN and cancer, we analyzed FASN expression in human cervical cancer cells, in pre-malignant lesions and cervical cancer samples of patients and the effects of FASN inhibition with Orlistat. However, orlistat has a poor water solubility and oral bioavailability, and therefore Poly(ε-caprolactone) nanocapsules containing ORL were developed and their antiproliferative effect in the cervical cancer cell line HeLa was evaluated. The expression of FASN was observed in all cervical cancer cell lines and patients samples with increased expression on more advanced stages. The treatment of cervical cancer cells with orlistat resulted is a decreased cell viability in a time-dependent manner and triggers apoptosis. Orlistat treatment also showed cell cycle arrest and autophagy in all cervical cancer cells. The nanoformulation of Orlistat showed an encapsulation efficiency of almost 100% and a higher antiproliferative effect against HeLa cells compared with the non-encapsulated drug in solution. Therefore, the results indicate that the inhibition of FASN could be proposed as a promising therapeutic strategy for cervical cancer.

Key words: Cervical Cancer, FASN, Nanocapsules, Orlistat.

SUMÁRIO

1.INTRODUÇÃO

O câncer é uma das principais causas de morbidade e mortalidade que atinge a população mundial, com aproximadamente 18,1 milhões de novos casos e 9,6 milhões de mortes em 2018 (BRAY *et al.*, 2018). Atualmente, está bem estabelecido que fatores genéticos, ambientais e de estilo de vida podem contribuir para o aparecimento do câncer. No entanto, estudos visando o melhor entendimento da biologia celular do câncer e como esse processo de transformação celular ocorre, tornam-se cada vez mais necessários e podem contribuir para a descoberta de novos alvos terapêuticos e tratamentos mais eficazes.

Entre os tipos de câncer que mais afetam as mulheres esta o câncer de colo de útero, com aproximadamente 570 mil novos casos e 311 mil mortes por ano no mundo. É a quarta neoplasia que mais afeta as mulheres e sua incidência é maior nos países menos desenvolvidos (BRAY *et al.*, 2018). Estima-se que cerca de 1,4 milhões de mulheres vivam com câncer cervical no mundo (MAHMOODI *et al.*, 2018). No Brasil, o câncer de colo de útero é o terceiro tumor mais frequente na população feminina, com uma estimativa de 16.370 novos casos em 2018 (INCA, 2017).

A descoberta de que alterações metabólicas poderiam estar envolvidas no desenvolvimento de neoplasias malignas revelou a importância da síntese *de novo* de ácidos graxos para a produção de lipídios. Os lipídios podem ser utilizados na construção da membrana plasmática, no fornecimento de energia para processos vitais, na sinalização e estimulação de fatores de transcrição. No tecido normal os lipídios são obtidos principalmente da alimentação diária, e a produção endógena de ácidos graxos encontra-se em níveis baixos (WEISS *et al.*, 1986). Por outro lado, as células neoplásicas são capazes de sintetizar endogenamente os ácidos graxos que necessitam (MEDES *et al.*, 1953; SWINNEN *et al.*, 2003). Além disso, estudos tem demonstrado que diferentes tumores apresentam níveis elevados da enzima ácido graxo-sintase (FASN), principal enzima envolvida na síntese *de novo* de ácidos graxos (MENENDEZ e LUPU, 2007). A alta expressão da FASN, também tem sido ligada a um pior prognóstico e resistência a quimioterapia (WU *et al.*, 2014). Estudos utilizando inibidores, mostraram que a inibição da FASN levou a uma diminuição na proliferação celular, com posterior morte por apoptose (PIZER *et al.*, 1998; LI *et al.*, 2001; ZHOU *et al.*, 2003). Apesar da superexpressão da FASN estar bem estabelecida para alguns tipos de neoplasias, nenhum estudo foi publicado até o momento correlacionado a FASN e o câncer de colo de útero.

O fármaco orlistate (Xenical®) foi originalmente desenvolvido para o tratamento da obesidade. No entanto, este composto mostrou ser um inibidor irreversível de FASN (KRIDEL *et al.*, 2004). O orlistate apresenta alguns problemas para sua utilização como medicamento antitumoral, pois é extremamente insolúvel em água, apresenta baixa permeabilidade intestinal e possui baixa biodisponibilidade oral (LUPU e MENENDEZ, 2006). Apesar disso, muitos estudos têm demonstrado que o orlistate tem poder de inibir a FASN diminuindo a proliferação e causando morte celular programada em diferentes linhagens tumorais (CARVALHO *et al.*, 2008; SEGUIN *et al.*, 2012; AGOSTINI *et al.*, 2014; MULLEN e YET, 2015; SOKOLOWSKA *et al.*, 2017; SCHCOLNIK-CABRERA *et al.*, 2018).

Diante da falta de estudo que caracterizem a expressão da FASN no câncer de colo de útero e os problemas que o fármaco orlistate apresenta para sua utilização como medicamento antitumoral, este trabalho terá como principais objetivos estudar o papel da FASN no desenvolvimento do câncer de colo de útero e as consequências de sua inibição com o fármaco orlistate, caracterizar a FASN como um possível alvo terapêutico para o câncer de colo de útero e desenvolver uma nanoformulação de orlistate que permita melhorar suas propriedades de biodisponibilidade e internalização celular.

2.ESTADO DA ARTE

2.1.Câncer de Colo de Útero

O câncer de colo de útero representa um grande problema de saúde pública, sendo a quarta neoplasia que mais afeta as mulheres no mundo (BRAY *et al.*, 2018) e a terceira no Brasil (INCA, 2017). Atualmente, já está bem estabelecido que o principal fator de risco para o desenvolvimento do câncer do colo do útero é a infecção pelo Papilomavírus Humano (HPV) (WALBOOMERS *et al.*, 1999). O HPV infecta células normais da camada basal da cérvice uterina, levando a uma transformação progressiva das células, que pode evoluir para lesões precursoras do câncer de colo de útero, as quais, se não diagnosticadas e tratadas, podem favorecer a carcinogênese (OSTOR, 1993). A maioria das mulheres sexualmente ativas, em algum momento de suas vidas, são infectadas pelo HPV; no entanto, 90% das infecções regridem espontaneamente em um ou dois anos (WHO, 2019).

Até o momento mais de 170 tipos de HPV foram identificados (DE VILLIERS *et al.*, 2004). Entretanto, somente cerca de 18 deles são considerados de alto risco e estão associados ao câncer de colo de útero. Dentre estes, podemos destacar os genótipos de HPV 16 e HPV 18 como sendo os de maior risco oncogênico e responsáveis por aproximadamente 70% dos casos de câncer de colo de útero (MUNOZ *et al.*, 2003; SMITH *et al.*, 2007).

O HPV possui em seu genoma 8 genes que codificam proteínas importantes para sua replicação (BAKER *et al.*, 1991), sendo os genes E6 e E7 os mais associados à transformação celular e consequente desenvolvimento do câncer (GHITTONI *et al.*, 2015). Em infecções persistentes, o HPV pode integrar seu genoma ao da célula hospedeira, levando à transcrição dos genes das oncoproteínas E6 e E7. A oncoproteína E6 causa a degradação e inativação da proteína p53, inibindo a apoptose (WHITE *et al.*, 2012). Já a oncoproteína E7 se liga à proteína do retinoblastoma (pRb), a qual é responsável por regular a progressão do ciclo celular (DARNELL *et al.*, 2007). Ao perder estes importantes mecanismos associados ao controle da proliferação, as células do epitélio uterino passam a proliferar descontroladamente acumulando mutações que contribuem para a carcinogênese (ZUR HAUSEN, 2002).

As alterações celulares que levam ao câncer de colo de útero podem contribuir para o diagnóstico precoce, permitindo que o tratamento seja iniciado antes do surgimento de um câncer invasor. O principal teste utilizado no rastreamento é o

exame citológico de Papanicolau. Este exame permite visualizar modificações celulares e classificar as mesmas. A classificação mais atual utiliza o Sistema de Bethesda, no qual as alterações citológicas do epitélio escamoso são categorizadas em lesão intraepitelial de baixo grau (*low grade intraepithelial lesion* - LSIL), lesão intraepitelial de alto grau (*high grade intraepithelial lesion* - HSIL), atipias celulares de significado indeterminado, como células escamosas atípicas de significado indeterminado (ASC-US) e células escamosas atípicas que não permitem excluir uma lesão de alto grau (ASC-H) e carcinoma invasor. Por sua vez, as lesões do epitélio glandular são classificadas como células glandulares atípicas de significado indeterminado (AGC), adenocarcinoma endocervical *in situ* (AIS) e adenocarcinoma invasor (BETHESDA, 2015). Com base nos resultados encontrados no Papanicolau, a paciente pode ser encaminhada para a realização de exame histopatológico. Neste caso, as lesões podem ser divididas em neoplasia intraepitelial cervical (NIC) de grau I, II ou III e carcinoma invasor (RICHART, 1972). A correta identificação das alterações é importante para que a conduta do tratamento seja a mais adequada.

O tratamento do câncer de colo de útero, por sua vez, é baseado no estadiamento da doença e sua classificação é realizada através do sistema FIGO (*International Federation of Gynecology and Obstetrics*). Para pacientes em estágio I, no qual a neoplasia ainda está restrita ao órgão, geralmente é realizada cirurgia, que na maioria dos casos é curativa. Já para pacientes em estágio II, no qual há crescimento extra-uterino sem disseminação, e III, no qual há crescimento com disseminação para as paredes da pelve ou vagina, o tratamento de escolha é a quimioterapia e a radioterapia, com taxas de sucesso menores que para pacientes em estágio I. Para pacientes em estágio IV, no qual ocorre a invasão da bexiga e/ou reto, o tratamento usualmente é paliativo e envolve quimioterapia, radioterapia e cirurgia (DENNY *et al.*, 2015; MARTH *et al.*, 2018). Apesar dos inúmeros benefícios, estas opções de tratamentos geralmente causam muitos efeitos adversos trazendo desconforto à paciente. Desta forma, estudos que possibilitem identificar novos alvos terapêuticos e que possam contribuir para o tratamento são ainda necessários.

2.2.Metabolismo Lipídico no Câncer

A proliferação descontrolada e acelerada de células anormais observada em neoplasias requer uma reprogramação metabólica para suprir as necessidades

energéticas utilizadas na divisão celular. Otto Warburg foi o primeiro autor a sugerir uma associação entre alterações metabólicas e o desenvolvimento de neoplasias malignas (WARBURG, 1956). Ele verificou que células neoplásicas eram capazes de obter energia através da glicólise, mesmo na presença de O_2. Esta observação foi chamada de glicólise aeróbica ou efeito de Warburg e durante muitos anos foi aceita como uma das principais alterações metabólicas necessárias para o desenvolvimento do câncer.

Entretanto, apesar do metabolismo aumentado da glicose ter sido inicialmente descrito como fator fundamental para a formação de neoplasias, muitos estudos têm demonstrado alterações em outras vias metabólicas e na expressão de diferentes enzimas, que por sua vez também contribuem para o fornecimento de energia e para a produção de macromoléculas importantes na divisão celular. Um exemplo é a síntese *de novo* de ácidos graxos que é fundamental para a produção de lipídios (SWINNEN *et al.*, 2003).

Os lipídios são essenciais para a divisão celular, pois além de serem utilizados na construção da membrana plasmática e fornecerem energia para processos vitais, sinalizam e estimulam fatores de transcrição (SANTOS e SCHULZE, 2012). No tecido normal, os lipídios são obtidos principalmente da alimentação diária, sendo que a produção endógena de ácidos graxos está restrita principalmente ao tecido adiposo, fígado e glândulas mamárias lactantes (WEISS *et al.*, 1986). Por outro lado, as células neoplásicas são capazes de sintetizar endogenamente os ácidos graxos, uma vez que a superexpressão de proteínas envolvidas na síntese de ácidos graxos tem sido encontrada em diversas neoplasias malignas (MEDES *et al.*, 1953; SWINNEN *et al.*, 2003).

A síntese *de novo* de ácidos graxos ocorre no citoplasma das células, onde unidades de dois carbonos são agrupadas a partir de uma molécula de acetil-CoA e uma molécula de malonil-CoA. A sequência de reações começa quando a glicose é convertida em acetil-CoA pela glicólise e em citrato na mitocôndria. O citrato, por sua vez, é transportado para o citoplasma e convertido novamente a acetil-CoA pela ATP citrato liase (ACLY). O acetil-CoA é então carboxilado a malonil-CoA pela acetil-CoA carboxilase (ACACA), possibilitando assim, que a ácido graxo-sintase (FASN), na presença de NADPH possa condensar o malonil-CoA com o acetil-CoA para produzir o palmitato, um ácido graxo de 16 carbonos que posteriormente pode ser

transformado em outros ácidos graxos de diferentes tamanhos e níveis de saturação (Figura 1) (COSTELLO e FRANKLIN, 2005).

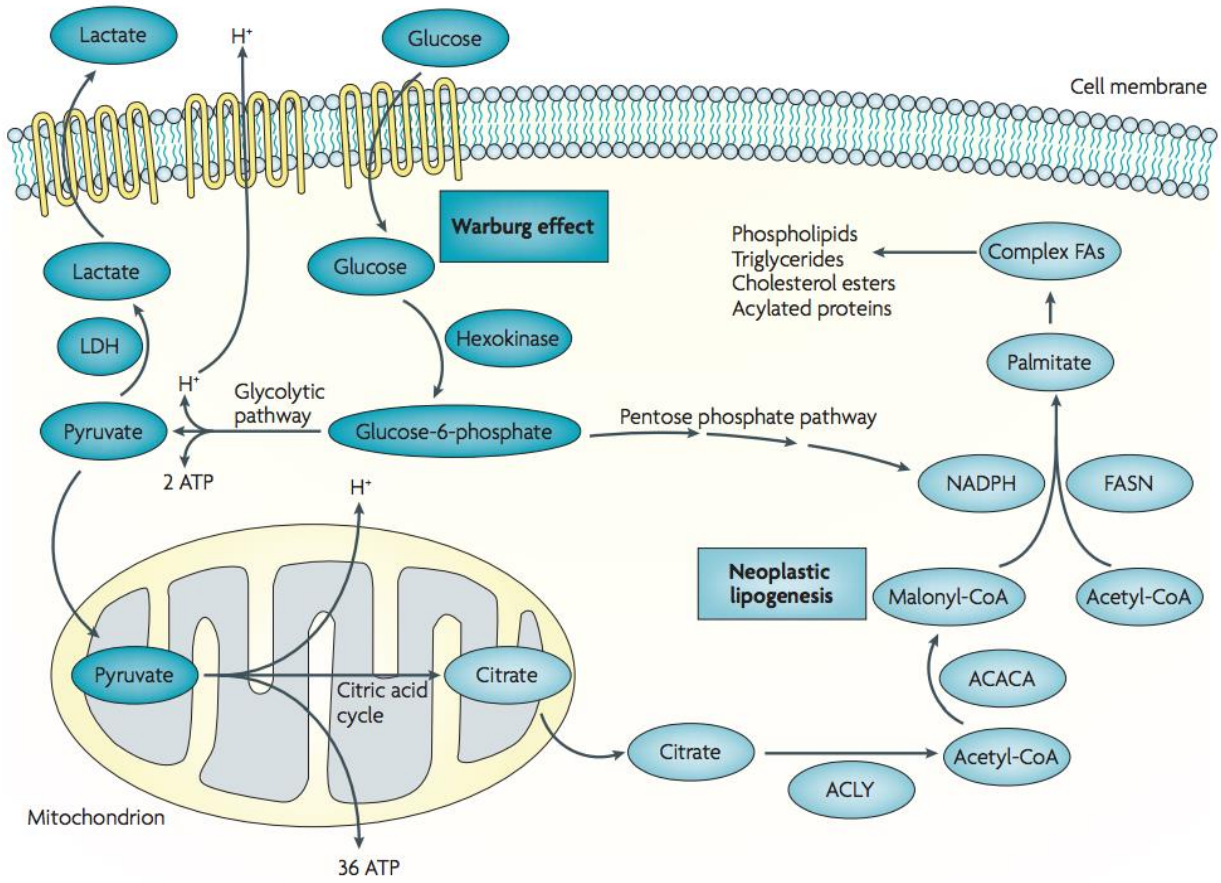

Figura 1. Conexão entre o metabolismo da glicose e síntese de ácidos graxos em células malignas. (MENENDEZ e LUPU, 2007). Cópia autorizada por Nature Reviews, número de licença 4482180193868.

Evidências de que a síntese *de novo* de ácidos graxos está ativada em células neoplásicas foi observada primeiramente com a utilização de C_{14}-glicose. Neste estudo, a maior parte dos ácidos graxos esterificados encontrados no tumor foram produzidos através da síntese *de novo* de ácidos graxos, mesmo na presença de lipídios circulantes (MEDES *et al.*, 1953). Posteriormente a este trabalho, vários estudos demonstraram elevada expressão das enzimas envolvidas na síntese em diferentes tipos de câncer.

A enzima ATP-citrato liase (ACLY), por exemplo, encontra-se superexpressa em células tumorais e sua inibição *in vivo* causou uma diminuição na proliferação tumoral (HATZIVASSILIOU *et al.*, 2005). Outra enzima importante desta via, acetil-CoA carboxilase (ACACA), também mostrou ser importante para o desenvolvimento

neoplásico ao estar expressa em elevados níveis em células de câncer de mama (CHAJES *et al.*, 2006). Posteriormente a estes trabalhos, outros estudos demonstraram elevada expressão da ácido graxo-sintase (FASN) em diferentes tipos de câncer (LITTLE e KRIDEL, 2008; SCHCOLNIK-CABRERA *et al.*, 2018).

Tendo em vista, a importância da produção de ácidos graxos endógenos para as células tumorais, o bloqueio da síntese de ácidos graxos se torna um mecanismo pelo qual podemos impedir o desenvolvimento tumoral. Como visto anteriormente, são necessárias várias etapas para que a produção de ácidos graxos ocorra. Enzimas como a citrato liase (ACLY), acetil-CoA carboxilase (ACACA) e ácido graxo-sintase (FASN) são alvos terapêuticos em potencial (STOIBER *et al.*, 2018). Além disso, muitas dessas enzimas encontram-se em níveis diminuídos em células não tumorais e sua associação com o desenvolvimento do câncer de colo de útero e seus potenciais tratamentos ainda não está descrita na literatura.

2.3.Ácido Graxo-Sintase (FASN)

A proteína FASN é a principal enzima responsável pela síntese *de novo* de ácidos graxos através da condensação do malonil-CoA com o acetil-CoA. Ela possui um papel importante na homeostase energética, pois converte o excesso de carbono ingerido em ácidos graxos que, por sua vez, são armazenados e utilizados quando necessários para produzir energia através da β-oxidação. Além disso, ela contribui para a produção de lipídios de membrana e é importante para a produção do leite materno e substância surfactante dos alvéolos pulmonares (CHIRALA e WAKIL, 2004).

