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Pharmacological fatty acid synthase inhibitors differently affect the malignant phenotype of oral cancer cells.

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ABSTRACT

Objective: Fatty acid synthase levels are associated with aggressiveness, prognosis, and risk of metastasis in oral squamous cell carcinomas. This enzyme contains seven catalytic domains and its inhibition by synthetic or natural drugs has antineoplastic properties such as C75, which is a synthetic inhibitor of the β- ketoacyl synthase domain, the antibiotic triclosan, ligand of the enoyl reductase domain, and the antiobesity drug orlistat, which inhibits the thioesterase domain. Here, we sought to investigate and compare the *in vitro* effects of C75, triclosan, and orlistat on malignant phenotypes of the cell line SCC-9: proliferation, cell cycle, apoptosis, adhesion, migration, and invasion.

Design: Half-maximal inhibitory concentration (IC_{50}) was determined using cell viability assays. Cell death and cell cycle progression were analyzed by Annexin V-PE/7-ADD-PerCP labeling and propidium iodide staining, respectively. Cell migration and invasion were assayed by transwells assays and cell adhesion using collagen and fibronectin.

Results: C75 showed the lowest IC_{50} and higher inhibition of lipid droplets at low concentrations and reduced cell motility. Triclosan showed the intermediate IC_{50} value, excellent reduction of lipid bodies at the IC_{50} when compared with C75 and orlistat. Also, triclosan reduced cell cycle progression, adhesion, migration, and invasion of SCC-9 and induced the highest levels of apoptosis. Orlistat promoted cell cycle arrest, but showed the lowest induction of apoptosis and did not affected invasion and adhesion of SCC-9.

Conclusion: Altogether, despite the particular effects of the analyzed fatty acid synthase inhibitors, triclosan showed to better interfere in tumorigenic phenotypes of SCC-9 cells.

Keywords: Fatty acid synthase, FASN inhibitors, oral cancer

INTRODUCTION

Head and neck cancer is the sixth most common cancer worldwide and annually accounts more than 650,000 cases and 330,000 deaths (Bray et al., 2018). More than 90% of all types of head and neck cancers are squamous cell carcinomas that affect the oral mucosa (Montero & Patel, 2015). Clinically, oral squamous cell carcinoma (OSCC) shows a locally aggressive behavior, frequent recurrences, and regional or distant metastases (Ho et al., 2017). Despite the efforts and new modalities of treatments, the mortality rates of OSCC remain unchanged for decades, with a 5-year survival rate of around 50% (Cohen Goldemberg et al., 2018).

The reprogramming of energy metabolism plays an important role in carcinogenesis and has been included as a hallmark of cancer by providing metabolic targets for cancer treatment (Hanahan & Weinberg, 2011). Accordingly, several studies demonstrate an aberrant expression of the enzyme fatty acid synthase (FASN) and increased endogenous lipogenesis in several human cancers and its precursors, including OSCC (Angeles & Hudkins, 2016; Menendez & Lupu, 2017; Silva et al., 2008; Silva et al., 2009, Silva et al., 2010). FASN is the multi-enzyme protein responsible for the *de novo* biosynthesis of long-chain fatty acids, especially palmitate, from acetyl-CoA and malonyl-CoA (Angeles & Hudkins, 2016; Kuhajda, 2000). Structurally, FASN is formed by two polypeptide chains (~270 kDa) containing seven different catalytic domains sequentially organized from the N-terminal to the C-terminal: β-ketoacyl synthase, acetyl-CoA transacylases and malonyl-CoA transacylases, dehydratase, enoyl reductase, β-ketoacyl reductase, and thioesterase site and the acyl carrier protein (Menendez & Lupu, 2017; Smith et al., 2003).

Under normal conditions, FASN levels in human tissues are usually low, since the cells utilize circulating lipids mainly from the diet (Menendez et al., 2004). In contrast, cancer cells highly express FASN to fuel membrane production, lipid-based post-translational protein modifications, redox balance maintenance, and energy metabolism (Menendez & Lupu, 2017; Röhrig & Schulze, 2016). In OSCC, FASN is overexpressed, associated with overall survival rates, and with the histological grade, lymphatic permeation, perineural infiltration, and lymph node metastasis (Silva et al., 2009; Silva et al., 2008).

