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RESEARCH

Cytotoxic evaluation of YSL-109 in a triple negative breast cancer cell line and toxicological evaluations

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Abstract

Breast cancer (BC) is the leading cause of cancer-related death in women worldwide. Triple negative breast cancer (TNBC) is the most aggressive form of BC being with the worst prognosis and the worst survival rates. There is no specific pharmacological target for the treatment of TNBC; conventional therapy includes the use of non-specific chemotherapy that generally has a poor prognosis. Therefore, the search of effective therapies against to TNBC continues at both preclinical and clinical level. In this sense, the exploration of different pharmacological targets is a continue task that pave the way to epigenetic modulation using novel small molecules. Lately, the inhibition of histone deacetylases (HDACs) has been explored to treat different BC, including TNBC. HDACs remove the acetyl groups from the ε-amino lysine resides on histone and non-histone proteins. In particular, the inhibition of HDAC6 has been suggested to be useful for the treatment of TNBC due to it is overexpressed in TNBC. Therefore, in this work, an HDAC6 selective inhibitor, the (S)-4-butyl-N-(1-(hydroxyamino)-3-(naphthalen-1-yl)-1-oxopropan-2-yl) benzamide (YSL-109), was assayed on TNBC cell line (MDA-MB231) showing an antiproliferative activity ($IC_{50} = 50.34 \pm 1.11$ μM), whereas on fibroblast, it was lesser toxic. After corroborating the in vitro antiproliferative activity of YSL-109 in TNBC, the toxicological profile was explored using combined approach with in silico tools and experimental assays. YSL-109 shows moderate mutagenic activity on TA-98 strain at 30 and 100 μM in the Ames test, whereas YSL-109 did not show in vivo genotoxicity and its oral acute toxicity (LD_{50}) in CD-1 female mice was higher than 2000 mg/kg, which is in agreement with our in silico predictions. According to these results, YSL-109 represents an interesting compound to be explored for the treatment of TNBC under preclinical in vivo models.

Keywords HDAC inhibitors · Triple negative breast cancer · Hydroxamic acid · Ames test · Oral acute toxicity · Micronucleus assays

Introduction

Breast cancer (BC) is the first leading cause of cancer-related death in women over the world (Sung et al. 2021). Approximately, 75% of the BCs express both estrogen receptor α (ERα) and progesterone receptor (PR), they are known as hormone positive. Hormone positive BCs are susceptible to endocrine therapies, such as ER modulators (tamoxifen) and aromatase inhibitors (letrozole and anastrozole), this type of cancer has better prognosis. There is another type of BC which expresses the human epidermal growth factor receptor 2 (HER2/ERBB2), a protooncogene receptor; it represents 15–20% of the total of BC types. For the treatment of HER2 positive ($HER2^+$) BC trastazumab, lapatinib or pertuzumab can be administered (Wu et al. 2015).

Almost 12–17% of BCs that do not express ERα, PR, and HER2 (ER^-, PR^-, $HER2^-$) are known as triple-negative

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BC (TNBC) (Almansour 2022). Other classification of BC is based on gene expression including luminal A, B, and $HER2^+$ (Al-Thoubaity 2020). TNBC is classified into four subtypes: basal cell-like type 1 (BL1), basal cell-like type 2 (BL2), mesenchymal subtype (M), and luminal androgen receptor subtype (LAR) (Lehmann et al. 2016). Overall, TNBC is characterized by poor prognosis; in fact, it is the worst in comparison to the other types of BC because it possesses a more clinically aggressive behavior with an early relapse (Fragomeni et al. 2018; Damaskos et al. 2019).

Until now, there is no clinically approved specific drug target for TNBC; there is only genotoxic chemotherapy which has a poor prognosis for the patients (Garmpis et al. 2017; Almansour 2022). Therefore, the search of effective therapies continues at preclinical and clinical level. In this sense, the exploration of different pharmacological targets is a continue task that pave the way to epigenetic modulation using novel small molecules. Lately, the inhibition of histone deacetylases (HDACs) has been explored to treat different BC, including TNBC (Maccallini et al. 2022). HDAC removes acetyl groups of Lys residues from histones and non-histone proteins. The non-histone proteins include α-tubulin, HF1, chaperone proteins, and transcription factors, among others. These enzymes regulate gene transcription, cell cycle progression, apoptosis, tumorigenesis, and angiogenesis (Seto and Yoshida 2014; Li and Seto 2016; Maccallini et al. 2022). HDACs are classified into four classes, the Zn-dependent class I (HDAC1-3, and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and 10), and class IV (HDAC11), and the NAD^+ dependent enzymes, known as sirtuins (Seto and Yoshida 2014).