Existem dois tipos de FASN, FASN I (encontrada em células eucarióticas) e FASN II (encontrada em bactérias), e ambas têm a capacidade de produzir ácidos graxos. A FASN I (referida neste trabalho apenas como FASN), encontrada em mamíferos, consiste em duas cadeias polipeptídicas idênticas de 270 kD, onde cada uma possui 6 sítios catalíticos: β-cetoacil-ACP-sintase (KS), malonil/acetil-CoA-ACP-transferase (MAT), β-hidroxiacil-ACP-desidratase (DH), enoil-ACP-redutase (ER), β-cetoacil-ACP-redutase (KR), tioesterase (TE) e um domínio transportador (proteína carreadora de acila - ACP) necessários para sua atividade enzimática (Figura 2) (MAIER *et al.*, 2006; MAIER *et al.*, 2008; MAIER *et al.*, 2010).

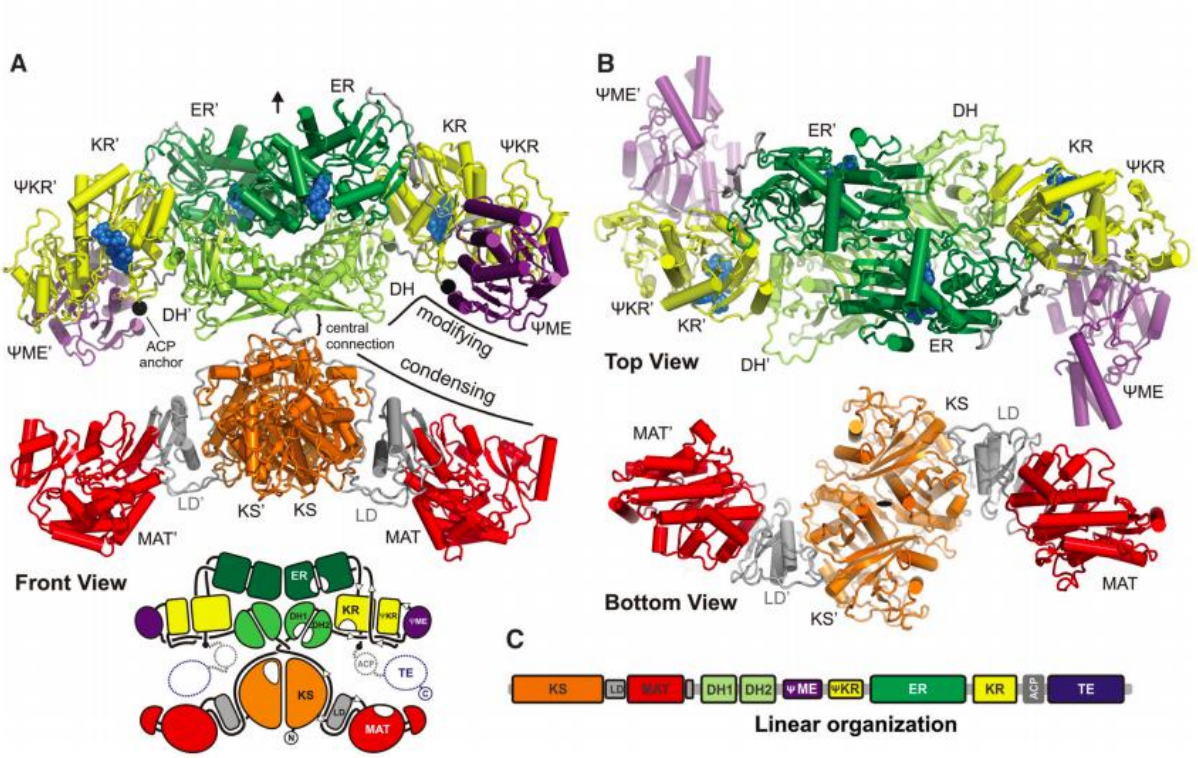

Figura 2. Desenho esquemático da enzima FASN em sua estrutura homodimérica. (A-B) Visão geral da estrutura. (C) Representação linear da organização estrutural. Domínios catalíticos: KS= β-cetoacil-ACP-sintase; MAT= malonil/acetil-CoA-ACP-transferase; DH= β-hidroxiacil-ACP-desidratase; ER= enoil-ACP-redutase; KR= β-cetoacil-ACP-redutase; TE= tioesterase; ACP= proteína carregadora de acila (esferas pretas). Cofatores NADP+ são demonstrados como esferas azuis (MAIER *et al.*, 2008). Cópia autorizada por Science, número de licença 451767448144.

Todos os sítios catalíticos participam na formação do palmitato, onde inicialmente a porção MAT transfere um grupo acetil oriundo do acetil-CoA para a porção ACP, que por sua vez transfere o grupo acetil para o sitio KS. Cada grupo malonil também é transferido para o sitio ACP pela porção MAT. Em seguida, o sítio KS realiza a condensação do malonil-ACP com o acetil-KS através de uma descarboxilação do grupo malonil. O resultado desta reação forma o acetoacetil-ACP, que é reduzido a butiril-ACP pelos sítios catalíticos DH, ER e KR. O butiril-ACP é então transferido para o sitio KS onde sofre uma nova condensação com outro grupo malonil. Cada molécula de malonil fornece dois carbonos para a reação e este ciclo de condensação e redução se repete até a formação do palmitato que é então liberado pela porção TE (LITTLE e KRIDEL, 2008).

Devido à dieta rica em ácidos graxos, a síntese *de novo* e a atividade da FASN encontram-se em níveis baixos na maioria dos tecidos de mamíferos, com exceção do fígado, tecido adiposo e glândulas mamárias de lactantes onde a lipogênese endógena é um importante processo (WEISS *et al.*, 1986; JAYAKUMAR *et al.*, 1995). Entretanto, estudos tem demonstrado que muitos tumores apresentam níveis elevados de FASN e que seus inibidores possuem atividade antitumoral promissora (MENENDEZ e LUPU, 2007; SCHCOLNIK-CABRERA *et al.*, 2018).

Além disso, estudos tem ligado a FASN com a replicação viral e entrada do vírus na célula hospedeira. Yang e colaboradores, foram os primeiros a demonstrar que a expressão da FASN estava aumentada na infecção pelo vírus da hepatite C e que sua presença facilitaria a replicação viral (YANG *et al.*, 2008). Outro estudo realizado com pacientes infectados pelo HIV, observou que a concentração de FASN no plasma desses pacientes era significativamente maior quando comparado com pacientes não infectados (ARAGONES *et al.*, 2010). Ainda, estudos posteriores relacionaram a FASN à infecção pelo vírus da Dengue, Hepatite B, pelo vírus sincicial respiratório e Chicungunha (HEATON *et al.*, 2010; ZHANG *et al.*, 2013; OHOL *et al.*, 2015; ZHANG *et al.*, 2018). No entanto, nenhum estudo foi publicado correlacionando a expressão da FASN e a infecção pelo HPV.

2.4.FASN e o câncer

De todas as enzimas envolvidas na síntese *de novo* de ácidos graxos, a FASN mostrou ser o melhor alvo terapêutico por sua distribuição, baixa expressão em células normais e importância para a sobrevivência e proliferação das células tumorais. Ainda, uma série de estudos tem demonstrado que sua expressão se encontra elevada em diferentes tipos tumorais como câncer de mama, próstata, bexiga, ovário, reto, esôfago, estomago, pulmão, tireóide, cabeça e pescoço, boca e endométrio (KUHAJDA, 2000; MENENDEZ e LUPU, 2007) e está associada ao prognóstico na maioria destas neoplasias malignas. No entanto, apesar de sua superexpressão estar bem estabelecida em células tumorais, os mecanismos pelos quais isso ocorre ainda não foram totalmente esclarecidos.

Diferentes estudos têm associado a expressão de FASN com a via de proliferação PI3K/AKT. Nesta cascata de sinalização, receptores de fatores de crescimento ao serem ativados, levariam à consequente ativação da cascata

PI3K/AKT. A AKT ativada, por sua vez, estimularia a síntese de fatores de transcrição, como por exemplo o SRBP1c, que então causaria um aumento na transcrição da FASN. Essa teoria tem como suporte o fato de que ao se inibir a cascata da PI3K/AKT, uma diminuição da expressão de FASN e de seu fator de transcrição também foram observadas (VAN DE SANDE *et al.*, 2002).

A necessidade de produção endógena de FASN tem sido atribuída ainda à manutenção dos níveis lipídicos necessários para os processos vitais de células altamente proliferativas como a palmitoilação de proteínas, restauração do potencial de oxidação através do consumo de NADPH em condições de hipóxia, além da regulação dos “lipid rafts”, estruturas da membrana plasmática que regulam a sinalização celular, tráfico intracelular de moléculas, polarização, migração e invasão de células tumorais (SWINNEN *et al.*, 2003; COLEMAN *et al.*, 2009).

A alta expressão de FASN também tem sido ligada a um pior prognóstico e resistência a quimioterapia (WU *et al.*, 2014; SHEN *et al.*, 2018). Estudos utilizando inibidores mostraram que sua inibição levou a uma diminuição na proliferação celular com posterior morte por apoptose (PIZER *et al.*, 1998; LI *et al.*, 2001; ZHOU *et al.*, 2003; SOKOLOWSKA *et al.*, 2017; PAPAEVANGELOU *et al.*, 2018). Ainda, ao se inibir a FASN, pode-se observar uma parada no ciclo celular e aumento na sobrevida de modelos xenográficos (FLAVIN *et al.*, 2010).

2.5.Inibidores da FASN

Muitos estudos têm demonstrado que a FASN pode se tornar um alvo terapêutico de grande relevância (STOIBER *et al.*, 2018). Atualmente, são conhecidas diversas substâncias com potencial para inibir a sua atividade, entre elas as mais conhecidas são: cerulenina, C75, orlistate e C93 (FLAVIN *et al.*, 2010; MULLEN e YET, 2015). A cerulenina é um produto metabólico do fungo *Cephalosporum caerulens* que tem poder de inibir a biossíntese de esteróis e ácidos graxos (FLAVIN *et al.*, 2010). A cerulenina foi o primeiro composto a mostrar atividade inibitória da FASN ligando-se ao domínio KS, e assim, impedindo a condensação entre o ácido graxo alongado e resíduos de acetil e malonil (MULLEN e YET, 2015). Este composto mostrou ter atividade antitumoral em células de câncer de mama, melanoma, câncer ocular e ovariano (PIZER *et al.*, 1996). No entanto, a cerulenina apresenta instabilidade química devido a um grupo epóxi altamente reativo e consequentemente

efeitos tóxicos que impedem sua utilização em modelos animais (LUPU e MENENDEZ, 2006).

Na tentativa de diminuir os efeitos adversos e melhorar a estabilidade da cerulenina, o composto C75 foi sintetizado (KUHAJDA *et al.*, 2000). Ele apresenta efeito inibitório da FASN ao se ligar aos domínios KS, ER e TE (RENDINA e CHENG, 2005). Assim como a cerulenina, apresenta atividade antitumoral em células de câncer de mama sendo o seu uso a primeira evidência *in vivo* da diminuição da progressão tumoral devido ao bloqueio da FASN. Apesar dos esforços em diminuir os efeitos adversos, o composto C75 promoveu perda de peso e anorexia em estudos *in vivo* (KUHAJDA *et al.*, 2000). Outro inibidor recentemente sintetizado foi a molécula C93 (ou FAS93). Este composto mostrou atividade antitumoral em modelos animais para câncer de pulmão de não-pequenas células e ovário, destacando-se não ter causado anorexia ou perda de peso nos animais (ORITA *et al.*, 2007).

O fármaco Orlistate (Xenical®) foi originalmente desenvolvido para o tratamento da obesidade devido a sua capacidade de inibir lipases pancreáticas no trato gastrointestinal e com isso evitar a absorção de gordura proveniente da alimentação. No entanto, este composto mostrou ser um inibidor irreversível de FASN ao se ligar ao domínio tioesterase (TE), o qual é responsável por terminar a síntese do palmitato (KRIDEL *et al.*, 2004). O Orlistate apresenta alguns problemas para sua utilização como droga antitumoral, pois é extremamente insolúvel em água, apresenta baixa permeabilidade intestinal e possui baixa biodisponibilidade oral (LUPU e MENENDEZ, 2006). Apesar disso, muitos estudos têm demonstrado que o Orlistate tem poder de inibir a FASN diminuindo a proliferação e causando morte celular programada em diferentes linhagens tumorais (CARVALHO *et al.*, 2008; SEGUIN *et al.*, 2012; AGOSTINI *et al.*, 2014; MULLEN e YET, 2015; SOKOLOWSKA *et al.*, 2017; SCHCOLNIK-CABRERA *et al.*, 2018).

Assim, diante do potencial antitumoral que os inibidores da FASN apresentam, grandes indústrias farmacêuticas como Glaxo, AstraZeneca e Merck já demonstraram interesse em desenvolver moléculas mais específicas, menos tóxicas e que causem menos efeitos adversos (MULLEN e YET, 2015). No entanto, até o momento, só existe uma molécula sendo testada em humanos. A molécula TVB-2640 está em fase I dos estudos clínicos em pacientes portadores de tumores sólidos em estágios avançados. Os objetivos do estudo são determinar a dose máxima e a segurança da molécula como monoterapia ou em conjunto com outros fármacos (BRENNER *et al.*, 2015;

BRENNER *et al.*, 2017). Contudo, ainda não existem moléculas inibidoras da FASN totalmente seguras e eficazes (MENENDEZ e LUPU, 2017).

Além disso, até o momento o mecanismo pelo qual a inibição da FASN retarda o desenvolvimento de neoplasias não foi totalmente esclarecido. Alguns estudos sugerem que ao inibir a FASN ocorra uma diminuição na disponibilidade de fosfatidilcolina, um dos principais lipídios afetados pela modulação da FASN, causando a escassez de fosfolipídios necessários para sintetizar a membrana celular de novas células (LUPU e MENENDEZ, 2006). Também, foi proposto que a inibição da FASN levaria a um a acúmulo de malonil-CoA, com consequente inibição da carnitina-palmitoil transferase I, que por sua vez levaria ao acúmulo de ceramida e indução dos genes pró-apoptóticos BNIP3, TRAIL e DAPK2 (PIZER *et al.*, 2000; BANDYOPADHYAY *et al.*, 2006). Além disso, uma grande quantidade de fosfolipídios é necessária durante a fase G1 e S do ciclo celular e consequentemente, o bloqueio da FASN levaria à alteração da replicação do DNA, causando assim uma parada no ciclo celular antes da fase G1 (ZHOU *et al.*, 2003).

2.6.Nanoformulações de Orlistate

Atualmente, a nanotecnologia também tem sido associada aos inibidores da FASN para melhorar suas propriedades, atraindo a atenção dos pesquisadores. Nanopartículas apresentam vantagens em relação ao fármaco livre. Elas possibilitam melhor a biodisponibilidade e internalização celular dos fármacos, controlar sua liberação, direcionar o fármaco para um alvo específico, e ainda, diminuir a dose terapêutica e o número de administrações pelo paciente (PEER *et al.*, 2007; GROSSEN *et al.*, 2017). Existem diversos tipos de nanopartículas que podem ser empregadas no desenvolvimento de formulações farmacêuticas, sendo as mais conhecidas e utilizadas: os lipossomas, as nanoemulsões, nanopartículas poliméricas, nanopartículas lipídicas sólidas, os vetores lipídicos nanoestruturados e as nanopartículas magnéticas (PANYAM e LABHASETWAR, 2003). Diferentes estudos têm sido publicados, demonstrando que a nanotecnologia pode ser uma alternativa promissora para as limitações que alguns inibidores da FASN apresentam. Hill e colaboradores verificaram que o tratamento de linhagens celulares de câncer de próstata e câncer de mama com nanopartículas derivadas de ácido hialurônico contendo Orlistate, levou a uma diminuição significativa na viabilidade celular em

comparação ao tratamento com Orlistate livre (HILL *et al.*, 2016). Outro estudo com nanopartículas de PLGA-b-PEG contendo Orlistate obteve resultados semelhantes em linhagens de câncer de mama triplo negativo (BHARGAVA-SHAH *et al.*, 2016). Além disso, estudos em células tumorais resistentes demostraram a possibilidade de utilizar nanopartículas de Orlistate em sinergismo com outros compostos para melhorar o tratamento do câncer (SOUCHEK *et al.*, 2017).

Desse modo, a possibilidade de utilizar nanotecnologia para melhorar as propriedades dos inibidores já existentes e aprovados para uso humano pelo FDA e outras agencias regulatórias, se torna uma opção bastante atrativa para pesquisadores e empresas farmacêuticas. Os sistemas nanoestruturados permitem direcionar e controlar a liberação dos fármacos, e buscam ainda, eliminar características indesejadas presentes nas apresentações convencionais.

3.OBJETIVOS

Este trabalho tem como objetivo geral a investigação da expressão da FASN e sua relação com o carcinogênese do câncer de colo de útero, propondo uma formulação tecnológica como estratégia terapêutica para o tratamento de câncer de colo de útero.

Assim, os seguintes objetivos específicos foram propostos:

- Avaliar a expressão da FASN em biópsias de lesões precursoras e de câncer de colo de útero, para correlacionar sua expressão com os diferentes estágios do desenvolvimento do câncer;
- Determinar e comparar a expressão da FASN nas linhagens de câncer de colo de útero HeLa, SiHa, C-33A e ME-180;
- Estudar e comparar o efeito do Orlistate na viabilidade e proliferação das linhagens HeLa, SiHa, C-33A e ME-180;
- Avaliar e comparar os efeitos da inibição da FASN após tratamento com Orlistate na indução da parada do ciclo celular, morte por apoptose e autofagia nas linhagens HeLa, SiHa, C-33A e ME-180;
- Desenvolver uma nanoformulação de Orlistate e testar sua citotoxicidade *in vitro* em linhagem de câncer de colo de útero (linhagem HeLa).

4.RESULTADOS

Os resultados dessa dissertação serão apresentados na forma de artigos científicos.

4.1. Artigo 1

Jéssica Nascimento; Camila Mariot; Débora Renz Barreto Vianna; Lúcia Maria Kliemann; Paula dos Santos Chaves; Massimo Loda; Andréia Buffon; Ruy Carlos Ruver Beck; Diogo André Pilger. *Fatty Acid Synthase on cervical cancer: a new therapeutic target?*

Este artigo foi elaborado de acordo com as normas da revista “Molecular Carcinogenisis”.

Fatty Acid Synthase on cervical cancer: a new therapeutic target?

[1]Jéssica Nascimento; [2]Camila Mariot; [1]Débora Renz Barreto Vianna; [3]Lúcia Maria Kliemann; [1]Paula dos Santos Chaves; [4,5]Massimo Loda; [1]Andréia Buffon; [1]Ruy Carlos Ruver Beck; [1*]Diogo André Pilger.

[1]Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
[2]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
[3]Departamento de Patologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul and Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.
[4]Department of Pathology and Laboratory Medicine, Weil Cornell Medicine, New York Presbyterian-Weill Cornell Campus and New York Genome Center Affiliate Member, New York, NY, USA.
[5]The Broad Institute of MIT and Harvard University, Cambridge, MA, USA.