In the last decade, several natural or synthetic FASN inhibitors have been described to promote anticancer effects by interfering in the lipogenic dependency (Angeles & Hudkins, 2016; Buckley et al., 2017; Menendez & Lupu, 2017; Chu et al., 2017). Among the most studied are cerulenin, and its synthetic derivative C75, the

antibiotic triclosan (TCS) and orlistat (ORL), a drug approved by FDA for the treatment of obesity (Liu, 2006; Lupu & Menendez, 2006; Rendina & Cheng, 2005; Wang et al., 2009). Cerulenin and C75 interact with the β-ketoacyl synthase domain and irreversibly inhibit the condensation reaction and (Rendina & Cheng, 2005). TCS blocks FASN activity through hydrogen bonding and hydrophobic interactions with specific amino acids in the enoyl reductase domain (Liu et al., 2002; Menendez & Lupu, 2017). On the other hand, ORL inhibits FASN activity through a covalent adduct with the thioesterase domain (Kridel et al., 2004; Menendez & Lupu, 2017).

Importantly, pharmacological or genetic inhibition of FASN selectively induces cancer cell death and reduction of tumor progression in mouse models (Angeles & Hudkins, 2016; Jones & Infante, 2015; Menendez & Lupu, 2017). Besides the classical FASN inhibitors, next-generation drugs have shown significant effects with high selectivity. IPI-9119, a specific non-commercial inhibitor of the thioesterase domain of FASN, is able to reduce the growth of prostate cancer xenografts and human organoids by inducing substantial metabolic reprogramming (Zadra et al., 2019). TVB-3166 is an imidazopyridine-based molecule which inhibits the β-ketoacyl reductase domain, reduces proliferation of several cancer cell lines by interfering in oncogenic pathways, especially PI3K-AKT-mTOR and β-catenin, and, when combined with paclitaxel, decreases the growth of the prostate xenografts (Heuer et al., 2017; Oslob et al., 2013; Ventura et al., 2015). Our group recently demonstrated that TVB-3166 decreases the viability and migration and induces apoptosis and cell cycle arrest of OSCC (Aquino et al., 2020). Moreover, TVB-2640 (ASC40), an analogue of TVB-3166, is the first FASN inhibitor included in clinical trials for patients with solid tumors (NCT02223247), as well as in phase II trials for colon (NCT02980029), KRAS mutated non-small cell lung carcinomas (NCT03808558), astrocytomas (NCT03032484), ErbB2 positive breast cancer (NCT03179904) and glioblastomas (NCT05118776). Finally, Omeprazole, an inhibitor of proton pump, was recently reported to inhibit FASN in gastric epithelial cells (Chen et al., 2020) and was safety when administrated for patients with triple negative breast cancer in dosis that inhibited FASN (Sardesai et al., 2021). In fact, clinical trials aiming to evaluate the response of this drug in castration resistant prostate cancer and breast cancer are also being conducted (NCT04337580; NCT02595372).

Here we describe for the first time the effects of the pharmacological inhibition of distinct catalytic domains of FASN with C75, TCS, and ORL in SCC-9 cells.

MATERIALS AND METHODS

Cell culture

Human OSCC (SCC-9) cells (American Type Culture Collection –ATCC, Manassas, VA, USA) were originally isolated from a tongue squamous cell carcinoma of a 25 years-old male and its tumorigenic phenotype is well documented in the literature by ours and other groups (Agostini et al., 2014; de Andrade et al., 2017; Liu et al., 2002; Ramos et al., 2002; Rheinwald & Beckett, 1981). SCC-9 were cultured in DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% or 10% FBS (Cultilab, Campinas, Brazil), 400 ng/mL hydrocortisone (hydrocortisone sodium succinate - Eurofarma, Brazil) and 1:100 antibiotic/antimycotic solution (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2. C75, TCS, ORL, and the vehicle DMSO (Dimethylsulfoxide, Sigma, St Louis, MO, USA) were added to the culture medium at the concentrations described in Table 1.

Proliferation assay and calculation of the IC_{50}

Cell viability was determined by seeding 8 x 10^4 SCC-9 cells in 12-well plates with DMEM/F-12 containing 10% FBS. After 24 hours, cells were serum-starved for additional 24 hours for cell cycle synchronization. After, the medium was replaced by 500 µL of DMEM/F-12 supplemented with 2% FBS containing increasing concentrations of C75, TCS or ORL (Sigma) for 24 or 48 hours. The volume of the vehicle DMSO was adjusted for each concentration. Control cells were treated with the maximum volume of DMSO. Cell viability was determined after the incubation with 25 mg/mL of MTT (4,5-dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide (Sigma) for 4 hours. After this period, formazan crystals produced by viable cells were eluted in absolute ethanol and the absorbance was determined with the aid of a microplate reader (Bio-Rad, Hercules, CA, USA) at 550nM and corrected at 650 nM. Inhibitory concentration 50% (IC_{50}) values were calculated by the dose-effect curve using the CompuSyn software (Cambridge, MA, USA) as previously described (Chou & Martin, 2005).