HDAC6 is mainly found in the cytoplasm achieving the acetylation of non-histone proteins, including α-tubulin, transcription factor such as p53, heat shock protein 90 (Hsp90), and cortactin. It possesses unique structural features, since it contains two deacetylase catalytic domains, a ubiquitin hydrolase-like zinc finger (ZnF-UBP) domain, a Ser-Glu-containing tetra decapeptide (SE14) repeat domain, and a conserved nuclear export signal at the N-terminus (Sixto-Lopez et al. 2019; Pulya et al. 2021). Moreover, HDAC6 plays a pivotal role in carcinogenesis by oncogenic cell transformation, cell migration, and tissue invasion (Li et al. 2018). A higher expression of HDAC6 has been observed on TNBC (Park et al. 2011). The inhibition of HDAC6 reduces glycolytic metabolism and prevents cell migration, invasion, and MMP-9 expression on MDA-MB-231 cells (Park et al. 2011; Dowling et al. 2021). The ability of HDAC6 to regulate tissue invasion of the TNBC cells would be linked to its tubulin and cortactin deacetylase activity, as well as to the regulation of Hsp90-aurora-A-cofilin-F-actin and cortactin pathway, being an important regulator of cytoskeletal remodeling and cell migration (Rey et al. 2011; Hsieh et al. 2019; Oba et al. 2021). Additionally, the selective inhibition of HDAC6 could produce fewer side effects and a selective tumor inhibition profile (Tang et al. 2019).

Previously, our work group reported a selective HDAC6 inhibitor, the (S)-4-butyl-N-(1-(hydroxyamino)-3-(naphthalen-1-yl)-1-oxopropan-2-yl) benzamide, hereafter referred as YSL-109. It was assayed on HDAC6, HDAC8, and HDAC1 with an $IC_{50} = 0.537$ nM, 2.24 μM, and 259.439 μM, respectively. YSL-109 showed antiproliferative properties against MCF-7 cell line ($IC_{50} = 3.41$ μM), which is express ER, PR, and glucocorticoid receptors, it is a noninvasive cell line and highly responsive to chemotherapy. Additionally, YSL-109 shows antiproliferative activity against HCC-1954 cell line ($IC_{50} = 3.77$ μM), which is a highly invasive, metastatic cell line that overexpresses HER-2 and is ER/PR negative (Sixto-Lopez et al. 2020).

In view that YSL-109 is a selective HDAC6 inhibitor with antiproliferative activity in lesser aggressive types of BC cells than TNBC, we have decided to explore the antiproliferative activity of the YSL-109 on TNBC cells (MDA-MB231). Additionally, with prospects to offer a novel compound as potential drug for TNBC, the in silico, in vivo, and in vitro toxicological profile of YSL-109 was also assayed.

Material and methods

Compounds

YSL-109 was synthesized in our laboratory following a previously reported method (Sixto-Lopez et al. 2020, 2021). Trichostatin A (TSA), a pan-HDAC inhibitor, was purchased from Enzo Life Sciences (Farmingdale, NY).

Cell culture

MDA-MB-231 and NIH-3T3 cell lines were cultured in 75 cm^2 bottles in Dulbecco's modified Eagle's medium (DMEM Gibco, Life Technologies, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Biowest, Kansas City, MO, USA) and antibiotic and antimycotic agents (Gibco). The cell cultures were kept in an incubator at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability by MTT assay

First, cell lines were cultured in 75 cm^2 bottles. Once the cells achieve an 80% of confluently, they were counted and 1×10^4 of cells were deposited per well in transparent 96-well plates. Cells were incubated for 24 h in DMEM medium supplemented with 10% of FBS, antibiotic/antimycotic. Then, cells were incubated with YSL-109 at 0.3, 10.4, 20.9, 41.7, 83.5, and 166.9 μM and TSA at 0.1, 0.2, 0.4, 0.8, and 1 μM, as previously reported (Vigushin et al. 2001; Rahimian and

Mellati 2017). After 48 h of incubation, the supernatant was removed, then 20 µL of MTT solution (0.5 mg/mL) was added, and the cells were incubated at 37 °C for 4 h. After, the MTT solution was removed, formazan salts were solubilized with 100 µL of DMSO, and the absorbance of each well was read at 550 nm on a Microplate Photometer (Multiskan EX, Thermo Scientific, Mexico).

Computational toxicology prediction

An in silico toxicological risk assessment was performed to predict the preclinical toxicological properties of YSL-109. To this end, ProTox-II (Banerjee et al. 2018), Lazar (lazy structure–activity relationships) (Maunz et al. 2013), T.E.S.T. (Toxicity Estimation Software Tool) (Martin 2016), Vega 1.5.1 (Benfenati et al. 2013), and ADMETlab 2.0 (Xiong et al. 2021) were used. These programs are the most used for evaluating the toxicological properties of new compounds. We have considered more than one software to get consensus results using different algorithms for the in silico prediction (Fuart Gatnik and Worth 2010, Rim 2020; Gupta et al. 2021).

ProTox-II includes molecular similarity, pharmacophore base, fragment propensities, most common features, and machine learning model to make the predictions. This software predicts rodent median lethal dose (LD_{50}) in mg/kg weight, toxicity class, as well as hepatotoxicity, carcinogenicity, immunotoxicity, and mutagenicity (Banerjee et al. 2018). Acute toxicity in rodents was predicted with Protox-II to gain information on the LD_{50} of YSL-109 that could guide us in the selection of the type of experimental test to be carried out following the guideline TG 423 of the Organization for Economic Cooperation and Development (OECD). The TG 423 suggest to consider available information about the probable non-toxicity of the compound or when it is above the regulatory limit dose, the maximum limit to test it should be administrating the highest initial dose level (2000 mg/kg body weight) (OECD 2002).

The Lazar employs data mining algorithms to predict toxicological endpoints from experimental training data, specifically it is based on chemical fragment similarities with well-known toxic fragments from the experimental database (Maunz et al. 2013). Furthermore, the Lazar was used to explore the carcinogenicity (mouse and rat), and mutagenicity (*Salmonella typhimurium*) predictions of the YSL-109.