*Corresponding Author: Diogo André Pilger

Laboratory of Biochemical and Cytological Analysis, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Av. Ipiranga 2752, Bairro Santana

CEP 90610-000, Porto Alegre, RS, Brazil.

Phone: + 55 51 33082102

E-mail: diogo.pilger@ufrgs.br

Acknowledgements

This work was supported by grants from Programa de Pós-Graduação em Ciências Farmacêuticas (PPGCF/UFRGS), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), and Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE/HCPA).

Abbreviations:

ANOVA: one-way analysis of variance
AO: acridine orange
DMEM: Dulbeccos's Modified Eagle`s Medium
EDTA: ethylenediaminetetraacetic acid
EMT: epithelial-mesenchymal transition
FASN: fatty acid synthase
HIV: Human Immunodeficiency Virus
HPV: Human Papillomavirus
HSIL: high-grade squamous intraepithelial lesions
LSIL: low-grade squamous intraepithelial lesions
NADPH: nicotinamide adenine dinucleotide phosphate
ORL: orlistat
PBS: phosphate-buffered saline
PE: plating efficiency
RPMI: Roswell Park Memorial Institute

Abbreviated title: FASN on cervical cancer: a new therapeutic target?

Key words: FASN, cervical cancer, orlistat

Abstract

Fatty acid synthase (FASN) is a rate-limiting enzyme responsible for *de novo* synthesis of fatty acids in the cytoplasm of tumor cells. Many tumors express high levels of FASN and its expression is associated with worse prognosis. Orlistat is a drug originally developed for the treatment of obesity. However, its antitumor properties have been shown as a tight-binding irreversible inhibitor of the FASN thioesterase domain. Cervical cancer represents a major public health problem and is the fourth most common cancer type that affects women worldwide. To date, only a few *in silico* studies correlated FASN expression with cervical cancer and the possibility exists of using it as a new therapeutic target. Therefore, in this study FASN expression in pre-malignant lesions and cervical cancer samples of patients was analyzed. In addition, the effects of FASN inhibition with Orlistat in different cervical cancer cell lines were evaluated. The FASN expression was observed in all patients' samples, with increased expression in more advanced stages. Treatment of cervical cancer cells with orlistat decreased cell viability in a time-dependent manner and triggered apoptosis. Orlistat treatment also showed cell cycle arrest and autophagy in all cervical cancer cells. Taken together, the results suggest that FASN is a potential therapeutic target for cervical cancer.

INTRODUCTION

Cervical cancer represents a major public health problem. It is the fourth most common cancer type that affects women worldwide[1]. The main risk factor for the development of cervical cancer is the persistent infection with high-risk human papillomavirus (HPV)[2]. The HPV infects cervical cells leading to cell transformation, which can progress to precursor lesions of cervical cancer[3]. Despite this, the presence of HPV infection alone is not sufficient to trigger the carcinogenesis and additional alterations are necessary[4].

Fatty Acid Synthase (FASN) is the main enzyme responsible for the *de novo* synthesis of fatty acids through the condensation of malonyl-CoA with acetyl-CoA and formation of palmitate, a 16-carbon fatty acid[5,6]. The *de novo* synthesis plays an important role in energy homeostasis because it converts excessive ingested carbon into fatty acids, which are stored and used when needed to produce energy through oxidation. In addition, it contributes to the production of membrane lipids that are essential for cell division and cell membrane formation. Because of availability of fatty acids from the diet, *de novo* synthesis and FASN activity are low in most normal tissues, with the exception of liver, adipose tissue, and lactating mammary glands[7,8]. On the other hand, studies have shown that many tumors have high levels of FASN and its expression is associated with worse prognosis[9].

Studies using FASN inhibitors have shown that their inhibition led to a decrease in cell proliferation, with subsequent death by apoptosis[10]. Also, when FASN is inhibited, cell cycle arrest occurs in vitro and tumor growth is inhibited in xenograph models[11]. There are many inhibitors for FASN activity and recently, a few have entered clinical trials with promising results[12,13]. The drug Orlistat (ORL) was originally developed for the obesity treatment. However, it has been shown to be an irreversible inhibitor of FASN with an important antiproliferative effect in different types of tumors[14,15].

Although overexpression of FASN is well established for some types of cancer, FASN expression in cervical cancer has not been studied. There are only a few studies of screening *in silico* showing that FASN may be a marker for cervical cancer[16,17].

The aim of this study was to evaluate FASN expression in Low- and High-grade squamous intraepithelial lesions and in cervical cancer patients' samples. In addition, the consequences of FASN inhibition using ORL *in vitro* were evaluated using different cervical cancer cell lines.

MATERIAL AND METHODS

Samples

The samples were obtained from 36 patients submitted to gynecological procedures at a reference hospital in the south of Brazil. The tissues were paraffin embedded and cut at a thickness of 4 µm. The sections were mounted on microscope slides. The sections were then fixed in 4 % formalin (pH 7.0) and stained using hematoxylin and eosin to determine the pathological type and grade according to the Bethesda System for Reporting Cervical Cytology[18] in four groups: 9 samples of cervicitis (classified as control), 9 samples of LSIL (Low-grade squamous intraepithelial lesions), 9 samples of HSIL (High-grade squamous intraepithelial lesions) and 9 samples of cervical cancer. It is important to emphasize that it was not possible to obtain samples of normal tissue since patients without malignant changes in cytology are not normally submitted to the biopsy procedure. This study was approved by the local institutional ethics committee (Approval protocol number: 2115382).

Immunohistochemistry

The FASN expression was analyzed using a kit Starr Trek Universal HRP Detection System (Biocare Medical, Pacheco, USA). In brief, the sections were dewaxed with xylene, gradually hydrated with gradient alcohol series and washed with phosphate-buffered saline (PBS). Then, the sections were incubated with citrate buffer and 5% hydrogen peroxide, and with background sniper reagent from the commercial kit. After, sections were incubated overnight with primary anti-FASN antibody at a 1:200 dilution (BD Transduction Laboratories, San Jose, USA). After washes, incubations with secondary biotinylated antibody, peroxidase enzyme and revealing solution of betazoid diaminobenzidine chromogen, from the commercial kit, were performed. Tissue sections were then counterstained with hematoxylin, dehydrated and mounted. The intensity of staining was graded semi-quantitatively as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The percentage of positive cells was quantified into four groups under a microscope as the extent of immunostaining: 0 (<10% positive cells), 1 (10-25% positive cells), 2 (26-75% positive cells), and 3 (>75% positive cells). The expression levels were defined as the multiplication of the percentage of positive staining and the intensity. The sections with a final score of 1 or less were considered as (-), between 2 and 4 as (+), between 5 and 8 as (++) and 9

was considered as (+++).

Cell Culture

In this study, four different cervical cancer cell lines (C-33A, ME-180, HeLa and SiHa) were used. They were obtained from American Type Culture Collection (Rockville, USA; ATCC®HTB-31™, ATCC®HTB-33™, ATCC®CCL-2™, ATCC®HTB-35™). The cell line C-33A is derived from cervical carcinoma and it doesn't contain copies of HPV integrated in its genome. The cell lines HeLa and SiHa contain HPV 18 and 16, respectively. The HeLa is derived from a uterine adenocarcinoma and the SiHa from a cervical stage II squamous cell carcinoma. The ME-180 is derived from a metastatic site in the peritoneum, it has copies of HPV with great homology to HPV 68 and it is derived from a cervical squamous cell carcinoma. Cervical carcinoma cell lines were maintained in culture flask in low-glucose DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA), 1% penicillin/streptomycin and 0.1% amphotericin (Sigma-Aldrich, St. Louis, USA) at 37^{o}C in a 5% CO_2 atmosphere. The ME-180 cell line was maintained at the same conditions but cultured with RPMI medium (Sigma-Aldrich, St. Louis, USA).

Preparation of Orlistat solutions

For cell culture experiments, Orlistat (ORL) was extracted from Xenical® (Roche, Basel, Switzerland) capsules according to Knowles *et al*[19]. Each pill was solubilized in 1 mL of ethanol, insoluble products removed by centrifugation (12,000g for 5 min) and the supernatant (250 mM of ORL) stored at -80^{o}C. The final concentration was confirmed by high-performance liquid chromatography[20]. Analyses were performed in a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SPD-20AV detector (UV). A C_{18} column was utilized as stationary phase and the mobile phase was composed of water containing 0.1% (v/v) of phosphoric acid (85%) and acetonitrile (5:95, v/v), run at a flow rate of 1.5 mL.min^{-1}. UV detection was carried out at 205 nm. The method was specific, linear (r=0.999, n=3) in the range of 10.00-30.00 µg.mL^{-1}, and precise (SD=1.64% and 3.13%) for intra and interday precision, respectively.

Cell counting

To evaluate the effects of FASN inhibition on cell viability, the cell lines were treated

with 100, 200, 300, 400, and 500 μM of ORL and incubated at 24, 48, and 72 h. These settings of ORL concentration were determined by preliminary tests from our research group and based on previous studies with FASN inhibition with ORL[21]. Cell lines ($1x10^4$ cells/well) were seeded on 24-well plates and after the incubation, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and 200 μL of 0.25% trypsin/EDTA was added to detach the cells, which were counted using a FACSVerse flow cytometer (BD Biosciences, San Jose, USA). Results are expressed as the percentage of control.

Clonogenic survival assay

Subconfluent cultures were exposed to 300 μM of ORL for 24, 48, and 72 h. Then the surviving adherent cells were washed with PBS, trypsinized, counted, and replated in 6-well plates (500 cells/well). After 10 days of incubation in a complete culture medium, the colonies formed from each cell plated were stained with crystal violet after fixation with methanol and counted manually. Plating efficiency (PE) was evaluated and the fraction of surviving cells was calculated.

Plating Efficiency (PE) = (No. of colonies counted/No. of cells plated) × 100

Survival Fraction = (PE of treated sample/PE of control) × 100.

Annexin V and propidium iodide staining

Apoptosis and necrosis induction were analyzed by Annexin V-FITC Apoptosis Kit (QuatroG, Porto Alegre, BR) according to the manufacturer`s instructions with some modifications. Briefly, the cell lines were plated in 24-well plates ($1x10^4$ cells), followed by the treatments with 300 and 400 μM of ORL. After, cells were harvested and incubated with 150 μL of annexin binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), annexin V at 0.75 μL/sample and propidium iodide (PI) at 15 μL/sample for 15 min at room temperature in the dark, and analyzed on FACSVerse flow cytometer (BD Biosciences, San Jose, USA).

Cell cycle assay

The effects of FASN inhibition on cell cycle phase distribution were assessed using flow cytometry. Cells were plated in a 6-well plate ($6\times10_4$ cells), followed by treatments with 300 and 400 μM of ORL. After treatments, cells were harvested and fixed in cold ethanol 70% v/v in PBS for 2 h. Fixed cells were washed with PBS and marked with a solution containing PI 12 μg/L, Triton X-100 and RNAse for 30 min, in the dark, at room temperature. DNA content was analyzed using a FACSVerse flow cytometer (BD Biosciences, San Jose, USA).

Quantification of acidic vacuolar organelles by AO staining

For acidic vacuolar organelles (a typical feature of autophagy) determination, cells were plate in a 24-well plate ($1x10_4$ cells) and exposed to 200, 300, and 400 μM of ORL. After the incubation, cells were trypsinized and incubated with AO (acridine orange) (2.7 mM) for 15 min at room temperature, and fluorescence emission was analyzed by flow cytometry using FACSVerse flow cytometer (BD Biosciences, San Jose, USA).

Statistical analysis

Statistical analysis was performed with Prism 5 (GraphPad, La Jolla, CA). Data are expressed as the percentage of control and presented as mean ± SD of at least three independent experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. Values were considered significant at $p < 0.05$.

RESULTS

FASN expression on cervical tissues

To evaluate FASN expression in patient tissues the immunohistochemistry technique was performed in cervicitis tissue (non-malignant control), LSIL, HSIL, and in cervical cancer. Immunochemical staining tests showed that FASN was mainly localized in the cytoplasm of cervical cells, while the stroma exhibited no staining or focal and weak positivity (**Figure 1**). Weak staining was detected in cervicitis tissues samples. In all samples of LSIL, HSIL, and carcinoma, FASN expression was more intense than in cervicitis tissue. LSIL showed moderate expression in 6/9 (66.7%) samples. High

expression was detected in 4/9 (44%) of HSIL and in 4/9 (44%) of carcinoma (**Table 1**), with a similar expression profile between these two groups. No significant correlation between the grade of lesion and FASN expression was observed, although a clear trend towards progressive increase with increase in stage of disease was noted.

FASN inhibition affects the growth of cervical cancer cells

To evaluate whether the inhibition of FASN activity can modify the growth rate of cervical cancer cells, the HeLa, SiHa, ME-180, and C-33A cell lines were treated with increasing concentrations of ORL. As shown in **Figure 2**, the inhibition with ORL reduced the number of viable cells in all cell lines. HeLa and SiHa had the highest reduction on the number of cells when compared to C-33A and ME-180 (**Figure 2A-D**). In addition, the incubation with 300 μM of ORL for 24, 48, and 72 h, showed a reduction of viable cells in a time-dependent manner (**Figure 2E**). Orlistat reduced significantly the viability already after 24 h of incubation. Furthermore, surviving cells had reduced long-term viability, since clonogenic survival of cells that survived 72 h treatment was reduced, indicating a slow mechanism of cell death (**Figure 2F**). To better characterize the antiproliferative properties of FASN inhibition on cervical cancer cells, flow cytometry analysis was performed to evaluate the cell cycle, which showed an increase in G0-G1 population as well as a decline in S phase at 400 μM of ORL in all cell lines, in comparison with untreated cells (**Figure 3**).

FASN inhibition induces Apoptosis and Autophagy

To investigate whether the decrease in viable cells was related to apoptosis and necrosis mechanisms, flow cytometry analysis for Annexin V and propidium iodide were performed (**Figure 4**). As shown in **Figure 4**, all cervical cancer cell lines showed an increase in the number of apoptotic cells for all treatments when compared to controls. The proportion of cells in apoptosis followed the increase of the concentration, with the exception of the C-33A cell. In addition, the proportion of cells marked by propidium iodide was not significant, showing that the decrease in cell viability is not occurring due to necrosis. The acridine orange was used to assess the acidic vacuolar organelles, which characterize autophagy. As indicated in **Figure 5**, the percentage of AO-positive cells increased in all cervical cancer cells when compared to controls after treatment with ORL.

DISCUSSION

Tumor cells synthesize most fatty acids that they need through *de novo* synthesis[22,23]. In turn, fatty acids are utilized for membrane lipids, provide energy and can stimulate signaling pathways by post-translational modification of targets. The FASN, the main enzyme of *de novo* synthesis of fatty acid, is responsible for producing palmitate through the condensation of malonyl-CoA and acetyl-CoA[24]. FASN is expressed at low levels or not at all in most normal tissues. On the other hand, most tumors have high FASN expression[9]. Furthermore, studies where FASN was inhibited showed a decrease in proliferation, cell cycle arrest, and death by apoptosis[11].

In this study, cytoplasmic FASN expression was found in all four patient sample groups tested. Even the cervicitis samples, that were used as non-malignant control, showed weak staining. These results are relevant because demonstrate that FASN may have an important role in inflammation as well. Indeed, some studies have observed that fatty acid synthesis is important for macrophages activation and for signaling the immune system[25,26]. In addition, FASN expression was progressively more intense in LSIL, HSIL, and carcinoma samples, which indicates a possible role of the enzyme in cervical carcinogenesis. Even though FASN expression did not show a statistical correlation with the grade of cervical lesions, our findings are consistent with many studies that showed FASN activation early in the neoplastic process of breast, gliomas, lung, colon, and prostat[27-31]. Moreover, esophageal squamous cell dysplasia, adenoma and metaplasia of the stomach, also showed overexpression of FASN[32,33]. The FASN overexpression in early stages of cancer is relevant for the carcinogenesis because in under hypoxia conditions, large amounts of lactate are produced by aerobic glycolysis, damaging the respiratory chain. FASN, in turn, has the ability to perform a redox balance by consuming excess NADPH, favoring oxidative respiration[34]. Moreover, FASN expression has been correlated with epithelial-mesenchymal transition (EMT). Li *et al.* showed that FASN mediated the EMT in breast cancer cells[35], and Hung *et al*. reported that FASN inhibition prevents EMT process in breast cancer [36]. The EMT allows the cells to spread more quickly and in a more aggressive way by the loss of epithelial features and acquisition of mesenchymal characteristic. These advantages give cervical cancer a greater ability to proliferate and survive[37,38]. Therefore, FASN activation in pre-malignant lesions and cancer suggests that FASN may be decisive for early neoplastic transformation.

To better understand the FASN role in cervical cancer carcinogenesis, the effect

of ORL was evaluated in cervical cell lines. Orlistat, a drug originally developed for the treatment of obesity, is an inhibitor of pancreatic lipases in the gastrointestinal tract. However, it has been shown to be an irreversible inhibitor of FASN binding to the thioesterase domain, which is responsible for the palmitate synthesis termination[14]. The inhibition of FASN with ORL was able to decrease cell viability, and colony formation, and cause apoptosis and cell cycle arrest in the human cervical cancer cell lines HeLa, SiHa, C-33A, and ME-180. However, SiHa and HeLa cells, which are HPV 16-positive and HPV 18-positive, respectively, showed a greater reduction in cell proliferation when compared to the C-33A and ME-180 cells. It is important to emphasize that infection by HPV 16 and 18 is the most commonly associated factor with the development of human cervical cancer[39,40]. However, to date, there is no study correlating FASN expression and HPV infection. Despite this, different studies are showing FASN expression in viral infection as Hepatitis C, HIV, and Respiratory Syncytial Virus[41-43]. Therefore, more studies are needed to establish a relationship between FASN and HPV infection.

Furthermore, it was possible to demonstrate that the decrease in viability occurred by apoptosis and not by necrosis, since after treatment with ORL the percentage of necrotic cells was low in all cell lines. These results are in agreement with many studies reporting a decrease in cell viability and death by apoptosis after treatment with ORL[14,21,29]. The ORL was also able to induce autophagy in all cervical cancer cell lines. Although further studies are still required to confirm the autophagy, our data are in agreement with Grube *et al.* that showed that ORL induced autophagy in glioma cells[29]. Also, Peng *et al.* showed that ORL can induce apoptosis and autophagy at the same time in ovarian cancer. However, when cells were treated with an autophagy inhibitor, the number of apoptotic cells increased, suggesting that the ORL has a cellular pro-survival role but could be a potential adjuvant in cancer treatment[44].