Bodipy staining for lipid droplets

In order to confirm the FASN inhibition by C75, TCS, and ORL, we performed BODIPY™ (Invitrogen) staining for neutral lipids by plating 2 X 10^3 SCC-9 cells in each well of 8-well chamber slides (Lab-tek, Thermofisher-Nunc). After 24h, the medium was replaced by 500 µL of DMEM-F12 without FBS and, following additional 24h, cells were incubated with the concentrations corresponding to the $IC_{12.5}$, IC_{25}, and IC_{50} of the studied drugs diluted in DMEM/F12 with 2% FBS for 24 and 48h. After this period, lipid droplets were stained by adding 3.8 µM of bodipy in the cell media (DMEMF/12 with 2% FBS) for 1

hour at 37°C. Cells were washed with PBS, fixed with absolute formaldehyde with 10% $CaCl_2$ for 30 minutes, washed again, and the slides mounted in aqueous media. At least 10 micrographies for each condition were captured and analyzed in an epifluorescence microscope (Leica DMR, Wetzlar, Germany). The number of lipid bodies was counted and normalized by the corresponding cell area with the aid of the Image J software (National Institute of Health, Bethesda, Maryland, USA).

Cell cycle and apoptosis analysis

For the flow cytometry analyses, cells were plated in 25cm^2 culture flasks, serum-starved for 24h, and treated for 48h with three different concentrations of the FASN inhibitors. Cells were then harvested by trypsinization and centrifuged at 900xg. To determine SCC-9 distribution in each phase of the cell cycle, cells were fixed in cold 70% ethanol for at least 16h at -20°C, and, after washing in cold PBS treated with 10 µg/ml RNAse (Sigma) at 37°C for 1h and incubated with 50 µg/mL propidium iodide at 4°C for 2 hours. To determine the percentage of apoptotic and necrotic cells, cells were washed in PBS and incubated with Annexin V-FITC (1:100) and 7-AADPerCP (1:200) in a binding buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl 2 and 1.8 mM $CaCl_2$ in the dark for 20 minutes at room temperature. Ten thousand events were acquired, analyzed by flow cytometry on FL-1 or FL-2 channel of a FACS Calibur (BD Biosciences, San Jose, CA, USA) equipped with an argon laser for apoptosis and cell cycle respectively. The distribution of cells in the cycle was evaluated with the software ModFit (Verity Software House) and the percentage of apoptotic or necrotic cells calculated by the software CellQuest (Becton Dickinson and Company, San Jose, CA, USA).

Cell adhesion assays

Four wells of a 96 well plate were sensitized with 10 µg/cm^2 of type I collagen or fibronectin diluted in 100 µL of PBS and as, negative controls, with 100 µL of PBS. The plates were maintained for 24 hours at 4°C and, after this period, each well was washed with PBS and blocked with 200 µL 3% BSA for 2h at 37°C. After the treatment with FASN inhibitors for 48h, cells were trypsinized and resuspended in DMEM/F12 supplemented with 3% BSA. For each condition, 3 X 10^4 cells diluted in 100 µL of DMEM/F12 supplemented with 3% BSA were plated in each well of the previously sensitized plates and incubated for 45 minutes to 1h. The period of adhesion was determined by observation in a phase contrast microscope. Non-adherent cells were washed 3 times with 100 µL of PBS and the adherent cells fixed with 4% formalin for 15 minutes at room temperature. Cells were then stained with an aqueous solution of 1% toluidine blue and

1% borax for 5 minutes. The excess of dye was removed by vigorous washing in distilled water. After elution in 100 µL of a 1% SDS, the absorbances were determined at 655nM in a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell migration and invasion assays