Regarding T.E.S.T., it allows to predict the compounds toxicity by means of Quantitative Structure Activity Relationships (QSARs) assays. In the present studies, the hierarchical method was used for making the predictions. The toxicity is estimated from the weighted average of the predictions of several models, which are gained through Ward's method that divides the training set in several structurally similar clusters. Finally, a genetic-based algorithm generates the QSAR models for each cluster. Then, predictions are made from the closest cluster from each step in the hierarchical clustering approach (Martin et al. 2008; Martin 2016).

Vega 1.1.5 platform includes QSAR predictive models that are derived from CAESAR, T.E.S.T., ISS, SARpy, KNN, and IRFMN (Benfenati et al. 2013). Vega combines QSAR predictive models with an extra tool, so-called Applicability Domain Index (ADI), which assess the reliability of the model prediction (Benfenati et al. 2013).

Finally, ADMETlab2.0 was used to complement the computational predictions. This software employs a quantitative structure–property relationship (QSPR) models trained by the multi-task graph attention framework supported by experimental administration, distribution, metabolism, excretion, and toxicological (ADMET) data (Xiong et al. 2021).

Animals

Six female CD-1 mice (20 ± 2 g) were obtained from the Facultad de Estudios Superiores Iztacala, UNAM (FES-Iztacala, UNAM). Animals were housed in a temperature and light controlled room (25 °C in a 12 h dark–light cycle) and provided with food and water ad libitum (Rodent Diet™). Before starting the experiments, the animals were acclimatized for 7 days. All the experiments were performed according to the Official Mexican Standard for the care and use of laboratory animals (NOM-062-ZOO-1999) and the standard for humanely killed (NOM-033-ZOO-1995). All experimental procedures were approved by the Institutional Bioethics Committee of the Escuela Nacional de Medicina y Homeopatía (ENMyH) number CBE/001/2020. The animals used were the same for the oral acute toxicity assay and micronucleus assay.

Preparation of YSL-109 compound for animal studies

A stock solution of YSL-109 in DMSO (D5879, Sigma Aldrich) was prepared at 7683 mM. One dose of 2000 mg/kg of YSL-109 in DMSO at 5% was used.

Mutagenic assay

To investigate the possible mutagenic effect of YSL-109, the Ames test was carried out as previously described by Maron and Ames (Ames et al. 1973). For this end, TA98 and TA100 strains of *Salmonella typhimurium* were used. Additionally, the rat S9 microsomal fraction was used to evaluate the mutagenic effect of the YSL-109 metabolites. S9 fraction was obtained from the liver of male Wistar rat induced with Aroclor 1254. The strains of *S. typhimurium* were cultured during 16 h at 37 °C in a shaking bath at 990 rpm. Next, 100 µL of bacterial cultures was transferred into sterile tubes with 2 mL of soft agar (45 °C), then to this tube, the YSL-109 compound was added at 0.3, 1, 3, 10, 30, 100, and 300 µM in

10 µL of DMSO. The concentrations of the YSL-109 analyzed in the Ames test were chosen based on the range in which the compound showed cytotoxic effect on previous and current assayed cancer cell lines (Sixto-Lopez et al. 2020). Finally, YSL-109 concentrations were placed in a Petri dish which also contains Vogel–Bonner minimal medium and were incubated during 48 h at 37 °C. As positive control, picrolonic acid and methyl N-nitrosoguanidine were used for TA98 and TA100, respectively, while 2-amino-anthracene was used for both strains combined with S9 fraction. Revertant colonies (His^+) were counted. As a negative control, DMSO was used. A test was considered positive when the spontaneous revertant colonies exceeded twice the basal revertant colonies.

Acute toxicity

The acute toxicity test was performed according to the guide TG 423 of the OECD, using the limit test at one dose level of 2000 mg/kg (OECD 2002). Prior to intragastric (IG) administration of YSL-109, female mice were fasted for 8 h and this was continued for 2 h after the treatment. Mice were randomly assigned to control ($n=3$, single dose of vehicle (DMSO 5%) and test group ($n=3$, IG administered with a single dose of 2000 mg/kg of YSL-109). Mice were observed after administration at 1, 2, 4, and 6 h, and once per day for 14 days. Body weight was weekly recorded to detect weight gain or loss, any toxicity sign (from skin, eyes, and mucous membrane changes as well as respiratory, circulatory, also somatomotor activity and behavior patterns) or even mice death. Mice were sacrificed according to the standards for humanely sacrificed (Boivin et al. 2017). Furthermore, all tested animals were subjected to gross necropsy. The organs (spleen, liver, kidneys, heart, and brain) were extracted, and macroscopic observations were made to detect pathological lesions. Lethal dose 50 (LD_{50}) was estimated according to the Globally Harmonized Classification System (GHS) (NATIONS U 2013).

Micronucleus test

Nine female CD-1 mice were used for the study (20 ± 2 g). Three mice were randomly assigned to each group (test, control, and positive control). The compound YSL-109 (2000 mg/kg) and vehicle (DMSO 5%) were prepared as above mentioned and were IG administered. As positive control, cyclophosphamide (CP) was used (50 mg/kg intraperitoneal administration, IP). For the test, flow cytometry-based micronucleus measurements were performed using an in vitro test kit MicroFlow™ (Litron Laboratories, Rochester, NY). Forty-eight hours after the IG administration, 120 µL of blood samples was collected from the mouse tail and processed according to MicroFlow™ (Litron Laboratories, Rochester, NY).