In summary, our results showed increased FASN expression in early cervical cancer development with expression in LSIL and HSIL samples. FASN inhibition by ORL decreases cell viability and triggers apoptosis, cell cycle arrest and autophagy in different cervical cancer cell lines. Taken together, the data reported here suggest that FASN is a potential therapeutic target for cervical cancer. However, further studies are needed to understand the role of FASN on cervical cancer development.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424.
2. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999;189(1):12-19.
3. Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol.* 1993;12(2):186-192.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-674.
5. Costello LC, Franklin RB. 'Why do tumour cells glycolyse?': from glycolysis through citrate to lipogenesis. *Mol Cell Biochem.* 2005;280(1-2):1-8.
6. Jayakumar A, Tai MH, Huang WY, et al. Human fatty acid synthase: properties and molecular cloning. *Proc Natl Acad Sci U S A.* 1995;92(19):8695-8699.
7. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer.* 2007;7(10):763-777.
8. Weiss L, Hoffmann GE, Schreiber R, et al. Fatty-acid biosynthesis in man, a pathway of minor importance. Purification, optimal assay conditions, and organ distribution of fatty-acid synthase. *Biol Chem Hoppe Seyler.* 1986;367(9):905-912.
9. Kuhajda FP. Fatty acid synthase and cancer: new application of an old pathway. *Cancer Res.* 2006;66(12):5977-5980.
10. Zhou W, Simpson PJ, McFadden JM, et al. Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. *Cancer Res.* 2003;63(21):7330-7337.
11. Buckley D, Duke G, Heuer TS, et al. Fatty acid synthase - Modern tumor cell biology insights into a classical oncology target. *Pharmacol Ther.* 2017;177:23-31.
12. Dean EJ, Falchook GS, Patel MR, et al. Preliminary activity in the first in human study of the first-in-class fatty acid synthase (FASN) inhibitor, TVB-2640. *Journal of Clinical Oncology.* 2016;34(15_suppl):2512-2512.
13. Brenner A, Falchook G, Patel M, et al. Abstract P6-11-09: Heavily pre-treated breast cancer patients show promising responses in the first in human study of

the first-In-class fatty acid synthase (FASN) inhibitor, TVB-2640 in combination with paclitaxel. *Cancer Research.* 2017;77(4 Supplement):P6-11-09-P16-11-09.

14. Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. *Cancer Res.* 2004;64(6):2070-2075.
15. Schcolnik-Cabrera A, Chavez-Blanco A, Dominguez-Gomez G, et al. Orlistat as a FASN inhibitor and multitargeted agent for cancer therapy. *Expert Opin Investig Drugs.* 2018;27(5):475-489.
16. Nisthul AA, Retnakumari AP, A S, Anto RJ, Sadasivan C. In silico screening for identification of fatty acid synthase inhibitors and evaluation of their antiproliferative activity using human cancer cell lines. *J Recept Signal Transduct Res.* 2018;38(4):335-341.
17. Xia L, Wang H, Cai S, et al. Integrated Analysis of a Competing Endogenous RNA Network Revealing a Prognostic Signature for Cervical Cancer. *Front Oncol.* 2018;8:368.
18. Bethesda. *The Bethesda System for Reporting Cervical Cytology - Definitions, Criteria and Explanatory Notes.* Third ed. New York: Springer International Publishing; 2015.
19. Knowles LM, Axelrod F, Browne CD, Smith JW. A fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating Skp2. *J Biol Chem.* 2004;279(29):30540-30545.
20. Dolenc A, Govedarica B, Dreu R, Kocbek P, Srcic S, Kristl J. Nanosized particles of orlistat with enhanced in vitro dissolution rate and lipase inhibition. *Int J Pharm.* 2010;396(1-2):149-155.
21. Carvalho MA, Zecchin KG, Seguin F, et al. Fatty acid synthase inhibition with Orlistat promotes apoptosis and reduces cell growth and lymph node metastasis in a mouse melanoma model. *Int J Cancer.* 2008;123(11):2557-2565.
22. Medes G, Thomas A, Weinhouse S. Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. *Cancer Res.* 1953;13(1):27-29.
23. Swinnen JV, Van Veldhoven PP, Timmermans L, et al. Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. *Biochem Biophys Res Commun.* 2003;302(4):898-903.

24. Little JL, Kridel SJ. Fatty acid synthase activity in tumor cells. *Subcell Biochem.* 2008;49:169-194.
25. Qian X, Yang Z, Mao E, Chen E. Regulation of fatty acid synthesis in immune cells. *Scand J Immunol.* 2018;88(5):e12713.
26. Carroll RG, Zaslona Z, Galvan-Pena S, et al. An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation. *J Biol Chem.* 2018;293(15):5509-5521.
27. Alo PL, Visca P, Trombetta G, et al. Fatty acid synthase (FAS) predictive strength in poorly differentiated early breast carcinomas. *Tumori.* 1999;85(1):35-40.
28. Ogino S, Nosho K, Meyerhardt JA, et al. Cohort study of fatty acid synthase expression and patient survival in colon cancer. *J Clin Oncol.* 2008;26(35):5713-5720.
29. Grube S, Dunisch P, Freitag D, et al. Overexpression of fatty acid synthase in human gliomas correlates with the WHO tumor grade and inhibition with Orlistat reduces cell viability and triggers apoptosis. *J Neurooncol.* 2014;118(2):277-287.
30. Piyathilake CJ, Frost AR, Manne U, et al. The expression of fatty acid synthase (FASE) is an early event in the development and progression of squamous cell carcinoma of the lung. *Hum Pathol.* 2000;31(9):1068-1073.
31. Migita T, Ruiz S, Fornari A, et al. Fatty acid synthase: a metabolic enzyme and candidate oncogene in prostate cancer. *J Natl Cancer Inst.* 2009;101(7):519-532.
32. Nemoto T, Terashima S, Kogure M, et al. Overexpression of fatty acid synthase in oesophageal squamous cell dysplasia and carcinoma. *Pathobiology.* 2001;69(6):297-303.
33. Kusakabe T, Nashimoto A, Honma K, Suzuki T. Fatty acid synthase is highly expressed in carcinoma, adenoma and in regenerative epithelium and intestinal metaplasia of the stomach. *Histopathology.* 2002;40(1):71-79.
34. Hosios AM, Vander Heiden MG. The redox requirements of proliferating mammalian cells. *J Biol Chem.* 2018;293(20):7490-7498.
35. Li J, Dong L, Wei D, Wang X, Zhang S, Li H. Fatty acid synthase mediates the epithelial-mesenchymal transition of breast cancer cells. *Int J Biol Sci.* 2014;10(2):171-180.

36. Hung CM, Kuo DH, Chou CH, Su YC, Ho CT, Way TD. Osthole suppresses hepatocyte growth factor (HGF)-induced epithelial-mesenchymal transition via repression of the c-Met/Akt/mTOR pathway in human breast cancer cells. *J Agric Food Chem.* 2011;59(17):9683-9690.
37. Baron A, Migita T, Tang D, Loda M. Fatty acid synthase: a metabolic oncogene in prostate cancer? *J Cell Biochem.* 2004;91(1):47-53.
38. Qureshi R, Arora H, Rizvi MA. EMT in cervical cancer: its role in tumour progression and response to therapy. *Cancer Lett.* 2015;356(2 Pt B):321-331.
39. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 2003;348(6):518-527.
40. Smith JS, Lindsay L, Hoots B, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer.* 2007;121(3):621-632.
41. Yang W, Hood BL, Chadwick SL, et al. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. *Hepatology.* 2008;48(5):1396-1403.
42. Aragones G, Alonso-Villaverde C, Oliveras-Ferraros C, et al. Infection with HIV and HCV enhances the release of fatty acid synthase into circulation: evidence for a novel indicator of viral infection. *BMC Gastroenterol.* 2010;10:92.
43. Ohol YM, Wang Z, Kemble G, Duke G. Direct Inhibition of Cellular Fatty Acid Synthase Impairs Replication of Respiratory Syncytial Virus and Other Respiratory Viruses. *PLoS One.* 2015;10(12):e0144648.
44. Peng H, Wang Q, Qi X, Wang X, Zhao X. Orlistat induces apoptosis and protective autophagy in ovarian cancer cells: involvement of Akt-mTOR-mediated signaling pathway. *Arch Gynecol Obstet.* 2018;298(3):597-605.

Table 1. FASN expression in cervical tissue.

Levels of staining	- (%)	+ (%)	++ (%)	+++ (%)
Control (9)	4 (44.4)	4 (44.4)	1 (11.1)	0 (0.0)
LSIL (9)	0 (0.0)	3 (33.3)	6 (66.7)	0 (0.0)
HSIL (9)	0 (0.0)	2 (22.2)	3 (33.3)	4 (44.4)
Carcinoma (9)	0 (0.0)	3 (33.3)	2 (22.2)	4 (44.4)

LSIL: Low-grade Squamous Intraepithelial Lesion; HSIL: High-grade Squamous Intraepithelial Lesion.

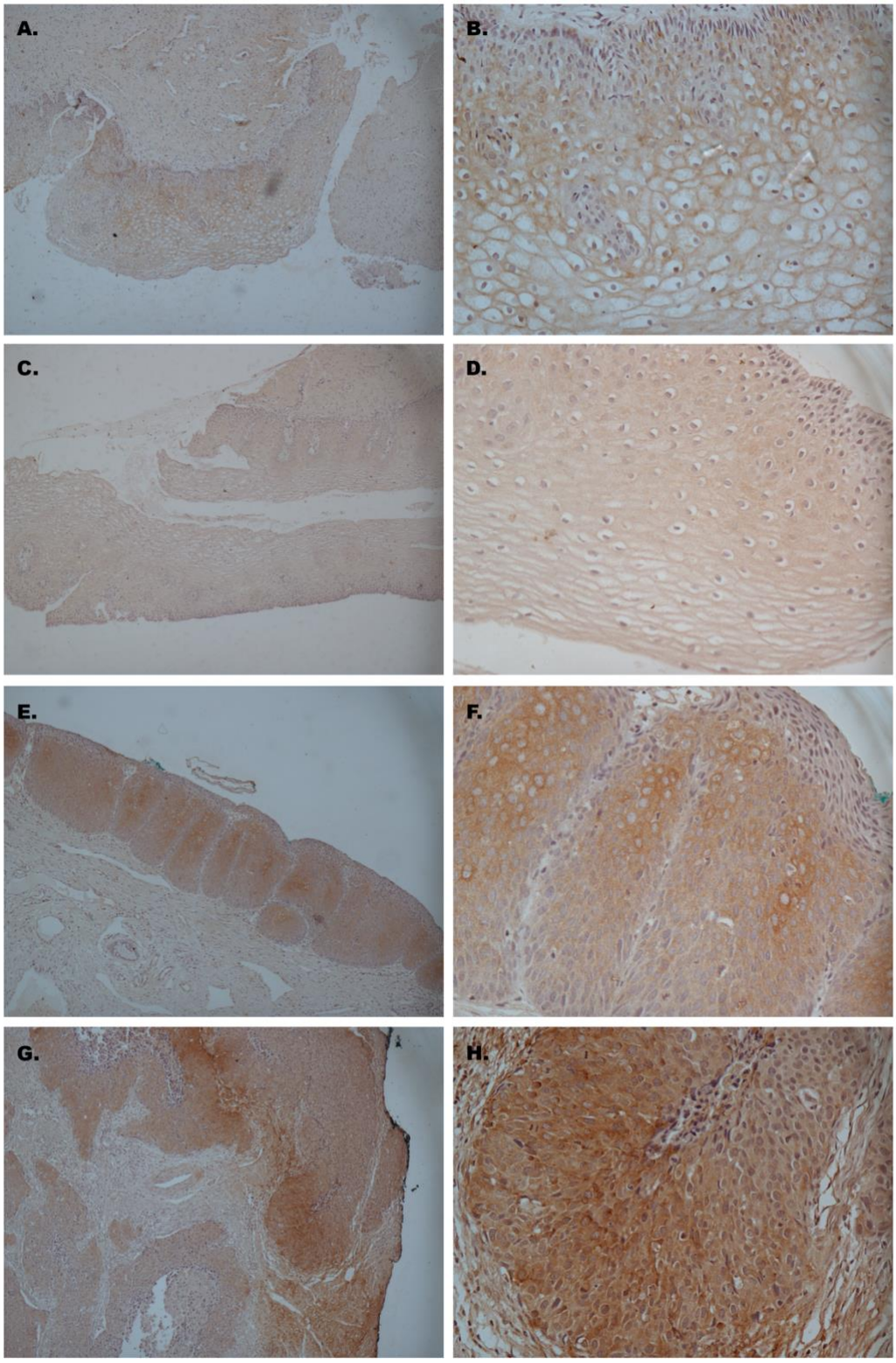

Figure 1. Representative immunohistochemistry pictures of FASN expression in cervical tissue. FASN was detected in cervicitis (A-B), LSIL (C-D), HSIL (E-F), and carcinoma (G-H). A, C, E, and G = original magnification, X 200; B, D, F, and H = original magnification, X 400.

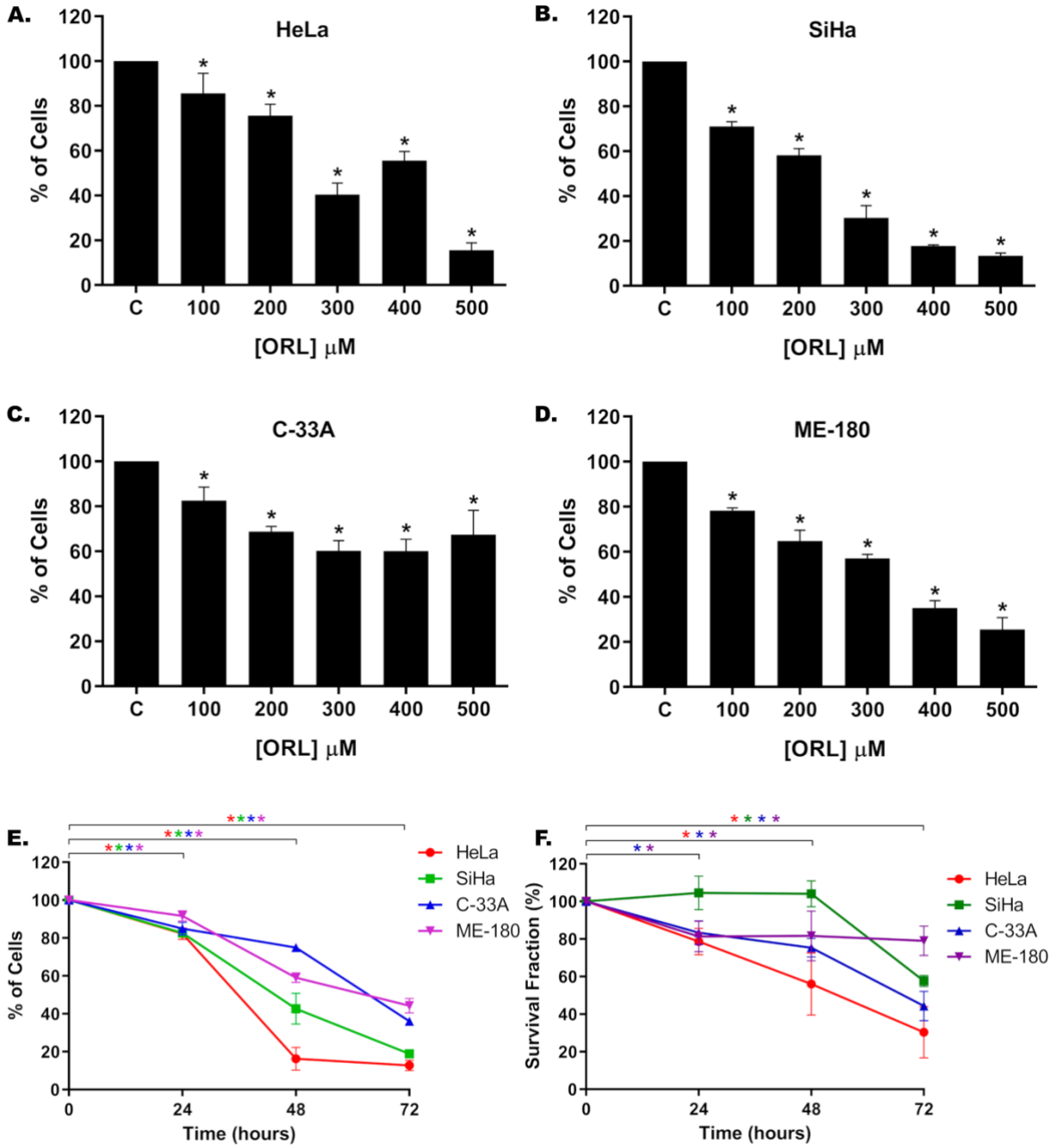

Figure 2. FASN activity is necessary for cervical cancer cell lines growth and survival. Treatment with ORL reduced the number of viable cells in all cervical cancer cell lines HeLa (A), SiHa (B), C-33A (C), and ME-180 (D), when compared with the respective controls. (E) Treatment with ORL showed a reduction of viable cells in a time-dependent manner. (F) ORL affects the cell lines capability of colony formation after treatment. Values refer to the average of three independent experiments ± SD. *p< 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

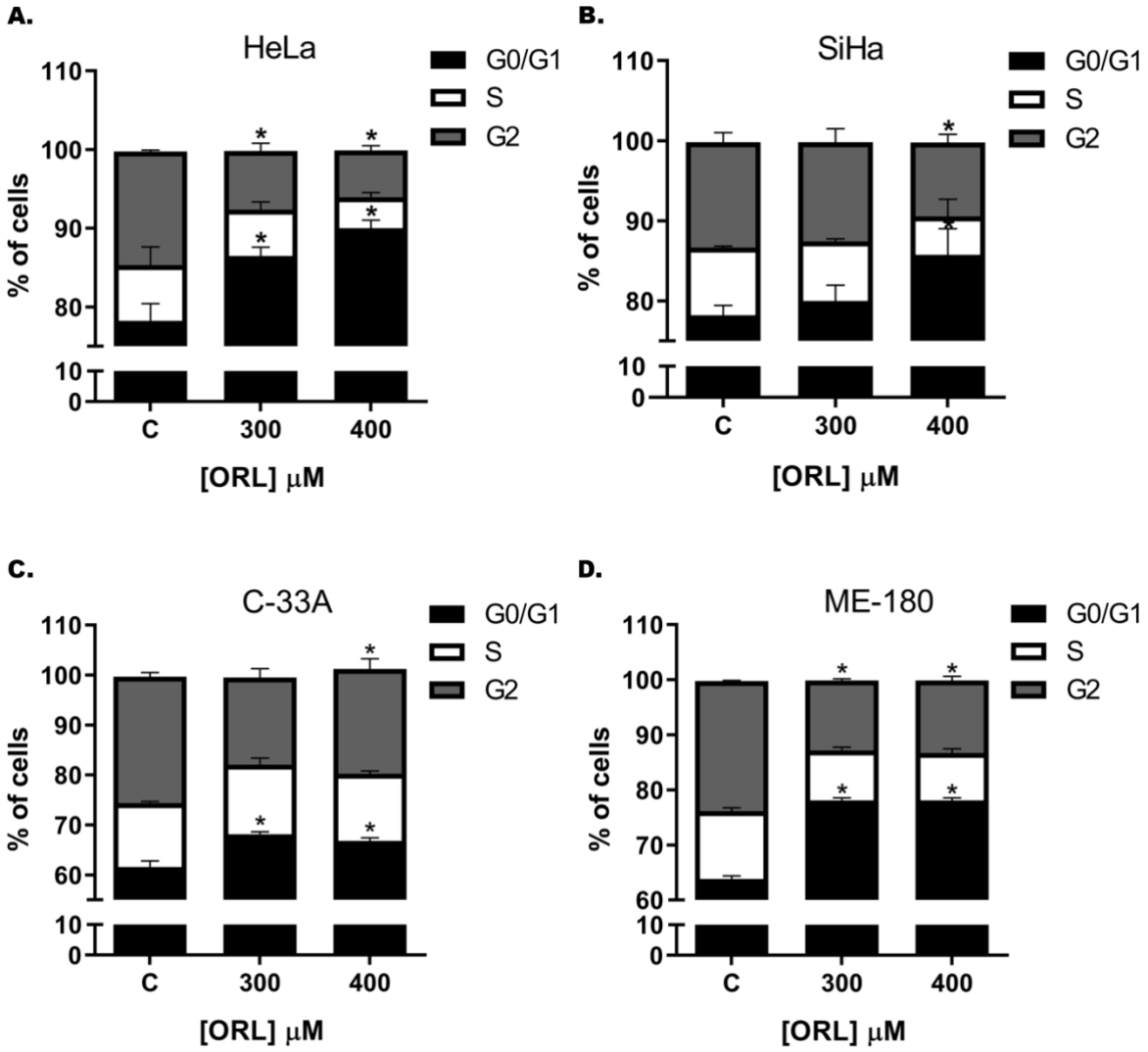

Figure 3. Orlistat treatment cause cell cycle arrest. Cell cycle analysis by flow cytometry showed that FASN inhibition with ORL enhances the G0-G1 population and reduces the number of cells in the S phase in cell lines HeLa (A), SiHa (B), C-33A (C), and ME-180 (D). Values refer to the average of three independent experiments ± SD. *p< 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