The effects of C75, TCS, and ORL in the migratory phenotype of SCC-9 cells were evaluated by transwell migration assays. Prior to the plating, cells were serum-starved for 24 hours and treated with the IC_{50} of C75, TCS, or ORL for additional 24 hours. Briefly, 8 X 10^4 SCC-9 cells were resuspended in 200 µL of FBS-free DMEM/ F-12 and added in the upper compartment of each 8 µM pore Transwell™ nylon filter membrane insert. In the lower compartment of each transwell were added 1 mL of DMEM/F-12 containing 2% FBS and FASN inhibitors or vehicle. After 24 hours, migrating cells were fixed in 10% formalin for 10 minutes and stained by 1% toluidine blue. The inserts were washed with distilled water and the cells of the upper compartment carefully removed with distilled water-soaked cotton swabs. After elution with 500 µL of 1% SDS, the absorbances were read at 655nM in a microplate reader (Bio-Rad). Invasion assays were performed as previously described (Mendonça et al., 2017; Salo et al., 2015). First, 60 µl of a 3.22 µg/mL myogel solution were added in the upper chamber of 8-µM pore Transwell™ inserts and solidified at 37°C for 30 minutes. After this period, serum-starved SCC-9 cells (3 X 10^5 for each condition) were resuspended in serum-free medium containing the IC_{50} concentrations of each FASN inhibitor or DMSO and seeded onto the insert with solidified myogel. The lower compartments of the transwells were filled with 1 mL of DMEM/F-12 supplemented with 2% FBS and the respective FASN inhibitors or the vehicle. After 48h, invasive cells were fixed with 10% formalin for 10 minutes and stained with 1% toluidine blue for 1 hour at room temperature. After washing the non-adherent cells and elution with 500 µL of 1% SDS, the absorbances were read at 650 nM in a microplate reader (Bio-Rad).

Statistical analysis

All experiments were performed in technical replicates and repeated at least three times independently. The appropriate statistical tests were applied according to the data distribution. Normal distribution was verified using Shapiro-Wilk or D'Agostino-Pearson (Bodipy quantification) tests and comparisons between groups were performed using one-way ANOVA with Tukey's post-hoc analysis. The significance level was 95% ($p < 0.05$).

RESULTS

C75, TCS, and ORL reduce SCC-9 proliferation and induce distinct morphologic changes

The IC_{50} for each drug (Table 1) was calculated based on the amount of viable cells by using dose-response curves with MTT assays (Figure 1). Cell death was measured by the treatment with increasing concentrations of C75, TCS, and ORL for 24h (Figures 1A-C) or 48h (Figure 1D-F) and, as expected, we observed a dose-dependent effect in all analyzed conditions. C75 reduced the SCC-9 viability in a dose-dependent manner in 24h (Figure 1A) and 48h (Figure 1D) in concentrations lower than 30 μM. On the other hand, TCS was not effective in SCC-9 cells in lower concentrations but highly affected the SCC-9 viability in concentrations above 36 μM for 24h (Figure 1B) and 18 μM for 48h (Figure 1E). ORL reduced SCC-9 viability in concentrations higher than 100 μM for 24h (Figure 1C) and 50 μM for 48h (Figure 1F). The morphology of SCC-9 treated with C75, TCS, or ORL for 24h (Figure 1 G-J) or 48h (data not shown) was monitored using phase contrast microscopy and compared with DMSO treated cells. Each drug promoted distinct morphologic changes in SCC-9 cells, however, the morphology was not affected by the period of treatment (24 or 48h). As seen in Figure 1G, SCC-9 cells incubated with the vehicle showed an accentuated pleomorphism with the predominance of polygonal shaped cells, large nuclei, and evident nucleoli. The treatment with C75 promoted an evident cytoplasmatic enlargement and a decrease of the nucleus/cytoplasm ratio (Figure 1H, arrows). SCC-9 cells treated with TCS showed an important reduction in cell size and an increase of cytoplasmic granules (Figure 1I, arrows). Finally, treatment with ORL promoted an evident cytoplasmic retraction and cell elongation compared to the control group (Figure 1J). All compounds increased the number of detached rounded cells characteristic of dead cells (Figures 1H-J, arrowheads).

FASN inhibition reduce lipid droplets in SCC-9 cells

In order to confirm that the effects of C75, TCS, and ORL on SCC-9 cells are associated with the reduction of FASN activity, we used bodipy to stain intracellular lipid droplets. These studies showed that, compared to the vehicle (Figure 2A and Figure S1A), all the concentrations of C75 reduced the lipids droplets of SCC-9 cells in 24h (Figure S1) and 48h (Figure 2), and the concentration corresponding to its IC_{50} had the highest effect on FASN inactivation in 24h (83% of inhibition, Figures S1B, E). Similar effects were found by treating SCC9 cells with C75 for 48h (Figure 2A-E). On the other hand, TCS showed a dose-dependent effect and maximum FASN inhibition with the dose relative to the IC_{50}

when SCC9 were treated for 24h (95% of lipid droplets reduction, Figures S1C, F). Similar results were obtained when the cells were treated for 48h (Figure 2F-J). ORL was the less effective and reduced 28% and 38% of lipid bodies in SCC-9 cells treated with its IC_{25} and IC_{50} for 24h, respectively (Figures S1D, G). We also did not observed statistical differences when SCC9 were treated with the $IC_{12.5}$ of ORL for 48h (Figure 2O). However, the treatment with the IC_{25} and the IC_{50} of ORL for 48h of significantly reduced the lipid bodies of SCC9 cells (Figure 2K-O).