Micronucleated reticulocytes (MN-RETs) and micronucleus in the population of mature normochromic erythrocytes (MN-NCE) were analyzed in peripheral blood samples (Schmid 1976).

The % RET, % MN-RET, and % MN-NCE were calculated using the below formula:

$$\%RET = (MN - RET + RET)/(MN - RET + RET + MN - NCE + NCE) * 100$$

$$\%\mathrm{MN} - \mathrm{RET} = (\mathrm{MN} - \mathrm{RET})/(\mathrm{MN} - \mathrm{RET} + \mathrm{RET}) * 100$$

$$\%\mathrm{MN} - \mathrm{NCE} = (\mathrm{MN} - \mathrm{NCE})/(\mathrm{MN} - \mathrm{NCE} + \mathrm{NCE}) * 100$$

If the compound produced a significant increment in MN frequency regarding the positive control group, it was considered genotoxic.

Statistical analysis

GraphPad Prism 5.0 (GraphPad software, Inc.) was used for statistical analysis. Mutagenicity test was evaluated using a two-way ANOVA test and Bonferroni post hoc analysis. To determine difference in body and organ mice weight, a *t* student was performed. MN was evaluated using one-way ANOVA non-parametric test and Dunnett post hoc analysis.

The differences of the means of the groups were compared with the non-treated group and were considered significant when $p < 0.05$.

Results

Antiproliferative activity on MDA-MB-231 and NIH-3T3 cell lines

The YSL-109 and TSA antiproliferative activity on MDA-MB-231 was determined at 48 h (Fig. 1). Figure 1A shows that YSL-109 caused dependent-concentration cell death (10.4 to 166.9 µM), the $IC_{50} = 50.34 \pm 1.11$ µM at 48 h (Table 1). In terms of morphology, nonmorphological changes were observed in the control group at 48 h (Fig. 1C), with YSL-109 at 41.7 µM produces death morphological changes on MDA-MB-231, such as rounded cell retraction, formation of islets, and cell shrinkage (Fig. 1D).

In addition, the IC_{50} of YSL-109 was 56.5 ± 2.24 µM on NIH-3T3 (Fig. 1E), (Table 1), whereas that TSA within the range of 0.1–1 µM does not show a significant decrease in cell viability (Fig. 1F).

In silico toxicological assessment

In silico toxicological assessment was done with five software (Table 2). For the mutagenic activity, YSL-109 was predicted as mutagenic by ProTox-II, Lazar, ADMETlab2.0, and Vega 1.5.1. Only T.E.S.T. and THE Model