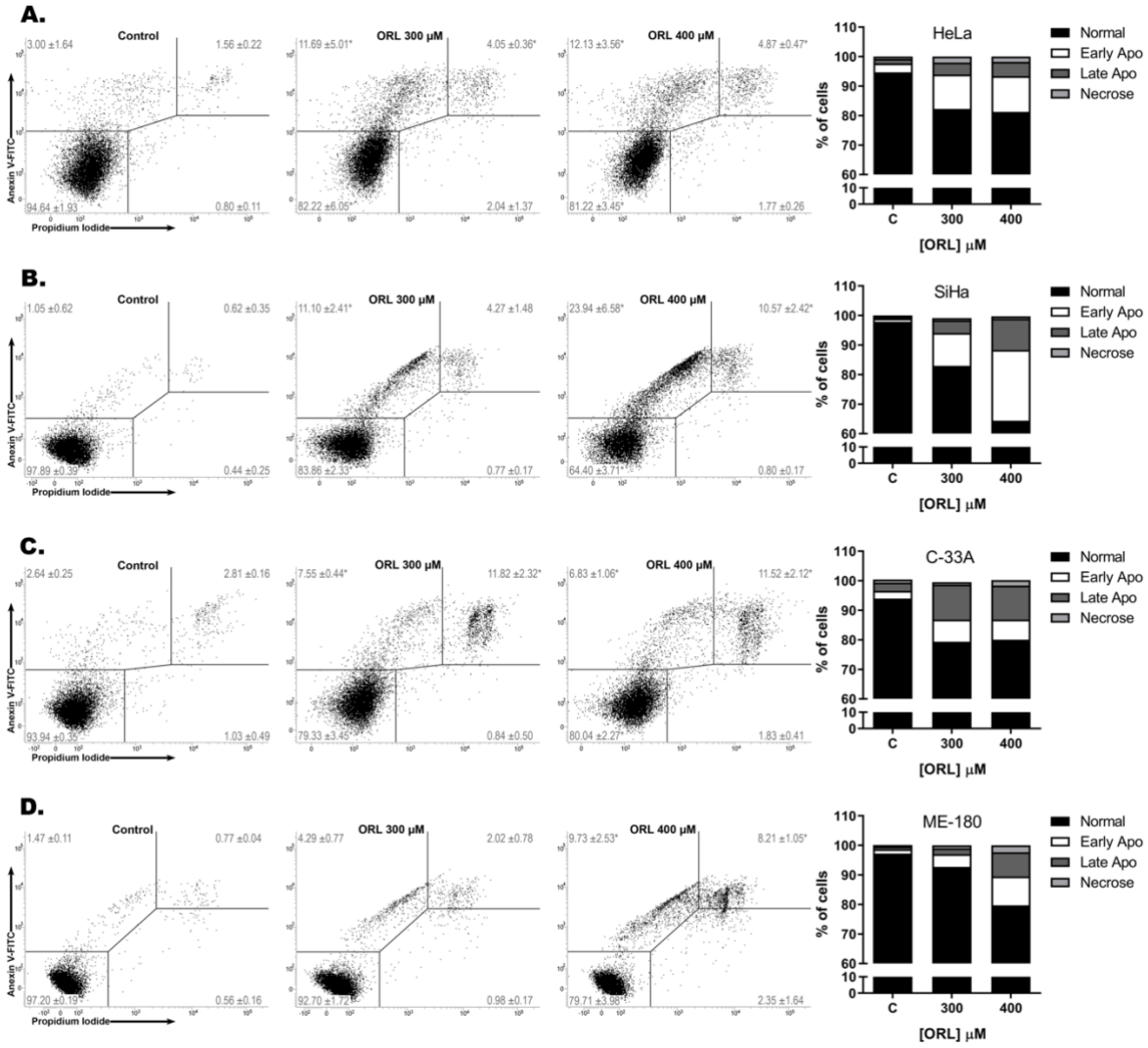

Figure 4. Orlistat triggers apoptosis in cervical cancer cell lines. Annexin V and PI experiment showing that ORL induces apoptotic cell death, but not necrosis in HeLa (A), SiHa (B), C-33A (C), and ME-180 (D) cell lines. Values refer to the average of the percentage of cells in each gate of three independent experiments ± SD. *p< 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

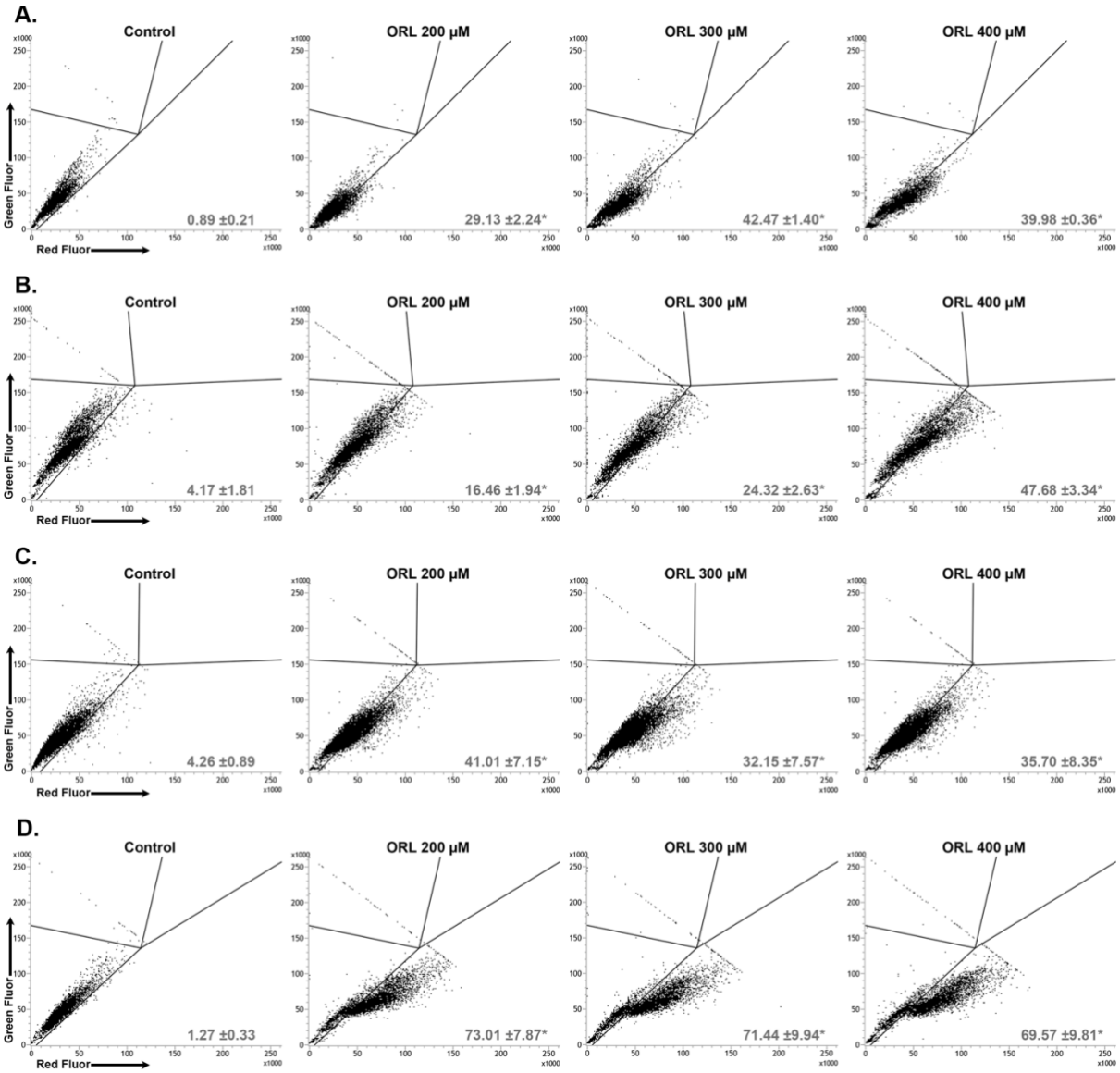

Figure 5. ORL triggers autophagy in cervical cancer cell lines. Flow cytometry showed an increase of AO-marked cells in HeLa (A), SiHa (B), C-33A (C), and ME-180 (D) cell lines. Values refer to the average of the percentage of cells in each gate of three independent experiments ± SD. *p< 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

4.2. Artigo 2

Jéssica Nascimento, Isadora do Canto Olegário, Camila Mariot, Paula dos Santos Chaves, Rafaela Oliveira, Andréia Buffon, Silvia Stanisçuaski Guterres, Diogo André Pilger, Ruy Carlos Ruver Beck. *Encapsulation of orlistat in biodegradable polymeric nanocapsules improves its antiproliferative effect against cervical cancer cells.*

Este artigo foi elaborado de acordo com as normas da revista “Biomedicine & Pharmacotherapy”.

Encapsulation of orlistat in biodegradable polymeric nanocapsules improves its antiproliferative effect against cervical cancer cells

Jéssica Nascimento[1a], Isadora do Canto Olegário[2a], Camila Mariot[2], Paula dos Santos Chaves[1], Rafaela Oliveira[2], Edilene Gadelha de Oliveira[1], Silvia Stanisçuaski Guterres[1,2], Andréia Buffon[1,2], Diogo André Pilger[1,2], Ruy Carlos Ruver Beck[1,2*].

[1]Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[2]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[a] These authors made equal contributions and are considered first co-authors.

[*]**Corresponding author:**
Dr. Ruy Carlos Ruver Beck
Departamento de Produção e Controle de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS)
Av. Ipiranga 2752, Santana
CEP 90610-000, Porto Alegre, RS, Brazil.
Phone: +55 (51) 3308-5951
E-mail: ruy.beck@ufrgs.br

Abstract

Orlistat is a drug that has been studied for having promising antitumor properties due to its inhibition of fatty acid synthase (FASN), the main enzyme responsible for *de novo* synthesis of fatty acids. FASN is overexpressed in different types of cancer, like cervical cancer cells as recently reported by our group. However, orlistat has a poor water solubility and oral bioavailability, limiting its formulation and uses. Therefore, its encapsulation in polymeric nanocapsules (NC) and the *in vitro* antiproliferative effect in the cervical cancer cell line HeLa was evaluated. NC containing or not orlistat (NC-ORL and NC-U, respectively) were prepared using Poly(ε-caprolactone) by the interfacial deposition of preformed polymer method. The *in vitro* antiproliferative effect of the non-encapsulated and encapsulated ORL was evaluated in HeLa cells. Cell counting by flow cytometry was used to evaluate antitumor effect after 48 h of treatment. The nanoformulations showed a mean Z-average between 210 and 220 nm regardless of the presence of ORL. An encapsulation efficiency of almost 100% was reached for NC-ORL and they were not toxic to HeLa cells in the experimental conditions. Moreover, NC-ORL had a more pronounced antiproliferative effect compared to non-encapsulated orlistat, mainly at the higher concentrations tested. No agglomeration of particles in the cell culture medium can have contributed for this. In conclusion, this novel nanoformulation of orlistat showed greater antiproliferative effect against HeLa cells over the non-encapsulated drug solution and seems to be a promising alternative for the treatment of cervical cancer overcoming their physicochemical properties like poor water solubility and low oral bioavailability.

Keywords: Fatty acid synthase; Cervical cancer; Orlistat; Nanocapsules.

Abbreviations: FASN, fatty acid synthase; MCT, medium chain triglycerides; NC-ORL, orlistat-loaded nanocapsules; NC-U, unloaded nanocapsules; ORL, non-encapsulated orlistat; PCL, poly(ε-caprolactone).

1. Introduction

Cancer proves itself to be a major concern in public health worldwide every year with very significant incidence rates. And even though cancer mortality rates have declined over the past decade, they still remain high [1]. Cervical cancer is the fourth most common cancer in women in the world, and the seventh overall [2]. Treatments available are based on the stage of the disease, with surgical therapy being a frequent choice for patients in early stages, and radiotherapy and chemotherapy being chosen at intermediate and advanced stages [3]. The options for chemotherapy treatment are not very extensive and can bring many adverse effects. Therefore, it is evident the urgency of new findings in therapeutic targets and anti-cancer drugs.

Altered metabolic pathways in cancer have been widely recognized, and lipid metabolism appears to be a promising target for further studies [4]. Fatty acids serve as an important substrate for a variety of cellular processes, and most normal cells and tissues obtain lipids from dietary intake, while *de novo* synthesis of fatty acids seems to be of minor importance, except for fetal lungs, adipose tissue, liver, cycling endometrium and lactating breasts [5]. However, when it comes to tumors, *de novo* synthesis may generate more than 93% of the fatty acids in the neoplastic cells [6]. This process of endogenous lipid biosynthesis is catalyzed by fatty acid synthase (FASN), the main enzyme involved in *de novo* synthesis of fatty acids [7]. A series of studies have already demonstrated high levels of FASN in different types of tumors, such as breast, prostate, ovarian, colorectal, stomach, bladder, lung, oral cavity and tongue, head and neck, thyroid and endometrial. However, the exact mechanisms responsible for overexpression and increased activity of FASN in cancers are not completely understood yet [6, 8, 9].

Taking into account, the possibility of using the higher expression of FASN in cancer as a target, researchers synthesized inhibitors of this enzyme with possible antitumor activities [10]. Indeed, it has been demonstrated that FASN inhibition can lead to stress and death of cancer cells [11, 12]. Currently, several substances are known to be potential FASN inhibitors: cerulenin, C75, orlistat, C93 and some natural polyphenols [6, 13]. Orlistat is a drug approved by Food and Drug Administration for treating obesity due to its property of inhibiting pancreatic and gastric lipases, but it has been discovered as an irreversible inhibitor of the thioesterase domain of FASN

[14]. Several studies have shown that orlistat has an antiproliferative effects and leads to cell death in different types of cancer cell lines [15-19]. On the other hand, as orlistat has poor water solubility and low oral bioavailability, its formulation in a suitable dosage form for its therapeutic use as an antitumor drug is still a challenge [20].

In order to overcome drawbacks in the administration of complex drugs, as those may have inadequate biopharmaceutical profiles, novel drug delivery systems have been designed in recent decades. Nanoparticles, especially those made of biocompatible polymers, attract great attention of researchers due to their potential applications in therapeutics [21]. Nanocapsules are a type of nanoparticles that consists of oily droplets surrounded by a thin polymeric membrane. The drug may be dissolved in the core and/or adsorbed on the polymer wall [22]. These nanocarriers systems seem to be very promising for cancer therapy, since they can offer highly desirable advantages over non-encapsulated drugs, including drug protection from premature degradation, early interaction with the biological environment, improved antiproliferative effect, enhanced absorption into a selected tissue, control of the drug's pharmacokinetics, improved intracellular penetration, and less adverse effects [23].

Previous studies proposed nanoformulations of orlistat for the treatment of cancer cells, but none of them targeting cervical cancer [24-27]. Moreover, studies from our group showed overexpression of FASN in cervical cancer cells [28]. Considering the overexpression of FASN and whereas the effects of orlistat in its non-encapsulated or nanoencapsulated form have not been evaluated in cervical cancer cells, the aim of this study was to produce orlistat-loaded nanocapsule suspensions, using poly(ε-caprolactone) as the biocompatible and biodegradable polymer, evaluating the antitumor efficacy of this formulation in cervical cancer cell line HeLa, compared to the non-encapsulated drug solution.

2. Material and methods

2.1Preparation of nanocapsule suspension

Orlistat-loaded nanocapsules were prepared by the interfacial deposition of preformed polymer method [29]. Poly(ε-caprolactone) 80.000 MW (0.100 g) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in acetone (27 mL) and maintained at 40°C under magnetic stirring for 1.5 h. Then, the oil MCT (165 µL) and the drug orlistat

(0.010 g) (Sigma-Aldrich, St. Louis, MO, USA) were added, composing the organic phase of the formulation. This solution was stirred for further 10 min. At the same time, the aqueous phase of the formulation was prepared by adding polysorbate 80 (0.077 g) in ultrapure water (54 mL), at 40°C and kept under magnetic stirring. The organic solution was injected into the aqueous solution, at 40°C. After 10 min of stirring, the suspension was concentrated by evaporation under reduced pressure at 40°C using a rotary evaporator. The final volume was adjusted to 10 mL in a volumetric flask. This formulation was named NC-ORL. Nanocapsule suspension containing no drug (unloaded nanocapsules, named NC-U) was prepared as described above, without adding orlistat to the organic phase. Three batches of each formulation were prepared and characterized. Formulations were kept protected from light and at room temperature until analysis.

2.2Physicochemical characterization of formulations

2.2.1Laser diffraction analysis

Particle size was evaluated by laser diffraction (LD) analysis (Mastersizer 2000, Malvern Instruments Ltd., UK). Volume-weighted mean diameters (*D* 4,3) and polydispersity (Span) were analyzed. Each sample was added directly into the aqueous disperser compartment of the equipment, containing about 150 mL of distilled water until the adequate obscuration index (2–8%) was reached.

2.2.2Dynamic light scattering analysis

Mean particle diameter (ʒ-average) and polydispersity index (PDI) were measured using dynamic light scattering (DLS) (ZetaSizer Nano ZS, Malvern Instruments Ltd., UK). For this analysis, the suspensions (20 µL) were diluted in water (10 mL) previously filtered (0.45 µm, Millipore®).