C75, TCS and ORL induces cell cycle arrest and apoptosis in SCC-9 cells

In order to better characterize the effects of FASN inhibitors on SCC-9 cells, we next performed cell cycle and apoptosis analysis by flow cytometry. The treatment with different concentrations of C75 (Figure 3A), TCS (Figure 3B), and ORL (Figure 3C) for 48 h resulted in the accumulation of cells in the G0/G1 phase and significant reduction of the S phase. The number of cells in the G2/M phases of the cell cycle was not affected by the studied drugs (Figure 3A-C). The IC_{50} of C75 increased in 23.19% the number of cells in G0/G1 (Control: 45.05%; C75 IC_{50}: 68.25%) and reduced in 17.62% the number of cells in the S phase (Control: 35.04%; C75 IC_{50}: 17.42%). The IC_{50} of TCS resulted in an accumulation of cells in G0/G1 of 35.81% (Control: 49.05%; TCS IC_{50}: 84.86%) while the S phase was reduced in 26.8% (Control: 32.39%; TCS IC_{50}: 5.59%). Finally, cells in G0/G1 were increased by ORL in 31.58% (Control: 52.08%; ORL IC_{50}: 83.67%) and the S phase reduced in 24.05% (Control: 29.82; ORL IC_{50}: 5.77%). To compare the effects of the studied drugs on proliferating SCC-9 cells, we performed statistical analyzes with the number of cells in S phase (Figures 3D-F) and found that TCS and ORL similarly affected cell cycle progression.

C75 (Figure 4A), TCS (Figure 4B), and ORL (Figure 4C) significantly reduce the number of viable cells and increase the number of apoptotic cells in a dose-dependent manner. As expected, the highest effect was observed in the concentration relative to the $IC_{50,}$ in which C75 (Figure 4A) and ORL (Figure 4C) increased the apoptotic levels in 5.5X and 4.58X, respectively (Control: 1.63; C75: 8.94 and Control: 1.13; ORL: 5.17). TCS showed the highest effect with an increase of the apoptotic cells in 21.89X (Control: 1.08; TCS IC_{50}: 21.89) (Figure 4B). At low concentrations, TCS was also effective promoting a dose dependent increase in apoptotic levels (1.75X for the $IC_{12.5}$ and 8.42X for the IC_{25}). Statistical analysis comparing the effects of the drugs using the concentration relative to the IC_{50} showed that TCS was the most effective drug in reducing the number of live cells (Figure 4D) and increasing the percentage of apoptotic cells (Figure 4E). Necrosis was not significantly affected by the drugs. Gate information were provided in the Figures S2 (cell cycle) and S3 (apoptosis).

Adhesion, migration, and invasion of SCC-9 cells were differently affected by C75, TCS, and ORL

In order to verify the effects of C75, TCS, and ORL on the adhesion of SCC-9 cells to extracellular matrix components, cells were treated for 48h with different concentrations of the drugs and allowed to adhere in previously sensitized plates with fibronectin or type I collagen. These studies showed a slight increase, not statistically significant, in the adhesion of SCC-9 cells treated with the $IC_{12.5}$. No significant reduction in the cell adhesion to the matrix components was observed in the IC_{25} and IC_{50} concentrations of C75 (Figure 5A). The adhesion of SCC-9 cells was significantly reduced with TCS in a dose-dependent manner (Figure 5B) while ORL did not affect the adhesion to both type I collagen and fibronectin (Figure 5C).

By using transwell cell migration (Figure 6A-D) and invasion (Figure 6F-I) assays, we observed that the treatment with the IC_{50} of C75, TCS, and ORL for 48h reduced in 56%, 65.7%, and 31% the migration of SCC-9 cells, respectively (Figure 6E). The invasive ability of SCC-9 cells were similarly reduced by C75 and TCS and not modified by ORL, when compared with the control cells (Figure 6J).