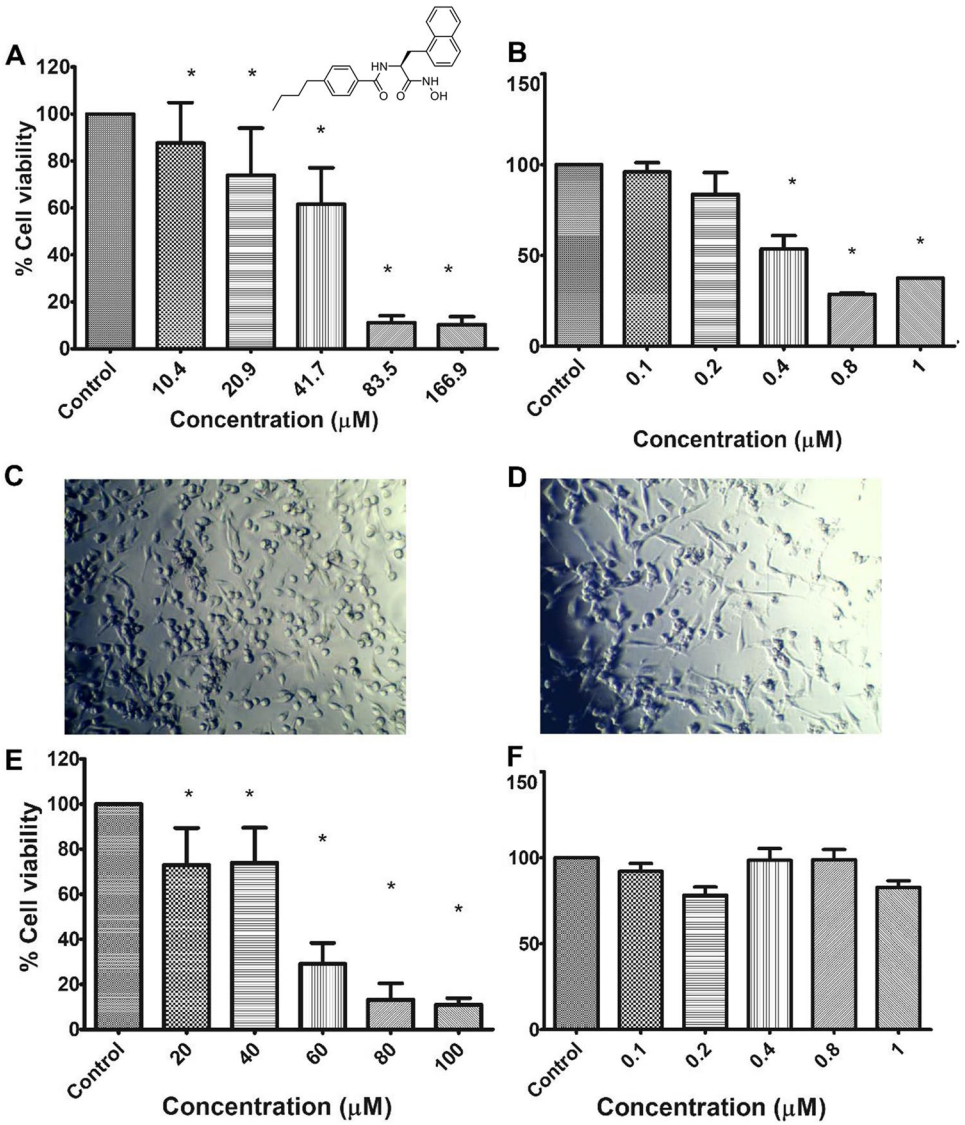

Fig. 1 Viability of treated cell lines evaluated by MTT assay. MDA-MB-231 cells treated with **A** YSL-109 and **B** TSA at 48 h. The morphology of MDA-MB-231 cells **C** in the control and **D** treatment with 41.7 μM of YSL-109. NIH-3T3 cells treated with **E** YSL-109 and **F** TSA at 48 h. The experiments were performed in triplicate with $n=8$ for each concentration. Data are presented as the means ± SE. *$p \leq 0.05$ statistically significant difference compared to the negative control. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test

Caesar 2.1.213 which is used by Vega 1.5.1 (Caesar 2.1.13) predicted YSL-109 as non-mutagenic.

In terms of oral acute toxicity, both ProTox-II and T.E.S.T. predicted a LD_{50} higher than 2500 mg/kg in rodents and rats, respectively. While, with ADMETlab2.0, YSL-109 was predicted as probable toxic at a dose <500 mg/kg but with a probability of 0.3 to 0.7.

According to ProTox-II predictions, YSL-109 may not produce immunotoxicity and hepatotoxicity, but Vega 1.5 and ADMETlab2.0 predict hepatotoxicity.

Regarding carcinogenicity, Protox-II, ADMETlab2.0, and Vega 1.5 by Caesar 2.1.9 and IRMF models predicted YSL-109 as carcinogenic. Contrastingly, Vega by ISS 1.0.2 model and Lazar in mouse and rat models predicted YSL-109 as non-carcinogen. YSL-109 was predicted as non-genotoxic in vivo but with positive activity in an in vitro model by Vega 1.5.1.

In addition, ADMETlab2.0 predicts YSL-109 as inactive hERG (human ether-a-go-go related gene) blocker, skin sensitization, eye corrosion, eye irritation, and respiratory toxicity.

Table 1 Determination of the 50% inhibitory concentration (IC_{50}) of the YSL-109 and TSA on MDA-MB-231 and NIH-3T3 cell lines

Compounds	IC_{50} (μM)	
	MDA-MB-231	NIH-3T3
YSL-109	50.34 ± 1.11	56.5 ± 2.24
TSA	0.5086 ± 0.0610	ND*

The experiments were performed in triplicate with $n=8$ for each concentration. Data are presented as the means ± SE. **ND*, not determined

Table 2 Toxicological results obtained by in silico studies and experimental assays of YSL-109

	In silico results					Experimental results
	Software					
Evaluated parameter	ProTox-II	Lazar	T.E.S.T	Vega 1.5.1 (probability)	ADMETlab 2.0 (probability)	
LD_{50} (rodent)	2500 mg/kg					>2000 mg/kg
Predicted toxicity class (GHS)	5					5
Hepatotoxicity	Inactive				Active (0.7–1)	Not observed
Carcinogenicity	Active				Active (0.5–0.7)	ND
Immunotoxicity	Inactive					ND
Mutagenicity	Active					Mutagenic
Carcinogenicity (mouse)		Non-carcinogenic				ND
Carcinogenicity (rat)		Non-carcinogenic				ND
Mutagenicity (*Salmonella typhimurium*)		Mutagenic	Non-mutagenic		Probable mutagenic (0.5–0.7)	Mutagenic
LD_{50} (oral rat)			4,483.31 mg/kg		Probable toxic (<500 mg/kg) (0.3–0.7)	ND
Mutagenic score (consensus)				Mutagenic (0.76)		Mutagenic
Model Caesar 2.1.13				Non-mutagenic		
ISS 1.0.2				Mutagenic		
SarPy 1.0.7				Mutagenic		
KNN 1.0.0				Mutagenic		
Carcinogenicity Caesar 2.1.9				Carcinogen (0.553)		ND
ISS 1.0.2				Non-carcinogen		ND
IRFMN/Antares 1.0.0				Carcinogen		ND
Hepatotoxicity (IRFMN) 1.0.0				Toxic		Not observed
In vitro micronucleus (IRFMN/VERMEER) 1.0.0				Active		Not genotoxic
In vivo micronucleus (IRFMN) 1.0.0 (Sarpy and KNN)				Non-genotoxic		
hERG Blocker					Inactive	
Maximum recommended daily dose in human					Nontoxic (dose >0.011 mmol/kg/day)	ND
Skin sensitization					Nontoxic	ND
Eye corrosion					Nontoxic	ND
Eye Irritation					Nontoxic	ND
Respiratory toxicity					Nontoxic	ND

Mutagenic assay

The Ames test was carried out to evaluate the mutagenic effect of YSL-109. YSL-109 showed a mutagenic effect in TA98 strain at 30 and 100 µM with and without S9 (Fig. 2A).