2.2.3Zeta potential analysis

Zeta potential was determined by electrophoretic mobility (ZetaSizer Nano ZS, Malvern Instruments Ltd., UK). For this analysis, samples (20 µL) were diluted in NaCl solution 10 mM (10 mL) previously filtered (0.45 µm, Millipore®).

2.2.4Analytical method

The orlistat assay was carried out by high performance liquid chromatography [30]. Analyses were performed in a Shimadzu LC system (Kyoto, Japan) equipped with a CBM-20A system controller, a LC-20AT pump, a DGU-20A5 degasser, a SIL-20A auto-sampler, and a SPD-20AV detector (UV). A Phenomenex Luna C_{18} column (250 mm x 4.6 mm I.D., with a particle size of 5 µm) was utilized as stationary phase. The mobile phase was composed of water containing 0.1% (v/v) of phosphoric acid (85%) and acetonitrile (5:95, v/v), run at a flow rate of 1.5 mL.min^{-1}. UV detection was carried out at 205 nm, run time was 12 min and an injection volume of 25 µL was used. The method had specificity, good linearity (r=0.999, n=3) in the range of 10.00 - 30.00 µg.mL^{-1}, and good intra (SD=1.64%) and interday (SD=3.13%) precision.

2.2.5Drug content and encapsulation efficiency

Orlistat content was assayed after dissolution of NC suspensions (1.0 mL) in acetonitrile at final volume of 10 mL followed by sonication during 10 min. This dispersion was centrifuged at 4,120 ×*g* for 10 min and an aliquot (2.0 mL) of the supernatant was diluted to 10 mL in mobile phase. The sample was filtered with hydrophilic membrane (0.45 µm, Millipore®) and analyzed. Encapsulation efficiency was calculated based on the difference between total drug and free drug contents in the ultrafiltrate. The ultrafiltrate was obtained by ultrafiltration/centrifugation technique (Ultrafree-MC 10,000 MW, Millipore, Billerica, USA) at 4,120 ×*g* for 10 min.

2.2.6pH measurement

The pH values were measured with a potentiometer (VB-10, Denver Instrument, USA) using the original, undiluted formulations directly.

2.2.7Particle number density

The number of nanoparticles in suspension (number per mL) was determined by turbidimetric technique (UV) as previously described by Andrade et al. [31]. Samples (n = 3) were analyzed using a spectrophotometer (Shimadzu VR-1800PC, Shimadzu, Japan).

2.3 In vitro *assays*

2.3.1Cell culture

Human cervical cancer cell line HeLa was obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown and maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin and 0.1% amphotericin (Sigma-Aldrich, St. Louis, MO, USA), in 5% CO_2 humidified atmosphere at 37°C.

2.3.2Cell treatments

A solution of non-encapsulated orlistat (named ORL), used for cell treatments, was previously prepared by dissolving orlistat powder in sterile DMSO, resulting in a stock solution of 250 mM. HeLa cells were seeded in 24-well plates, at a density of 10.0 x 10_3 cells per well, and incubated for 48 h before treatments. After, cells were treated with ORL at concentrations of 10, 25, 50, 75, 100 and 150 µM of NC-ORL at the same drug concentrations corresponding to the following particle number densities: 0.03 x 10_{12}, 0.07 x 10_{12}, 0.14 x 10_{12}, 0.22 x 10_{12}, 0.29 x 10_{12}, and 0.43 x 10_{12} particles.mL^{-1}. The controls used were DMEM (10% FBS) as a negative control, DMSO at the same volume used for ORL 150 µM, and NC-U at the same particle number density (particles.mL^{-1}) used for all concentrations of NC-ORL. Cells were incubated with the treatments for 48 h. Before starting these experiments, the particle behavior of the nanocapsules (n = 3) in the cell culture medium was evaluated adding the nanocapsules suspension (1 mL) to the cell culture medium (13.5 mL) at 37 ± 0.5°C followed by laser diffraction analysis after 0, 24 and 48 h.

2.3.3Cell counting

Flow cytometry (Attune Flow Cytometer, Thermo Fisher Scientific, USA) was used for counting cell number. After 48 h, treatments were withdrawn from the wells and cells were rinsed with phosphate-buffered saline (PBS), then 200 µL of trypsin was added into each well. After the cells detachment, 400 µL of DMEM (10% FBS) was added and the cell suspensions were collected into eppendorfs. Cells were analyzed by flow cytometry, with an acquisition time set to 30 s, generating a value of events per µL of cell suspension. The results for treated cells, expressed in percentage of cell

number, were normalized to control cells (treated with DMEM 10% FBS), which represents 100% of cell number.

2.4Statistical analysis

The results are expressed as mean ± SD (standard deviation), calculated from at least three independent measurements. Statistical analysis was performed by One-Way ANOVA followed by Tukey test to compare the effect of different particle number density of NC-U on the cell viability. Two-Way ANOVA followed by Bonferroni test was performed to compare the data from NC-ORL and ORL in the study of the *in vitro* antiproliferative effect. All statistical analyses were carried out using the Graphpad Prism software. The level of significance was set to $p \leq 0.05$.

3.Results and discussion

3.1Nanoformulations

Nanocapsules were prepared in this study using a biodegradable polymer (PCL) because its compatibility with different routes of administration, such as oral, intravenous, and local/topical [32], giving different possibilities to formulate them as final dosage forms. Nanocapsule suspensions (NC-ORL and NC-U) had a liquid, milky white and homogeneous aspect, regardless of the presence of the drug. Their particle size distribution profiles are shown in **Fig. 1**. They had a unimodal and submicrometric particle size distribution profile, refuting any particle agglomeration during the production process. NC-ORL and NC-U showed *D*4,3 values of 334 ± 55 nm and 325 ± 35 nm, respectively, with Span values lower than 2.0, confirming their narrow size distribution. After these first analysis showing the exclusive presence of particles at the nanoscale, further characterization steps were carried out. The detailed physicochemical characteristics of the nanoformulations are summarized in **Table 1**. They had mean particle diameter (ʒ-average) ranged between 210 and 220 nm according to the dynamic light scattering (DLS) analysis. The polydispersity indices (PDI) measured by DLS were below 0.20 and confirmed the narrow particle size distribution observed by the laser diffraction analysis, as discussed above. Zeta potential values were slightly negative and similar for both formulations. These values are expected for nanocapsules made of poly(ε-caprolactone) and having a non-ionic stabilizing agent (in this case, polysorbate 80), since this polymer presents terminal

carboxylic groups that give a negative characteristic to nanocapsule surface [33]. NC showed slightly acid pH values which can also be explained by the properties of the polymer. The drug content of the NC-ORL (0.96 ± 0.05 $mg.mL^{-1}$) was very close to the initial set concentration (1.00 $mg.mL^{-1}$). In addition, orlistat showed an excellent encapsulation efficiency of 99.8%. This result may be explained by the lipophilic nature of the drug (log P = 8.5), indicating that it has a high affinity for the oily core of the nanocapsules.

All the physicochemical properties of NC-ORL and NC-U discussed above are in agreement with data shown in literature for this type of formulation [32, 33]. Therefore, these nanocapsules suspensions have suitable characteristics for further *in vitro* studies. However, prior performing the *in vitro* studies, the particle number densities were calculated. This analysis has highly importance in order to compare the behavior of loaded and unloaded nanocapsules in the cell viability assay. Moreover, classical pharmacology uses only the total mass of a drug as a parameter of dose. But for drug-loaded in nanoparticles, the surface area of the particles (related to the particle number) may induce the interactions with biological systems and the total mass may not be the only parameter of dose. Therefore, it is highly recommended to evaluate the effect of this kind of formulations considering the particle number density (in this case, number of NC per ml) [34]. The particle number density of the loaded and unloaded nanocapsules were calculated by turbidimetry. The particle number densities were similar ($p>0.05$) for both formulations NC-ORL (7.30×10^{12} $particles.mL^{-1}$) and NC-U (6.24×10^{12} $particles.mL^{-1}$).

3.2In vitro assays

Orlistat has been shown to be a drug with promising antitumor properties in different types of cancer. It could decrease cell viability and trigger apoptosis, as well as induced cell cycle arrest and cell stress in glioma, endometrial, prostate and oral cancer [17, 35-37]. Also, orlistat could reduce the volume of primary tumors and the number of lymphnode metastasis in melanoma xenograft models and oral squamous cell carcinoma orthotopic models [15, 16]. However, orlistat presents many negative characteristics for its adequate use, such as low cell permeability, low water solubility and poor oral bioavailability [8, 9]. In an attempt to overcome these issues, some

studies have already tried to develop different formulations with orlistat, but none of them used nanocapsules targeting cervical cancer [24-27].

In our study, we developed a nanocapsule formulation with biodegradable polymer to improve orlistat efficacy. In this aim, we used the cervical cancer cell HeLa to access the effect of orlistat nanocapsules. Cell counting by flow cytometry was used to evaluate the effect of the treatments on the number of viable cells, thus estimating cell death and antiproliferative effect. However, prior experiments were carried out to evaluate the nanocapsules behavior in the cell culture medium. As showed in Fig. 2, laser diffraction analysis showed that the mean particle size of NC remained in the nanometric scale even after 48 h and similar to the original NC suspension. Similar results were previously reported by Rigo et al [34] for PCL nanocapsules and RPMI and MEM cell culture media. Therefore, in agreement with this previous report, no agglomeration behavior of nanocapsules in the cell culture media could be explained by no interaction between the polymer and/or the non-ionic surfactant present at the nanocapsules surface and the components of the DMEM medium. Afterwards, a cell counting study using NC-U was conducted to estimate the toxicity of the unloaded nanocapsule suspension to HeLa cells after 48 h of treatment. Only at the highest tested concentration (0.43×10^{12} particles.mL^{-1}) there was a significant decrease in viable cell number compared to the negative control (**Fig. 3**). This result was somewhat expected, considering that the number of particles is this case is very large. These results are in agreement with the previous report by Rigo et al. [34], showing that the cytotoxic effects by unloaded NC are not cell-specific, and suggesting that this effect is a consequence of the physical effect of the nanocapsules due to the *in vitro* overexposure of cells to a high number of particles. On the other hand, the percentage of living cells in the concentration of 0.43×10^{12} $\mu mol.L^{-1}$ (about 80%) can still be considered quite good, even though this statistical difference was observed. Consequently, it is possible to conclude that the unloaded nanocapsule suspension was not toxic to HeLa cells in the experimental condition.

Regarding the treatments with ORL and NC-ORL (**Fig. 4**), the non-encapsulated drug (ORL) had little effect on the number of viable cells after 48 h in all concentrations tested. Even at the highest concentration (150 µM) of ORL was able to reduce cell number only in about 20%. The percentage of remaining cells ranged between from about 97% for the lowest concentration (10 µM) and 80% for the highest concentration

(150 µM) of ORL. On the other hand, the treatment with NC-ORL at the concentrations of 75, 100 and 150 µM, corresponding to 0.22 x 10^{12}, 0.29 x 10^{12}, and 0.43 x 10^{12} µmol.L^{-1}, respectively, had a notable and significant effect on cell number, decreasing it about 40, 55 and 70%, respectively. NC-ORL at the drug concentration of 25 and 50 µM (particle concentration of 0.07 x 10^{12} and 0.14 x 10^{12} µmol.L^{-1}, respectively) did not show difference in the cell number compared with the corresponding drug dose in the treatment with the drug solution (ORL). However, at the lowest dose and particle concentration tested (10 µM and 0.03 x 10^{12} µmol.L^{-1}, respectively), NC-ORL treatment had a statistically significant difference on viable cell number compared with the corresponding ORL dose. This may have happened because the ORL at 10 µM did not have effect on cell viability, remaining almost the total number of viable cells as in the control. On the other hand, the NC-ORL shows its antiproliferative effect even at this lowest dose and particle concentration. So, although the percentage of living cells in NC-ORL at 10 µM (0.03 x 10^{12} µmol.L^{-1}) was relatively high (about 80%), when it was compared to the corresponding ORL dose, the difference between these treatments was statically significant. In other words, we could see an anticipation (regarding the drug dose) on orlistat effect when it was nanoencapsulated, suggesting its future uses at lower effective doses by its nanoencapsulation. Moreover, no agglomeration of the nanocapsules in the culture medium can have contributed to ensure the best antiproliferative effect of the nanoencapsuled orlistat.

These data represent an important contribution to the literature, as a novel strategy for the treatment of cervical cancer using the higher expression of FASN in this cell type as an effective target [28]. Some previous studies reported the nanoencapsulation of orlistat as an alternative for the treatment of different cancers, using breast and prostate cancer cell lines [24-27]. However, our recent study reporting the FASN overexpression in cervical cancer cells opens the possibility of widening the future application of nanoencapsulated orlistat in the cancer therapy.

4.Conclusions

Orlistat was effectively encapsulated in polymeric nanocapsules as an aqueous drug delivery systems suitable to formulate the drug in different pharmaceutical dosage forms. *In vitro* studies demonstrated that orlistat-loaded nanocapsules has greater antiproliferative effects against HeLa cell line when compared to the non-encapsulated

drug solution of ORL. Therefore, it may be assumed that this novel formulation using a biodegradable polymer can become an attractive alternative for the treatment of cervical cancer in the future. Further studies should be carried out to evaluate the *in vivo* effects of nanoencapsulated orlistat for the treatment of cervical cancer, alone or even in combination with standard treatment.

Acknowledgments

The authors thank to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPERGS (Fundação de Amparo a Pesquisa do Rio Grande do Sul) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Finance Code 001) for the financial support.

Declaration of interest: The authors declare that they have no conflict of interest.

References

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2018.* CA Cancer J Clin, 2018. **68**(1): p. 7-30.
2. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
3. Marth, C., et al., *Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2018. **29**(Supplement_4): p. iv262.
4. Medes, G., A. Thomas, and S. Weinhouse, *Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro.* Cancer Res, 1953. **13**(1): p. 27-9.
5. Weiss, L., et al., *Fatty-acid biosynthesis in man, a pathway of minor importance. Purification, optimal assay conditions, and organ distribution of fatty-acid synthase.* Biol Chem Hoppe Seyler, 1986. **367**(9): p. 905-12.
6. Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.
7. Chirala, S.S. and S.J. Wakil, *Structure and function of animal fatty acid synthase.* Lipids, 2004. **39**(11): p. 1045-53.

8. Flavin, R., et al., *Fatty acid synthase as a potential therapeutic target in cancer.* Future Oncol, 2010. **6**(4): p. 551-62.
9. Liu, H., et al., *Biochemistry, molecular biology, and pharmacology of fatty acid synthase, an emerging therapeutic target and diagnosis/prognosis marker.* Int J Biochem Mol Biol, 2010. **1**(1): p. 69-89.
10. Kuhajda, F.P., et al., *Synthesis and antitumor activity of an inhibitor of fatty acid synthase.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3450-4.
11. Orita, H., et al., *Selective inhibition of fatty acid synthase for lung cancer treatment.* Clin Cancer Res, 2007. **13**(23): p. 7139-45.
12. Little, J.L., et al., *Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells.* Cancer Res, 2007. **67**(3): p. 1262-9.
13. Menendez, J.A. and R. Lupu, *Fatty acid synthase (FASN) as a therapeutic target in breast cancer.* Expert Opin Ther Targets, 2017. **21**(11): p. 1001-1016.
14. Kridel, S.J., et al., *Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity.* Cancer Res, 2004. **64**(6): p. 2070-5.
15. Carvalho, M.A., et al., *Fatty acid synthase inhibition with Orlistat promotes apoptosis and reduces cell growth and lymph node metastasis in a mouse melanoma model.* Int J Cancer, 2008. **123**(11): p. 2557-65.
16. Agostini, M., et al., *The fatty acid synthase inhibitor orlistat reduces the growth and metastasis of orthotopic tongue oral squamous cell carcinomas.* Mol Cancer Ther, 2014. **13**(3): p. 585-95.
17. Grube, S., et al., *Overexpression of fatty acid synthase in human gliomas correlates with the WHO tumor grade and inhibition with Orlistat reduces cell viability and triggers apoptosis.* J Neurooncol, 2014. **118**(2): p. 277-287.
18. Peng, H., et al., *Orlistat induces apoptosis and protective autophagy in ovarian cancer cells: involvement of Akt-mTOR-mediated signaling pathway.* Arch Gynecol Obstet, 2018. **298**(3): p. 597-605.
19. Sokolowska, E., et al., *Orlistat Reduces Proliferation and Enhances Apoptosis in Human Pancreatic Cancer Cells (PANC-1).* Anticancer Res, 2017. **37**(11): p. 6321-6327.
20. Schcolnik-Cabrera, A., et al., *Orlistat as a FASN inhibitor and multitargeted agent for cancer therapy.* Expert Opin Investig Drugs, 2018. **27**(5): p. 475-489.
21. Alonso, M.J., *Nanomedicines for overcoming biological barriers.* Biomed Pharmacother, 2004. **58**(3): p. 168-72.

22. Peer, D., et al., *Nanocarriers as an emerging platform for cancer therapy.* Nat Nanotechnol, 2007. **2**(12): p. 751-60.
23. Brigger, I., C. Dubernet, and P. Couvreur, *Nanoparticles in cancer therapy and diagnosis.* Adv Drug Deliv Rev, 2002. **54**(5): p. 631-51.
24. Paulmurugan, R., et al., *Folate Receptor-Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple-Negative Breast Cancer.* Mol Cancer Ther, 2016. **15**(2): p. 221-31.
25. Hill, T.K., et al., *Development of a Self-Assembled Nanoparticle Formulation of Orlistat, Nano-ORL, with Increased Cytotoxicity against Human Tumor Cell Lines.* Mol Pharm, 2016. **13**(3): p. 720-8.
26. Bhargava-Shah, A., et al., *Orlistat and antisense-miRNA-loaded PLGA-PEG nanoparticles for enhanced triple negative breast cancer therapy.* Nanomedicine (Lond), 2016. **11**(3): p. 235-47.
27. Souchek, J.J., et al., *Combination Treatment with Orlistat-Containing Nanoparticles and Taxanes Is Synergistic and Enhances Microtubule Stability in Taxane-Resistant Prostate Cancer Cells.* Mol Cancer Ther, 2017. **16**(9): p. 1819-1830.
28. Nascimento, J., et al., *Fatty Acid Synthase expression on cervical cancer and inhibition with Orlistat.* 2019.
29. H.Fessi, et al., *Nanocapsule formation by interfacial polymer deposition following solvent displacement.* International Journal of Pharmaceutics, 1989. **55**(1): p. R1-R4.
30. Dolenc, A., et al., *Nanosized particles of orlistat with enhanced in vitro dissolution rate and lipase inhibition.* Int J Pharm, 2010. **396**(1-2): p. 149-55.
31. Andrade, D.F., et al., *Redispersible spray-dried lipid-core nanocapsules intended for oral delivery: the influence of the particle number on redispersibility.* Pharm Dev Technol, 2018. **23**(4): p. 414-425.
32. Pohlmann, A.R., et al., *Poly(-caprolactone) microcapsules and nanocapsules in drug delivery.* Expert Opin Drug Deliv, 2013. **10**(5): p. 623-38.
33. Mora-Huertas, C.E., H. Fessi, and A. Elaissari, *Polymer-based nanocapsules for drug delivery.* Int J Pharm, 2010. **385**(1-2): p. 113-42.
34. Rigo, L.A., et al., *Nanoencapsulation of a glucocorticoid improves barrier function and anti-inflammatory effect on monolayers of pulmonary epithelial cell lines.* Eur J Pharm Biopharm, 2017. **119**: p. 1-10.