DISCUSSION

FASN is the key enzyme of the endogenous lipid metabolism and its activity is associated with signaling pathways and maintenance of the structure and function of cancer cell membranes (Buckley et al., 2017). Upregulation of FASN was demonstrated in several human malignancies, including oral cancer (Agostini et al., 2014; Aquino et al., 2020; Kelber et al., 2010; Ricklefs et al., 2020; Sadowski et al., 2014; Sippel et al., 2014). FASN has emerged as a therapeutic target due to its differential expression in normal and malignant tissues and its association with survival rates and prognosis (Röhrig & Schulze, 2016; Witkiewicz et al., 2008). Importantly, the multiple catalytic domains of FASN allowed the development of several FASN inhibitors through the last decade (Röhrig & Schulze, 2016). The first generation of FASN inhibitors provided the basis for the development and testing of new FASN-targeted drugs and clarifying the role of FASN and lipid metabolism in cancer cells (Menendez & Lupu, 2017). Classic FASN inhibitors, such as C75, TCS (triclosan), and ORL (orlistat), were reported to promote cell death *in vitro* and *in vivo* in several types of cancer cells, including OSCC (Agostini et al., 2014; Bastos et al., 2017; Menendez & Lupu, 2017; Zadra et al., 2019).

SCC-9, a model of OSCC shows a tumorigenic phenotype, documented in the literature by ours and other groups (Rheinwad and. Beckett, 1981; Ramos et al., 2001; Liu et al., 2002; De Andrade et al., 2017; Agostini et al., 2014). Here, we demonstrated that ORL has the highest IC_{50} when compared with C75 and TCS. Our group has previously shown the anti-tumoral effects of ORL in melanoma (Bastos et al., 2017; Carvalho et al., 2008; Seguin et al., 2012) and OSCC cells lines (Agostini et al., 2014) by using similarly high concentrations. On the other hand, a 50% reduction of the SCC-9 viability was found at smaller concentrations of C75 and TCS in both, 24h and 48h. There is no data in the literature regarding the effects of C75 and TCS in oral cancer cells. Though, our IC_{50} concentrations for C75 were similar to the results of previous studies in cells lines derived from breast, lung, and prostate cancers (Chen et al., 2012; Giró-Perafita et al., 2016; Menendez et al. , 2016; Puig et al., 2008; Relat et al., 2012; Sadowski et al., 2014). Also, the IC_{50} for TCS in retinoblastoma cells is 60 μM and between 4.5 to 6.9 μM in different prostate cancer cell lines (Deepa et al., 2012; Sadowski et al., 2014; Vandhana et al., 2013).

In our work, C75 reduced lipid droplets similarly at lower and higher TCS showed a dose-dependent reduction in lipogenesis. ORL also reduced the lipid bodies at its IC_{25} and IC_{50}. Despite their distinct rates of FASN inhibition, all drugs reduced the S phase

and increased the number of SCC-9 cells in the G0/G1 phases. Accordingly, several studies have demonstrated that FASN inhibitors promote a cell cycle arrest in several human cancer cell lines (Veigel et al., 2015). Agostini et al. (2014) demonstrated that the S phase of SCC-9 cells treated with ORL extracted from Orlistate (NeoQuímica Farmacêutica, Brazil) capsules for 72 h was reduced by approximately 75%. Similarly, Aquino et al. (2020) showed that TVB-3166, a new imidazopyridine-based FASN inhibitor, reduces the number of cells in the S phase by 57% in a highly metastatic OSCC carcinoma cell line LN-1A. The mechanisms by which FASN inhibitors affect the cell cycle progression are not well described. Zadra et al. (2019) demonstrated that the treatment of prostate cancer cell lines with IPI-9119 reduced the cell cycle kinetics by decreasing the levels of cyclin A2. Other studies have shown that the inhibition of FASN in cancer cell lines may affect several proteins involved in cell cycle control (Agostini et al., 2014; Deepa et al., 2013; Sangeetha et al., 2015; Ventura et al., 2015). Scaglia et al. (2014) observed with the incorporation of ^{14}C choline that the levels of fatty acids in cell membranes increase during cell cycle progression and reached the peak in G2/M. According to the same authors, the presence of a lipogenic checkpoint in the G2/M phase could explain, at least in part, the reduction of the cell cycle progression in FASN-inhibited tumor cells.