With S9, three-fold higher revertant colonies were observed for both 30 and 100 µM in comparison with negative control (DMSO), and without S9 (−S9), two and three-fold revertant colonies for 30 and 100 µM were observed, respectively. The positive control for TA98 strain without S9 produced 7.5 and 12.5-fold more

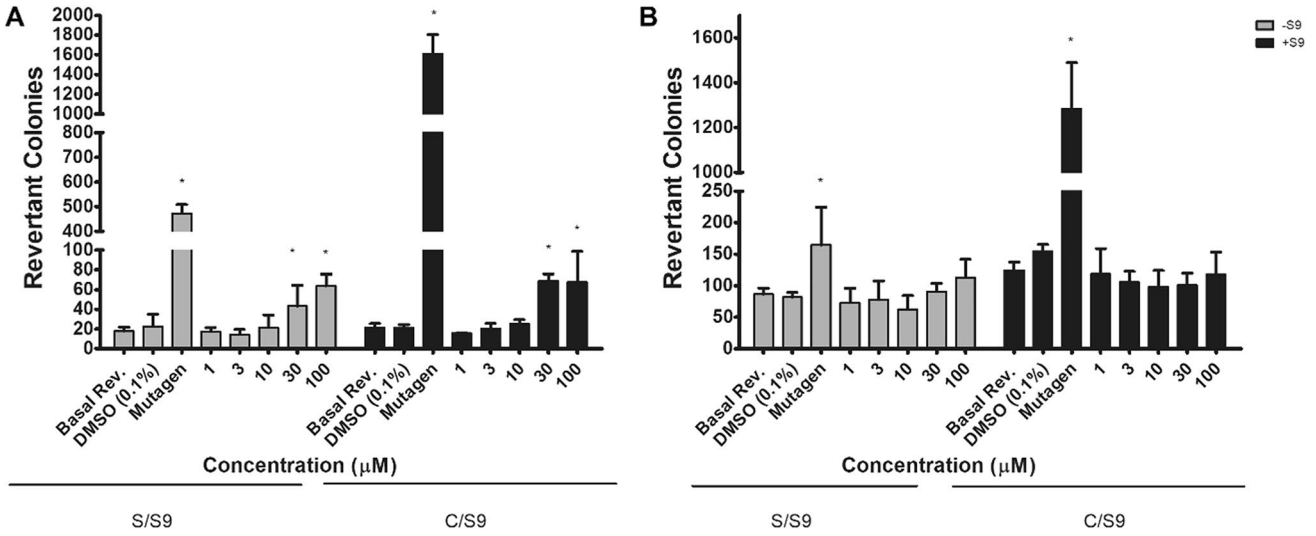

Fig. 2 Mutagenicity assay by Ames test of compound YSL-109, **A** in strain TA-98 and **B** in strain TA-100. Two independent experiments were carried out in triplicate in the presence of S9 (C/S9) and in the absence of S9 (S/S9), which is the microsomal fraction of rat liver induced with Aroclor 1254. Picrolonic acid (AP) was used as mutagen for strain TA98 S/S9 and 2-aminoanthracene (10 μg/plate, 2-AA) for strain TA98 S/S9, and for TA100 S/S9 and C / S9, methyl-N-nitrosoguanidine (MNNG) was used and 2-AA, respectively. *$p < 0.05$, two-way ANOVA test and Bonferroni post hoc analysis were carried out

revertant colonies than 30 and 100 μM concentration of YSL-109, while the positive control for TA98 with S9 fraction, the 2-aminoanthracene (AA), produced about 20-fold more revertant colonies than YSL-109, whereas YSL-109 does not show any mutagenic effect in the TA100 strain (Fig. 2B).

Oral acute toxicity

To determine the oral acute toxicity, the OECD test guidelines 423 was followed. This method is a stepwise procedure that use three animals of a single sex per step, generally females because several studies suggest that females are slightly more sensitive than males (Miller 2001; OECD 2002; Gandhi et al. 2004; Gochfeld 2007). Adopting this method, enough information is obtained that allows the classification of the substance as well as to estimate an LD_{50} value (OECD 2002). Three female CD-1 mice received a single dose of 2000 mg/kg, and no signs or symptoms of toxicity (such as piloerection, irritation of mucous membranes, or alterations in motor activity) or deaths were found during the period of study (days 1 to 14). Weights of the treated mice did not show statistically significant differences regarding the control group during the treatment period (Table 3). The organs spleen, pancreas, liver, kidneys, heart, and brain did not show any type of macroscopic morphological lesion or significant weight variation (Table 4). Therefore, the LD_{50} of YSL-109 is greater than 2000 mg/kg in CD-1female mice.

According to our results, YSL-109 is found in category 5 of the GHS with an LD_{50} greater than 2000 mg/kg in CD-1 female mice (OECD 2002).