35. Wysham, W.Z., et al., *Effects of Fatty Acid Synthase Inhibition by Orlistat on Proliferation of Endometrial Cancer Cell Lines.* Target Oncol, 2016. **11**(6): p. 763-769.

36. Wright, C., et al., *Anti-Tumorigenic Potential of a Novel Orlistat-AICAR Combination in Prostate Cancer Cells.* J Cell Biochem, 2017. **118**(11): p. 3834-3845.

37. Xiao, X., H. Liu, and X. Li, *Orlistat treatment induces apoptosis and arrests cell cycle in HSC-3 oral cancer cells.* Microb Pathog, 2017. **112**: p. 15-19.

Table 1: Physicochemical characterization of nanocapsule formulations

	NC-ORL (mean ± SD)	**NC-U (mean ± SD)**
Z-average diameter (nm)	211 ± 12	217 ± 9
PDI	0.15 ± 0.02	0.13 ± 0.02
Zeta potential (mV)	-6.62 ± 0.79	-7.20 ± 2.03
pH	5.78 ± 0.13	5.17 ± 0.27
Drug content (mg.mL^{-1})	0.96 ± 0.05	-
PND (particles.mL^{-1})	(7.30 ± 0.61) x 10^{12}	(6.24 ± 0.26) x 10^{12}

NC-ORL: orlistat-loaded nanocapsule suspension; NC-U: unloaded nanocapsule suspension; PDI: polydispersity index; PND: Particle number density.

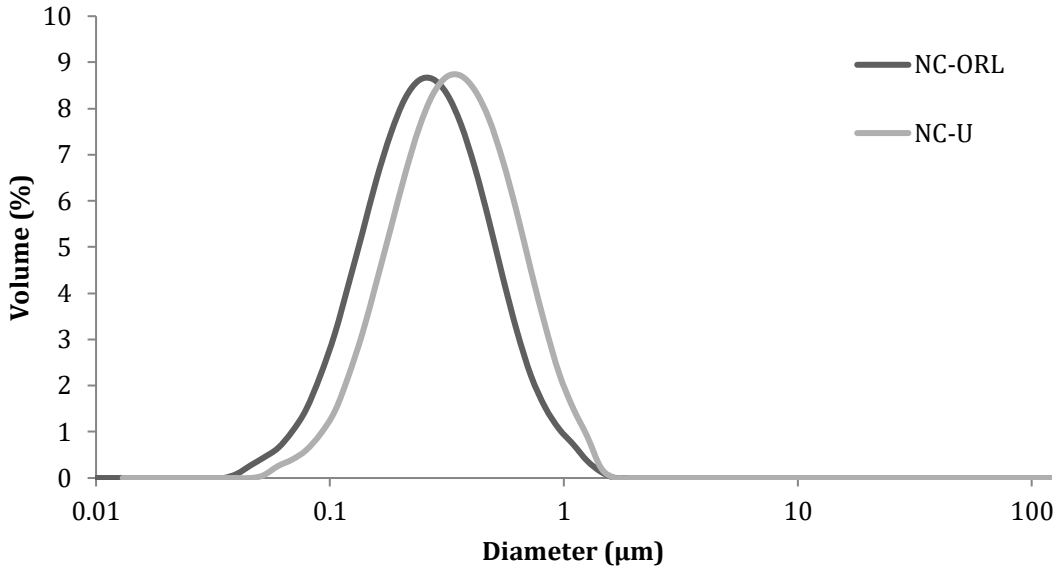

Figure 1: Particle size distribution profiles obtained by laser diffraction analysis for NC-ORL and NC-U (n=3).

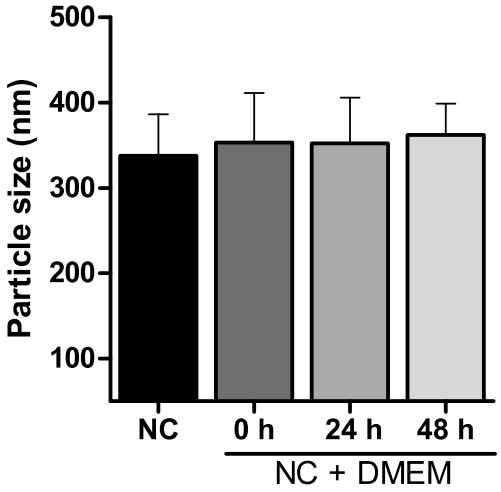

Figure 2: Nanocapsule aggregation on DMEM medium. The particle behavior of NC in the cell culture medium was evaluated by laser diffraction analysis after 0, 24, and 48 hours. The mean particle size of NC remained in the nanometric scale and similar to the original NC suspension. Data are expressed as mean ± SD. Differences were considered no significant at $p>0.05$ (One-Way ANOVA followed by Tukey test).

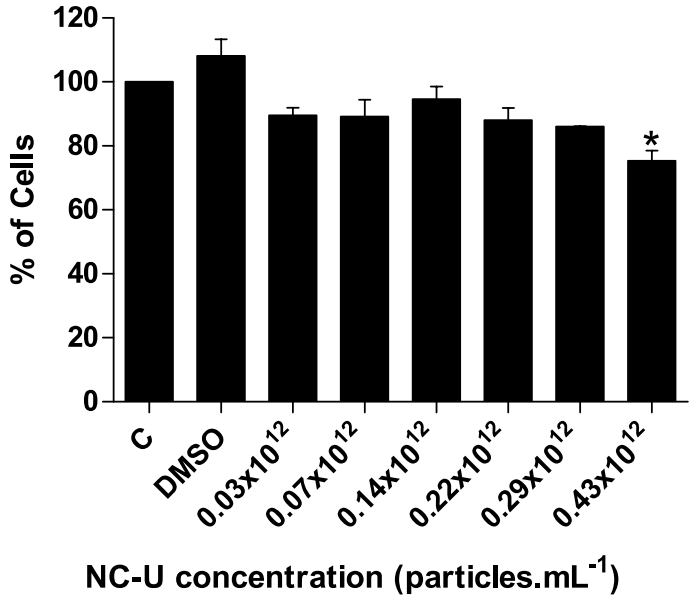

Figure 3: Toxicity evaluation of NC-U by cell counting. HeLa cells were incubated with NC-U at concentrations of $0.03x10_{12}$, $0.07x10_{12}$, $0.14x10_{12}$, $0.22x10_{12}$, $0.29x10_{12}$, and $0.43x10_{12}$ particles.mL_{-1} during 48 h. The particles concentration used are equivalents to the volume required to reach the NC-ORL tested concentration (10 to 150 μM). Data are expressed as mean ± SD. *Indicates significant difference compared to the control. Differences were considered significant at $p<0.05$ (One-Way ANOVA followed by Tukey test).

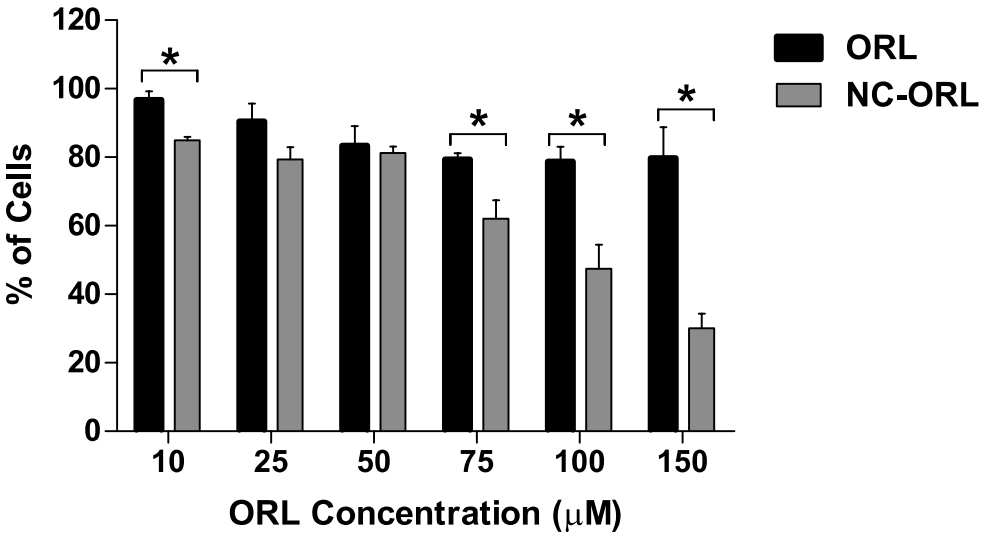

Figure 4: HeLa cell counting evaluated by flow cytometry after 48h of treatment. The cells HeLa were incubated with ORL and NC-ORL at concentrations of 10, 25, 50, 75, 100, and 150 μM. The added volume of NC-ORL to reach each concentration corresponds of 0.03 x 10^{12}, 0.07 x 10^{12}, 0.14 x 10^{12}, 0.22 x 10^{12}, 0.29 x 10^{12}, and 0.43 x 10^{12} μmol.L^{-1}, respectively. Data are expressed as mean ± standard error. *Indicates significant differences compared to the control. Differences were considered significant at $p<0.05$ (Two-Way ANOVA followed by Bonferroni test).

5.CONSIDERAÇÕES FINAIS

5.1.Discussão geral

O câncer é uma das principais causas de morbidade e mortalidade que atinge a população mundial, com aproximadamente 18,1 milhões de novos casos e 9,6 milhões de mortes em 2018 (BRAY *et al.*, 2018). Entre os tipos de câncer que mais afetam a população feminina no Brasil, encontramos o câncer de colo de útero, com uma estimativa de 16.370 novos casos em 2018 (INCA, 2017). Ainda o câncer de colo de útero é responsável por aproximadamente 570 mil novos casos e 311 mil mortes por ano no mundo (BRAY *et al.*, 2018). Trata-se, portanto, de um problema de saúde pública e carece ainda de alternativas mais eficazes e menos invasivas para seu tratamento.

A descoberta de que alterações metabólicas poderiam estar envolvidas no desenvolvimento de neoplasias revelou a importância da síntese *de novo* de ácidos graxos como potencial alvo terapêutico. Como principal enzima da síntese *de novo*, encontra-se a ácido graxo-sintase (FASN), responsável por produzir palmitato através da condensação de malonil-CoA e acetil-CoA (LITTLE e KRIDEL, 2008). A FASN apresenta baixa expressão na maioria dos tecidos normais e elevada expressão em tecidos tumorais (KUHAJDA, 2000). Ainda, estudos onde a FASN foi inibida mostraram uma redução na proliferação, parada no ciclo celular e morte por apoptose (LUPU e MENENDEZ, 2006).

Tendo em vista que até o momento não existem trabalhos demonstrando a expressão da FASN em câncer de colo de útero, a fase inicial deste trabalho foi dirigida ao estudo da expressão da FASN em linhagens celulares e em amostras de pacientes. Foi possível verificar que diferentes linhagens de células de câncer de colo de útero apresentam expressão de FASN. Interessantemente, as linhagens HeLa e SiHa, que possuem cópias do HPV 18 e 16, respectivamente, inseridos no seu genoma, e que são considerados de alto risco (MUNOZ *et al.*, 2003; SMITH *et al.*, 2007), foram também as linhagens com maior expressão de FASN. No entanto, até o momento, não há estudos correlacionando a infecção pelo HPV com a expressão da FASN. Apesar disso, vários estudos já relataram que a expressão da FASN está aumentada em infecções pelo vírus da Hepatite C, Hepatite B, HIV, Dengue, Vírus Sincicial Respiratório e Chicungunha. Portanto, estudos futuros são necessários para estabelecer também o papel da FASN na infecção pelo HPV (YANG *et al.*, 2008; ARAGONES *et al.*, 2010; HEATON *et al.*, 2010; ZHANG *et al.*, 2013; OHOL *et al.*,

2015; ZHANG *et al.*, 2018). Além disso, foi possível verificar que a FASN se encontra expressa em todos os estágios de lesões precursoras (LSIL e HSIL) e também em casos da neoplasia instalada. Este resultado está de acordo com outros trabalhos que também demonstraram expressão desta proteína em estágios iniciais da progressão tumoral (ALO *et al.*, 1999; PIYATHILAKE *et al.*, 2000; OGINO *et al.*, 2008; GRUBE *et al.*, 2014). Ainda, as amostras de cervicite que foram utilizadas como controle também demonstraram fraca expressão da FASN. A cervicite é um processo inflamatório que muitas vezes pode ser causado por microrganismos como a *Chlamydia trachomatis* e que levam a presença de infiltrado linfocitário. Recentemente, estudos verificaram que a FASN esta relacionada com a sinalização do sistema imunológico (CARROLL *et al.*, 2018; QIAN *et al.*, 2018). Apesar disso, a expressão da FASN foi progressivamente mais intensa nas amostras de LSIL, HSIL e carcinoma do que na cervicite.

Na sequência, foram estudados os efeitos da inibição da FASN em diferentes células de câncer de colo de útero. Para tanto, utilizamos o fármaco orlistate, já conhecido por levar a uma diminuição na viabilidade celular e morte por apoptose em diferentes tipos tumorais (KRIDEL *et al.*, 2004; CARVALHO *et al.*, 2008; PALLASCH *et al.*, 2008; CHUANG *et al.*, 2011; GRUBE *et al.*, 2014). Nossos resultados indicaram que a inibição da FASN pelo ORL foi capaz de diminuir a proliferação celular, tendo em vista que o número de células viáveis diminuiu em todas as concentrações testadas. Além disso, foi possível observar que as células que sobreviveram ao tratamento de 72 h com ORL, apresentaram uma menor capacidade de formar novas colônias. No entanto, as linhagens SiHa e HeLa, que apresentaram uma maior expressão da FASN, também mostraram uma maior redução na proliferação celular quando comparadas com as linhagens C-33A e ME-180. O tratamento com ORL foi capaz ainda de causar apoptose e parada no ciclo celular e autofagia em todas as linhagens de câncer de colo de útero. Embora mais estudos ainda sejam necessários para confirmar a autofagia, nossos dados estão de acordo com Grube et al. que mostrou que o tratamento com ORL foi capaz de induzir autofagia em culturas de células de glioma (GRUBE *et al.*, 2014). Além disso, Peng et al. mostrou que o ORL pode induzir apoptose e autofagia simultaneamente em culturas de células de câncer de ovário. No entanto, quando eles trataram as células com um inibidor de autofagia, o número de células apoptóticas aumentou, sugerindo que o ORL teria um papel pró-sobrevivência, podendo assim ser utilizado juntamente com outros fármacos para melhorar a terapia oncológica (PENG *et al.*, 2018).

No entanto, apesar dos resultados com o ORL serem promissores, este fármaco apresenta limitações para sua utilização como antitumoral. O ORL é extremamente insolúvel em água, tem baixa permeabilidade intestinal e possui baixa biodisponibilidade oral (LUPU e MENENDEZ, 2006). Tendo em vista estas características, no segundo capítulo desta dissertação, foi proposta e desenvolvida uma nanoformulação de ORL com o objetivo de melhorar sua capacidade de inibição da FASN. A nanotecnologia aplicada à farmacologia é uma ferramenta que possui vantagens em relação à utilização de fármacos na sua forma livre. Ela permite melhorar a biodisponibilidade e internalização celular dos fármacos pelas células, controlar sua liberação e direcionar o fármaco para um alvo específico. Através dessas características, a nanotecnologia permite diminuir a dose de medicamentos e com isso levar a uma diminuição da toxicidade e na frequência de efeitos adversos, melhorando a adesão pelos pacientes e tornando a terapia mais segura e eficaz (SUN *et al.*, 2017). Dessa forma, foram formuladas nanocápsulas de poli(ε-caprolactona) (PCL), um polímero biodegradável que possui alta biocompatibilidade e estabilidade. Além disso, sistemas de administração de fármacos baseados em nanocápsulas de PCL permitem melhorar a distribuição e aumentar a tempo de liberação dos fármacos, trazendo benefícios para o tratamento. A utilização de PCL é aprovada pelo FDA e possui compatibilidade com diferentes vias de administração, tais como a via oral, intravenosa, subcutânea e tópica (POHLMANN *et al.*, 2013; GROSSEN *et al.*, 2017).

Neste sentido, os efeitos das nanocápsulas de ORL na viabilidade celular foram avaliados utilizando-se a linhagem de cultura de câncer cervical HeLa. Foi possível verificar que as nanocápsulas contendo ORL foram capazes de diminuir o número de células viáveis de uma maneira mais acentuada que o fármaco livre (não-encapsulado). Estes resultados estão de acordo com estudos anteriores que demonstraram que nanoformulações de ORL podem ser uma alternativa para o tratamento de diferentes tipos de câncer (BHARGAVA-SHAH *et al.*, 2016; HILL *et al.*, 2016; PAULMURUGAN *et al.*, 2016).

Estes resultados, em conjunto, representam uma importante contribuição para que novas formas de tratamento do câncer de colo de útero sejam desenvolvidas. A nanoencapsulação do ORL pode ser uma alternativa viável para o aumento da sua biodisponibilidade oral ou para o desenvolvimento de implantes intrauterinos, requerendo estudos adicionais. Além disso, a FASN se mostrou um alvo terapêutico importante já que está presente nas lesões precursoras do câncer de colo de útero.

Como mencionado anteriormente, quando não tratadas essas lesões podem progredir para o câncer invasor e, desta forma, estratégias que permitam impedir a progressão tumoral são extremamente importantes.

5.2.Limitações e perspectivas

Este trabalho apresentou algumas limitações e que podem ser ainda melhor exploradas em estudos futuros. Nosso trabalho apresentou o grande mérito de avaliar, concomitantemente, a expressão da FASN tanto em modelos celulares quanto em amostras de pacientes com lesões envolvidas na carcinogênese cervical. Entretanto, uma dificuldade comum neste tipo de trabalho é o baixo tamanho amostral, bem como o estabelecimento de um controle adequado para comparação das análises. Sabe-se que a expressão de proteínas em amostras biológicas pode variar muito e, assim, seria interessante verificar a expressão da FASN em um número maior de amostras e ainda correlacionar a expressão com achado epidemiológicos e clínicos das pacientes.

Outra questão interessante que ainda pode ser verificada é a correlação da FASN com o HPV. Como comentando anteriormente, muito estudos tem demostrado que a FASN pode ser importante para que a infeção viral ocorra mais facilmente e o vírus se replique de forma mais efetiva. Tendo em vista que a infecção pelo HPV é um dos principais fatores de risco para o desenvolvimento do câncer de colo de útero, seria de extrema importância verificar se a FASN contribui neste mecanismo. A investigação da relação da FASN com as proteínas oncogênicas do HPV podem novamente proporcionar alternativas de tratamento, eventualmente mais relacionadas à infecção e menos às modificações teciduais acarretadas.