We also observed here that the accumulation of SCC-9 cells in G0/G1 and inhibition of S phase was accompanied by apoptosis. Although the treatment with the low concentrations of C75 and ORL promotes cell cycle arrest, apoptotic levels were slightly or not affected. Conversely, IC_{50} concentration of these drugs significantly increased apoptosis. In fact, TCS was the most effective FASN inhibitor in order to increase apoptosis. TCS was effective even at low concentrations and produced a dose-dependent increase in apoptotic levels. TCS reduces cell growth and viability of tumor cell lines, such as breast cancer (MCF-7 and SKBr-3) end retinoblastoma (Y79) (Deepa et al., 2012; Liu et al., 2002). According to our findings, Sadowski et al., (2014) compared the inhibitory effect of TCS, C75, and ORL in normal and prostate cancer cell lines and found that TCS has a greater cytotoxic effect and the lowest IC_{50}. The biological mechanisms that underlie the cell cycle arrest and apoptosis promoted by the FASN inhibitors need future mechanistic investigation. However, previous studies of our group has shown that FASN activity is necessary for proliferation and survival of OSCC cells considering that both, pharmacological or specific FASN inhibition, reduced the total lipid biosynthesis in OSCC cells (by incorporation studies of 14C-acetate).

Here we found that while ORL did not affect cell adhesion and invasion and slightly decreases migration of SCC-9 cells, C75 and TCS reduced in more than 50%

migration and invasion. In addition, C75 did not significantly affected the adhesion of SCC-9 to type I collagen and fibronectin and TCS promoted a dose-dependent reduction of cell adhesion to the studied extracellular matrix components. The role of FASN in adhesion, migration, and invasion processes are still not clear. Our group has shown that TVB-3166 significantly increases adhesion of SCC-9 and LN-1A cells to myogel, a matrix prepared from human uterine leiomyoma composed mainly by fibronectin, laminin, integrins and different collagens but reduced migration of SCC-9 cells. Zaytseva et al., (2012) showed that FASN inhibition with siRNA in colon cancer cells reduces the levels of MET, Akt, FAK, and paxillin, which are involved in cell adhesion and invasion. Jafari et al., (2019) suggested that the inhibition of FASN by TVB-3664 decreases the number of adhesion plaques in primary and metastatic colorectal cancer cells by immunofluorescence reactions for p-FAK and p-paxillin. Recently, De Piano et al., (2020) observed that FASN depletion using shRNA increases cell adhesion to matrigel and type I collagen in prostate cancer cell lines (CPTX 1542 and PC3) by increasing the paxillin levels and decreasing the palmitoylation of RhoU. Considering the controversial results in the literature and the different effects of C75, TCS, and ORL it is possible to speculate that cell type and the method used for the FASN inhibition can modulate the effect on cell adhesion. Despite the promising results *in vitro* and/or *in vivo,* is important to emphasize that C75, TCS and ORL are not available for clinical trials of cancer and demonstrated limitations of pharmacological properties and side-effects (Menendez & Lupu, 2017). In fact, new formulations using nanoparticles would be helpful to improve the drug permeability, absorption and bioavailability of the compounds (Chu et al., 2016).

In summary, we found that C75, TCS, and ORL have distinct effects in the tumorigenic phenotype of SCC-9 cells. C75 has the lowest IC_{50}, better inhibition of FASN at low concentrations, and an important reduction of the cell motility phenotypes, especially migration and invasion. TCS has the intermediate IC_{50} and an excellent reduction of lipid bodies at its IC_{50}. TCS also significantly reduces cell cycle progression, adhesion, migration, and invasion while induces high levels of apoptosis in SCC-9 cells. ORL is effective in promoting cell cycle arrest, but induces low levels of apoptosis and did not affect invasion and adhesion. These results indicate that TCS interfere in all the tumorigenic phenotypes of SCC-9 cells.

Author contributions

Willian Peter Boelcke – planned and performed experiments, analyzed data, wrote and approved the final version of the manuscript.

Isadora Ferrari Teixeira – planned and performed experiments, analyzed data, wrote and approved the final version of the manuscript.

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Ricardo Della Coletta – analyzed data, discussed the results, revised and approved the final version of the manuscript.

Edgard Graner - analyzed data, planned the experiments, discussed the results, revised and approved the final version of the manuscript.

Débora Campanella Bastos – conceived, planned and performed experiments, analyzed data, wrote, revised and approved the final version of the manuscript.