Micronucleus test

Through the micronucleus test, it was observed that YSL-109 did not induce a significant decrease in %RET (Fig. 3A), nor an increase in %MN-RET (Fig. 3B) and % MN-NCE (Fig. 3C), compared to CP. While the positive control, CP induced a significant decrease in %RET and increment in %MN-RET and MN-NCE (Fig. 3) (Table 5).

Table 3 Weight of female CD-1 mice (g), administered with YSL-109 at 2000 mg/kg

	Day 1		Day 14		Weight gained at day 14	
Group	Control	YSL-109	Control	YSL-109	Control	YSL-109
Weight	21.9 ± 2.7	23.2 ± 0.7	27.3 ± 1.8	29.3 ± 0.6	5.5 ± 1.5	6.1 ± 0.5

Values are expressed as average ± standard error of 3 animals per group. Student's *t* analysis was performed between two groups. GraphPad Prism 5.0 software (GraphPad Software, Inc.) was used to perform statistical analysis. Control: animals administered with 5% DMSO

Table 4 Organ weight of female CD-1 mice (g), administered with YSL-109 at 2000 mg/kg

Group	Brain	Heart	Liver	Spleen	Kidney 1	Kidney 2
Vehicle DMSO 5%	0.46 ± 0.05	0.17 ± 0.01	1.74 ± 0.16	0.11 ± 0.01	0.21 ± 0.02	0.20 ± 0.03
YSL-109 2000 mg/kg	0.47 ± 0.01	0.19 ± 0.02	1.90 ± 0.05	0.12 ± 0.01	0.22 ± 0.01	0.22 ± 0.02

Values are expressed as average ± standard error of 3 animals per group. Student's *t* analysis was performed between two groups. GraphPad Prism 5.0 software (GraphPad Software, Inc.) was used to perform statistical analysis. Control: animals administered with 5% DMSO

Discussion

TNBC is a type of BC with poor prognosis, an aggressive clinical behavior, early relapsed, and few therapeutic options (Fragomeni et al. 2018; Damaskos et al. 2019). There are no specific approved therapeutic options for TNBC (Hwang et al. 2019). Moreover, HDACs have been explored as potential targets to treat TNBC. Specifically, HDAC6 is considered a target since it is overexpressed on TNBC and its inhibition produce a detriment on cell invasion and migration (Rey et al. 2011; Hsieh et al. 2019). Therefore, in the current work, the TNBC antiproliferative activity of YSL109, a selective HDAC6 inhibitor was investigated.

According to our results, YSL-109 showed moderate antiproliferative activity in MDA-MB-231 cell line at 48 h (IC_{50} = 50.34 ± 1.11 µM) in comparison with the pan-HDAC inhibitor, TSA (IC_{50} = 0.5086 ± 0.0610 µM) (Table 1). The antiproliferative effect observed due to YSL-109 administration is comparable to other hydroxamic acid derivatives with promissory potential against TNBC, such as N-hydroxycinnamamide analog which showed activity at µM ranges (Shukla et al. 2018), panobinostat, and vorinostat depicted activities at nM ranges (920 and 100 nM, respectively), while valproic acid showed antiproliferative activity at 1.6 mM (Terranova-Barberio et al. 2016).

In terms of morphological changes, no changes were detected in MDA-MB-213 cells treated with the vehicle (DMSO 0.1%). The cells showed adherent properties and an elongated form with normal proliferation rates (Fig. 1C). While with at 41.7 µM of YSL-109, there were morphological changes, such as rounded cells, retraction, and formation of islets. These changes were more evident at higher concentrations (Fig. 1D), suggesting that the cells underwent an apoptotic process (Van Cruchten and Van Den Broeck 2002).

To compare with a cell line with normal proliferation rates, non-cancer murine fibroblast cells NIH-3T3 was also used to evaluate the activity of YSL-109 (Fig. 1E) showing an IC_{50} = 56.50 ± 2.24 µM (Table 1). YSL-109 showed a selectivity index (SI) of 1.12, indicating that YSL-109 inhibited the proliferation in both cell lines with better potency in TNBC. If one compound depicted higher SI by cancer cell lines over non-cancer cell line, it is considered that it would show fewer side effects; in this case, YSL-109 showed lower selectivity between cancer and non-cancerous cell line, but this behavior has been also observed in drugs that are currently clinical administered in humans,