Ainda, em relação ao ORL, é necessário realizar mais estudos para investigar o papel da autofagia na diminuição da viabilidade celular. A autofagia apresenta-se de forma ambígua, com trabalhos indicando sua importância na carcinogênese e outros deixando seu papel de forma secundária. (KRIEL e LOOS, 2019). Por isso, seria importante entender melhor a ligação entre a inibição da FASN e este processo.

Com relação às nanocápsulas de ORL, seria necessário aprofundar os estudos do papel na nanoencapsulação no controle da liberação do fármaco e na internalização pelas células. Além disso, apesar de já termos conhecimento de alguns mecanismos pelos quais o ORL leva a uma diminuição da viabilidade celular das

linhagens de câncer de colo de útero, seria necessário confirmar se os mesmos mecanismos ocorrem quando as células são tratadas com as nanocápsulas de ORL.

Finalmente, ambos os estudos, foram realizados em culturas de células. Embora tenhamos utilizado diferentes linhagens, com características diferentes e que nossos resultados sejam promissores, sabemos que podem existir diferenças quando os mesmos testes são avaliados *in vitro*. Portanto, seria interessante testar o ORL e as nanocápsulas em modelos animais.

Apesar das limitações citadas, os resultados encontrados podem servir de base para que mais estudos correlacionando a FASN e o câncer de colo de útero sejam realizados, estabelecendo uma abordagem promissora para estudos futuros.

5.3.Conclusões gerais

Nossos resultados comprovam que a FASN está expressa no câncer de colo de útero e que parece ser importante para progressão tumoral, já que está presente em lesões intraepiteliais de baixo grau (LSIL) e alto grau (HSIL). Estas lesões quando não diagnosticadas e tratadas podem favorecer a carcinogênese. Além disso, as linhagens de câncer de colo de útero HeLa, SiHa, C-33A e ME-180 apresentaram expressão de FASN de maneira diferente, sendo as linhagens HeLa e SiHa, as que apresentaram maior expressão.

Ainda, nosso trabalho demonstra que o ORL é capaz de inibir a FASN e assim impedir o desenvolvimento tumoral em linhagens celulares de câncer cervical, uma vez que sua utilização leva a diminuição na viabilidade celular, morte por apoptose, parada no ciclo celular e autofagia.

Além disso, as nanocápsulas contendo ORL que foram desenvolvidas como estratégia para melhorar os aspectos biofarmacêuticos do fármaco, demonstraram um maior efeito na diminuição da viabilidade celular da linhagem HeLa quando comparadas ao fármaco livre.

Sendo assim, os resultados apresentados indicam que a inibição da FASN pode ser uma estratégia terapêutica promissora para tratamento do câncer de colo de útero.

REFERÊNCIAS

AGOSTINI, M. et al. The fatty acid synthase inhibitor orlistat reduces the growth and metastasis of orthotopic tongue oral squamous cell carcinomas. **Mol Cancer Ther,** v. 13, n. 3, p. 585-95, Mar 2014.

ALO, P. L. et al. Fatty acid synthase (FAS) predictive strength in poorly differentiated early breast carcinomas. **Tumori,** v. 85, n. 1, p. 35-40, Jan-Feb 1999.

ARAGONES, G. et al. Infection with HIV and HCV enhances the release of fatty acid synthase into circulation: evidence for a novel indicator of viral infection. **BMC Gastroenterol,** v. 10, p. 92, Aug 13 2010.

BAKER, T. S. et al. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. **Biophys J,** v. 60, n. 6, p. 1445-56, Dec 1991.

BANDYOPADHYAY, S. et al. Mechanism of apoptosis induced by the inhibition of fatty acid synthase in breast cancer cells. **Cancer Res,** v. 66, n. 11, p. 5934-40, Jun 1 2006.

BETHESDA. **The Bethesda System for Reporting Cervical Cytology - Definitions, Criteria and Explanatory Notes**. Third. New York: Springer International Publishing, 2015.

BHARGAVA-SHAH, A. et al. Orlistat and antisense-miRNA-loaded PLGA-PEG nanoparticles for enhanced triple negative breast cancer therapy. **Nanomedicine (Lond),** v. 11, n. 3, p. 235-47, Feb 2016.

BRAY, F. et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. **CA Cancer J Clin,** v. 68, n. 6, p. 394-424, Nov 2018.

BRENNER, A. et al. Abstract P6-11-09: Heavily pre-treated breast cancer patients show promising responses in the first in human study of the first-In-class fatty acid synthase (FASN) inhibitor, TVB-2640 in combination with paclitaxel. **Cancer Research,** v. 77, n. 4 Supplement, p. P6-11-09-P6-11-09, 2017.

BRENNER, A. J. et al. First-in-human investigation of the oral first-in-class fatty acid synthase (FASN) inhibitor, TVB-2640. **Journal of Clinical Oncology,** v. 33, n. 15_suppl, p. TPS2615-TPS2615, 2015.

CARROLL, R. G. et al. An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation. **J Biol Chem,** v. 293, n. 15, p. 5509-5521, Apr 13 2018.

CARVALHO, M. A. et al. Fatty acid synthase inhibition with Orlistat promotes apoptosis and reduces cell growth and lymph node metastasis in a mouse melanoma model. **Int J Cancer,** v. 123, n. 11, p. 2557-65, Dec 1 2008.

CHAJES, V. et al. Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. **Cancer Res,** v. 66, n. 10, p. 5287-94, May 15 2006.

CHIRALA, S. S.; WAKIL, S. J. Structure and function of animal fatty acid synthase. **Lipids,** v. 39, n. 11, p. 1045-53, Nov 2004.

CHUANG, H. Y.; CHANG, Y. F.; HWANG, J. J. Antitumor effect of orlistat, a fatty acid synthase inhibitor, is via activation of caspase-3 on human colorectal carcinoma-bearing animal. **Biomed Pharmacother,** v. 65, n. 4, p. 286-92, Jul 2011.

COLEMAN, D. T.; BIGELOW, R.; CARDELLI, J. A. Inhibition of fatty acid synthase by luteolin post-transcriptionally down-regulates c-Met expression independent of proteosomal/lysosomal degradation. **Mol Cancer Ther,** v. 8, n. 1, p. 214-24, Jan 2009.

COSTELLO, L. C.; FRANKLIN, R. B. 'Why do tumour cells glycolyse?': from glycolysis through citrate to lipogenesis. **Mol Cell Biochem,** v. 280, n. 1-2, p. 1-8, Dec 2005.

DARNELL, G. A. et al. Human papillomavirus E7 requires the protease calpain to degrade the retinoblastoma protein. **J Biol Chem,** v. 282, n. 52, p. 37492-500, Dec 28 2007.

DE VILLIERS, E. M. et al. Classification of papillomaviruses. **Virology,** v. 324, n. 1, p. 17-27, Jun 20 2004.

DENNY, L. et al. Cervical Cancer. In: GELBAND, H.;JHA, P.*, et al* (Ed.). **Cancer: Disease Control Priorities, Third Edition (Volume 3)**. Washington (DC), 2015. ISBN 9781464803499
9781464803697.

FLAVIN, R. et al. Fatty acid synthase as a potential therapeutic target in cancer. **Future Oncol,** v. 6, n. 4, p. 551-62, Apr 2010.

GHITTONI, R. et al. Role of human papillomaviruses in carcinogenesis. **Ecancermedicalscience,** v. 9, p. 526, 2015.

GROSSEN, P. et al. PEG-PCL-based nanomedicines: A biodegradable drug delivery system and its application. **J Control Release,** v. 260, p. 46-60, Aug 28 2017.

GRUBE, S. et al. Overexpression of fatty acid synthase in human gliomas correlates with the WHO tumor grade and inhibition with Orlistat reduces cell viability and triggers apoptosis. **J Neurooncol,** v. 118, n. 2, p. 277-287, Jun 2014.

HATZIVASSILIOU, G. et al. ATP citrate lyase inhibition can suppress tumor cell growth. **Cancer Cell,** v. 8, n. 4, p. 311-21, Oct 2005.

HEATON, N. S. et al. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. **Proc Natl Acad Sci U S A,** v. 107, n. 40, p. 17345-50, Oct 5 2010.

HILL, T. K. et al. Development of a Self-Assembled Nanoparticle Formulation of Orlistat, Nano-ORL, with Increased Cytotoxicity against Human Tumor Cell Lines. **Mol Pharm,** v. 13, n. 3, p. 720-8, Mar 7 2016.

INCA, I. N. D. C. J. A. G. D. S.-. **Estimativa 2018: incidência de câncer no Brasil** Rio de Janeiro: 2017. 128.

JAYAKUMAR, A. et al. Human fatty acid synthase: properties and molecular cloning. **Proc Natl Acad Sci U S A,** v. 92, n. 19, p. 8695-9, Sep 12 1995.

KRIDEL, S. J. et al. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. **Cancer Res,** v. 64, n. 6, p. 2070-5, Mar 15 2004.

KRIEL, J.; LOOS, B. The good, the bad and the autophagosome: exploring unanswered questions of autophagy-dependent cell death. **Cell Death Differ**, Jan 18 2019.

KUHAJDA, F. P. Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. **Nutrition,** v. 16, n. 3, p. 202-8, Mar 2000.

KUHAJDA, F. P. et al. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. **Proc Natl Acad Sci U S A,** v. 97, n. 7, p. 3450-4, Mar 28 2000.

LI, J. N. et al. Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. **Cancer Res,** v. 61, n. 4, p. 1493-9, Feb 15 2001.

LITTLE, J. L.; KRIDEL, S. J. Fatty acid synthase activity in tumor cells. **Subcell Biochem,** v. 49, p. 169-94, 2008.

LUPU, R.; MENENDEZ, J. A. Pharmacological inhibitors of Fatty Acid Synthase (FASN)--catalyzed endogenous fatty acid biogenesis: a new family of anti-cancer agents? **Curr Pharm Biotechnol,** v. 7, n. 6, p. 483-93, Dec 2006.

MAHMOODI, P. et al. Early detection of cervical cancer based on high-risk HPV DNA-based genosensors: A systematic review. **Biofactors**, Nov 29 2018.

MAIER, T.; JENNI, S.; BAN, N. Architecture of mammalian fatty acid synthase at 4.5 A resolution. **Science,** v. 311, n. 5765, p. 1258-62, Mar 3 2006.

MAIER, T.; LEIBUNDGUT, M.; BAN, N. The crystal structure of a mammalian fatty acid synthase. **Science,** v. 321, n. 5894, p. 1315-22, Sep 5 2008.

MAIER, T. et al. Structure and function of eukaryotic fatty acid synthases. **Q Rev Biophys,** v. 43, n. 3, p. 373-422, Aug 2010.

MARTH, C. et al. Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. **Ann Oncol,** v. 29, n. Supplement_4, p. iv262, Oct 1 2018.

MEDES, G.; THOMAS, A.; WEINHOUSE, S. Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. **Cancer Res,** v. 13, n. 1, p. 27-9, Jan 1953.

MENENDEZ, J. A.; LUPU, R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. **Nat Rev Cancer,** v. 7, n. 10, p. 763-77, Oct 2007.

_____. Fatty acid synthase (FASN) as a therapeutic target in breast cancer. **Expert Opin Ther Targets,** v. 21, n. 11, p. 1001-1016, Nov 2017.

MULLEN, G. E.; YET, L. Progress in the development of fatty acid synthase inhibitors as anticancer targets. **Bioorg Med Chem Lett,** v. 25, n. 20, p. 4363-9, Oct 15 2015.

MUNOZ, N. et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. **N Engl J Med,** v. 348, n. 6, p. 518-27, Feb 6 2003.

OGINO, S. et al. Cohort study of fatty acid synthase expression and patient survival in colon cancer. **J Clin Oncol,** v. 26, n. 35, p. 5713-20, Dec 10 2008.

OHOL, Y. M. et al. Direct Inhibition of Cellular Fatty Acid Synthase Impairs Replication of Respiratory Syncytial Virus and Other Respiratory Viruses. **PLoS One,** v. 10, n. 12, p. e0144648, 2015.

ORITA, H. et al. Selective inhibition of fatty acid synthase for lung cancer treatment. **Clin Cancer Res,** v. 13, n. 23, p. 7139-45, Dec 1 2007.

OSTOR, A. G. Natural history of cervical intraepithelial neoplasia: a critical review. **Int J Gynecol Pathol,** v. 12, n. 2, p. 186-92, Apr 1993.

PALLASCH, C. P. et al. Targeting lipid metabolism by the lipoprotein lipase inhibitor orlistat results in apoptosis of B-cell chronic lymphocytic leukemia cells. **Leukemia,** v. 22, n. 3, p. 585-92, Mar 2008.

PANYAM, J.; LABHASETWAR, V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. **Adv Drug Deliv Rev,** v. 55, n. 3, p. 329-47, Feb 24 2003.

PAPAEVANGELOU, E. et al. The effect of FASN inhibition on the growth and metabolism of a cisplatin-resistant ovarian carcinoma model. **Int J Cancer,** v. 143, n. 4, p. 992-1002, Aug 15 2018.

PAULMURUGAN, R. et al. Folate Receptor-Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple-Negative Breast Cancer. **Mol Cancer Ther,** v. 15, n. 2, p. 221-31, Feb 2016.

PEER, D. et al. Nanocarriers as an emerging platform for cancer therapy. **Nat Nanotechnol,** v. 2, n. 12, p. 751-60, Dec 2007.

PENG, H. et al. Orlistat induces apoptosis and protective autophagy in ovarian cancer cells: involvement of Akt-mTOR-mediated signaling pathway. **Arch Gynecol Obstet,** v. 298, n. 3, p. 597-605, Sep 2018.

PIYATHILAKE, C. J. et al. The expression of fatty acid synthase (FASE) is an early event in the development and progression of squamous cell carcinoma of the lung. **Hum Pathol,** v. 31, n. 9, p. 1068-73, Sep 2000.

PIZER, E. S. et al. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. **Cancer Res,** v. 58, n. 20, p. 4611-5, Oct 15 1998.

PIZER, E. S. et al. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. **Cancer Res,** v. 56, n. 12, p. 2745-7, Jun 15 1996.

PIZER, E. S. et al. Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. **Cancer Res,** v. 60, n. 2, p. 213-8, Jan 15 2000.

POHLMANN, A. R. et al. Poly(-caprolactone) microcapsules and nanocapsules in drug delivery. **Expert Opin Drug Deliv,** v. 10, n. 5, p. 623-38, May 2013.

QIAN, X. et al. Regulation of fatty acid synthesis in immune cells. **Scand J Immunol,** v. 88, n. 5, p. e12713, Nov 2018.

RENDINA, A. R.; CHENG, D. Characterization of the inactivation of rat fatty acid synthase by C75: inhibition of partial reactions and protection by substrates. **Biochem J,** v. 388, n. Pt 3, p. 895-903, Jun 15 2005.

RICHART, R. M. Proceedings: An assessment of the biology of cervical intraepithelial neoplasia. **Proc Natl Cancer Conf,** v. 7, p. 219-22, 1972.

SANTOS, C. R.; SCHULZE, A. Lipid metabolism in cancer. **FEBS J,** v. 279, n. 15, p. 2610-23, Aug 2012.

SCHCOLNIK-CABRERA, A. et al. Orlistat as a FASN inhibitor and multitargeted agent for cancer therapy. **Expert Opin Investig Drugs,** v. 27, n. 5, p. 475-489, May 2018.

SEGUIN, F. et al. The fatty acid synthase inhibitor orlistat reduces experimental metastases and angiogenesis in B16-F10 melanomas. **Br J Cancer,** v. 107, n. 6, p. 977-87, Sep 4 2012.

SHEN, M. et al. FASN-TGF-beta1-PD-L1 axis contributes to the development of resistance to NK cell cytotoxicity of cisplatin-resistant lung cancer cells. **Biochim Biophys Acta Mol Cell Biol Lipids,** v. 1863, n. 3, p. 313-322, Mar 2018.

SMITH, J. S. et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. **Int J Cancer,** v. 121, n. 3, p. 621-32, Aug 1 2007.

SOKOLOWSKA, E. et al. Orlistat Reduces Proliferation and Enhances Apoptosis in Human Pancreatic Cancer Cells (PANC-1). **Anticancer Res,** v. 37, n. 11, p. 6321-6327, Nov 2017.

SOUCHEK, J. J. et al. Combination Treatment with Orlistat-Containing Nanoparticles and Taxanes Is Synergistic and Enhances Microtubule Stability in Taxane-Resistant Prostate Cancer Cells. **Mol Cancer Ther,** v. 16, n. 9, p. 1819-1830, Sep 2017.

STOIBER, K. et al. Targeting de novo lipogenesis as a novel approach in anti-cancer therapy. **Br J Cancer,** v. 118, n. 1, p. 43-51, Jan 2018.

SUN, Z. et al. Recent Advances in Application of Poly-Epsilon-Caprolactone and its Derivative Copolymers for Controlled Release of Anti-Tumor Drugs. **Curr Cancer Drug Targets,** v. 17, n. 5, p. 445-455, 2017.

SWINNEN, J. V. et al. Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. **Biochem Biophys Res Commun,** v. 302, n. 4, p. 898-903, Mar 21 2003.

VAN DE SANDE, T. et al. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. **Cancer Res,** v. 62, n. 3, p. 642-6, Feb 1 2002.

WALBOOMERS, J. M. et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. **J Pathol,** v. 189, n. 1, p. 12-9, Sep 1999.

WARBURG, O. On respiratory impairment in cancer cells. **Science,** v. 124, n. 3215, p. 269-70, Aug 10 1956.

WEISS, L. et al. Fatty-acid biosynthesis in man, a pathway of minor importance. Purification, optimal assay conditions, and organ distribution of fatty-acid synthase. **Biol Chem Hoppe Seyler,** v. 367, n. 9, p. 905-12, Sep 1986.

WHITE, E. A. et al. Comprehensive analysis of host cellular interactions with human papillomavirus E6 proteins identifies new E6 binding partners and reflects viral diversity. **J Virol,** v. 86, n. 24, p. 13174-86, Dec 2012.

WHO, W. H. O.-. Human papillomavirus (HPV) and cervical cancer. 2019. Disponível em: < https://www.who.int/en/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer >.

WU, X. et al. Molecular mechanisms of fatty acid synthase (FASN)-mediated resistance to anti-cancer treatments. **Adv Biol Regul,** v. 54, p. 214-21, Jan 2014.

YANG, W. et al. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. **Hepatology,** v. 48, n. 5, p. 1396-403, Nov 2008.

ZHANG, H. et al. Differential regulation of host genes including hepatic fatty acid synthase in HBV-transgenic mice. **J Proteome Res,** v. 12, n. 6, p. 2967-79, Jun 7 2013.

ZHANG, N.; ZHAO, H.; ZHANG, L. Fatty acid synthase promotes the palmitoylation of Chikungunya virus nsP1. **J Virol**, Nov 7 2018.

ZHOU, W. et al. Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. **Cancer Res,** v. 63, n. 21, p. 7330-7, Nov 1 2003.

ZUR HAUSEN, H. Papillomaviruses and cancer: from basic studies to clinical application. **Nat Rev Cancer,** v. 2, n. 5, p. 342-50, May 2002.