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Figure Legends:

Figure 1 - FASN inhibition with C75, TCS, and ORL reduces proliferation and induces distinct morphologic changes in SCC-9 cells. The IC50 was calculated using MTT viability assays after treating SCC-9 cells with FASN inhibitors for 24h (A-C) and 48h (D-F). C75 (A and D), TCS (B and E) and ORL (C and F) induced a dose-dependent SCC-9 cell death, allowing the determination of IC50. Compared to control cells (G), treatment with the IC50 of C75 (H) induced cytoplasmic retraction (arrow) and predominantly polygonal cells with abundant cytoplasm (arrow). (I) TCS reduced the cell size and increased the number of rounded cells (arrow) and the amount of cytoplasmic granules (arrow). ORL (J) induced a spindle pattern in SCC-9 cells (arrow). Highlighted rounded cells, characteristics of dead cells (arrowheads). Error bar = mean ± SD of at least 3 independent experiments. * $p < 0.05$ ANOVA and Tukey tests. Original 200X magnification.

Figure 2 – C75, TCS, and ORL reduces the amount of lipid bodies in SCC-9 cells. Compared with the control cells (A, F and K), the treatment with C75 (B-D), TCS (F-I) and ORL (L-N) significantly reduce the number of lipid bodies normalized by the cell area in comparison with to control cells. (E,J and O) number of lipid droplets stained by bodipy were normalized by the area of each cell. Error bars = mean ± SD of at least 50 cells in 10 microscopic fields (original magnification 400X). # different from all, * $p < 0.05$, ** $p < 0.0001$ ANOVA and Tukey tests (ORL) and Kruskall-Wallis (C75 and TCS).

Figure 3 – FASN inhibition promotes cell cycle arrest in G0/G1 and increases the S phase in SCC-9 cells. Treatment of SCC-9 cells with $IC_{12.5}$, IC_{25}, and IC_{50} for 48 hours of C75 (A) and ORL (C) induces a cell cycle arrest in the G0/G1 phase and reduces the number of cells in the S phase in a dose dependent manner. The treatment of SCC-9 cells with TCS (B) reduces S phase and increases the percentage of cells in the G0/G1at the IC_{25} and IC_{50}. The compounds equally affected the S-phase progression at the $IC_{12.5}$ (D) but atthe IC_{25} (E) and IC_{50} (F), TCS and ORL show higher inhibitory effect onS-phase, when compared to the C75. Error bars = mean ± SD of the experimental triplicate. * $p < 0.05$, ANOVA and Tukey tests.

Figure 4 – Apoptosis is induced by FASN inhibitors. The treatment of SCC-9 with the IC_{50} of C75 (A) and the IC_{25} and IC_{50} of TCS (B) reduces the number of viable cells and increases apoptotic cells. All tested concentrations of ORL (C) increase the number of apoptotic cells. TCS was the most effective when compared with C75 and ORL in order to reduce the number of living cells (D) and induce apoptosis (E). Error bar = mean ± SD of the experimental triplicate. * $p<0.05$, ** $p<0.005$, *** $p<0.0001$, ANOVA and Tukey tests.

Figure 5 - Cell adhesion is differently modulated by pharmacological FASN inhibitors. The treatment with C75 (A) resulted in a slight increase, not statistically significant, in SCC-9 adhesion at the $IC_{12.5}$ and IC_{50}. TCS significantly reduces SCC-9 adhesion to fibronectin and type I collagen in all the analyzed concentrations (B) and ORL (C) did not significantly affect the adhesion. Error bar = mean ± SD of the experimental triplicate. # different from all, * $p<0.05$, ANOVA and Tukey tests

Figure 6 – Migration and invasion phenotypes of SCC-9 cells are affected by the inhibition of FASN. Transwell migration (A-E) and invasion (F-J) assays with SCC-9 cells treated with the IC_{50} of C75, TCS and ORL. C75 (B) and TCS (C) showed a significant inhibition (E) of cell migration when compared to the control (A) and to the ORL (D). The invasiveness of the cells were significantly affected by C75 (G) and TCS (H) but not modulated by ORL (I-J). Original magnification 5X. Error bar = mean ± SD of the experimental triplicate. ANOVA and Tukey tests, # different from all, * $p<0.05$, ** $p<0.005$.

Supplementary file

Figure S1: C75, TCS, and ORL reduces the amount of lipid bodies in SCC-9 cells in 48h. The treatment with C75 (A-E), TCS (F-J) and ORL (K-O) significantly reduce the number of lipid bodies normalized by the cell area in comparison with to control cells. (E,J and O) number of lipid droplets satained by bodipy were normalized by the area of each cell. Error bars = mean ± SD of at least 50 cells in 10 microscopic fields (original magnification 400X). # different from all, * $p<0.05$, **$p<0.0001$ ANOVA and Tukey tests (ORL) and Kruskall-Wallis (C75 and TCS).

Figure S2: Cell cycle gate information.

Figure S3: Apoptosis gate information.