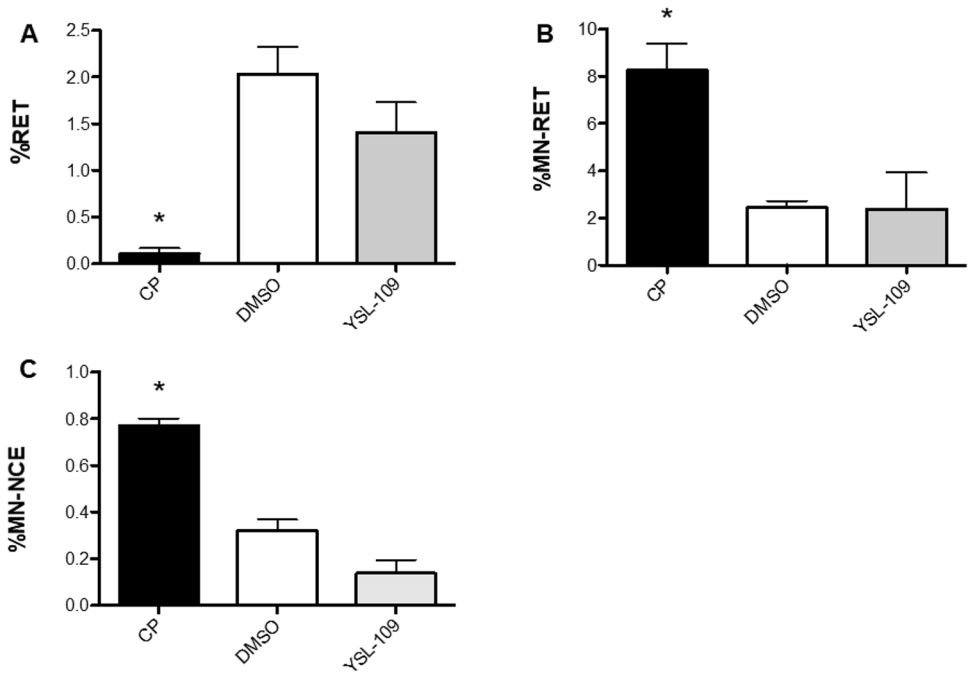

Fig. 3 Micronucleus test. **A** Percentage of reticulocytes (% RET), **B** percentage of micronucleated reticulocytes (% MN-RET), and **C** percentage of micronucleated normochromatic erythrocytes (% MN-NCE) after 48 h of treatment. CP (cyclophosphamide); DMSO (5% DMSO). *$p \leq 0.05$ statistically significant difference compared to the negative control (DMSO 5%). One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test

Table 5 Percentages of RET, MN-RET, and MN-CNE in mouse peripheral blood obtained by flow cytometry ($n=3$)

Treatment	Concentration	% RET	% MN-RET	% MN-NCE
Positive control (CP)	50 mg/kg (i. p.)	0.11 ± 0.06*	8.25 ± 1.15*	0.77 ± 0.03*
Negative control DMSO 5%	300 µL/mouse	2.03 ± 0.30	2.44 ± 0.29	0.32 ± 0.05
YSL-109	2000 mg/kg	1.41 ± 0.32	2.36 ± 1.58	0.14 ± 0.05

*$p \leq 0.05$ statistically significant difference compared to the negative control (DMSO 5%). One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. *i.p.* intraperitoneal

such as doxorubicin (Dante et al. 2019). TSA depicted an $IC_{50} = 0.5086 \pm 0.0610$ µM in MDA-MB-231 cells which is in agreement with previous reports (Fig. 1B) (Vigushin et al. 2001; Rahimian and Mellati 2017), whereas TSA at 0.1–1 µM does not significantly decrease the proliferation of NIH-3T3 cell lines (Fig. 1F).

Drug discovery is a complex process, and many compounds fail to become drugs due to lack of efficacy and safety (Guan et al. 2019; Xiong et al. 2021). Therefore, the determination of the toxicological properties of a compound in preclinical stages is important to determine the potential harmful effects (Raies and Bajic 2016). In the current work, we have determined the toxicological profile of YSL-109 combining computational and experimental tools.

In vivo and in vitro models are widely extended to perform toxicological assays; however, recently, in silico models are covering relevance, since the use of computational tools, including machine learning algorithms. The use of in silico models represents an alternative to reduce time and costs as well as the use of animals for the experiments. In silico toxicology predictions are still being used as complement to other models, and useful tools have been developed to assist toxicology predictions saving time in activities such as drug screening (Raies and Bajic 2016; Parthasarathi and Dhawan 2018). The employment of several software with different algorithms for biological predictions is recommendable since it increases the accuracy of the predictions.

In this work, four software were used to assist in toxicology predictions, ProTox-II, Lazar, Vega, ADMETlab2.0, and T.E.S.T. The mutagenic effect of YSL-109 on *Salmonella typhimurium* was predicted as positive by ProTox-II, Lazar, ADMETlab2.0, and Vega 1.5.1, whereas T.E.S.T. predicted that the compound would not have this effect (Table 2). Thus, T.E.S.T. fails to predict YSL-109 as a mutagenic compound, since according to our experimental studies YSL-109 can produce a mutagenic concentration-dependent effect on TA98 strain at 30–100 µM.

According to oral acute toxicity in silico predictions, the LD_{50} was higher than 2500 mg/kg in rodents and rat, respectively (Table 2), these results agree with our experimental results, further discussed. In such case, ADMETlab2.0 predicts as a toxic agent in rats, but contrastingly, it predicts the YSL-109 as nontoxic for humans at doses higher than 0.011 mmol/kg/día. Hence, further studies using other species should be performed to better characterize the acute toxicity of the compound.

Contradictory results were found in hepatotoxicity and carcinogenicity endpoint predictions since some predictions point out that YSL-109 might not have these effects while others indicate the opposite. Regarding immunotoxicity, according to ProTox-II, YSL-109 may not produce this effect. Finally, many other toxicological data were assayed on ADMETlab2.0 suggesting that the compound is inactive as hERG blocker skin sensitization, eye corrosion, eye irritation, and respiratory toxicity. In view of the above results, further experimental studies need to be done to test these activities on YSL-109.

Vega 1.5.1 successfully predicts the non-genotoxic effect of YSL-109 on an in vivo model and suggests a positive activity in an in vitro model. The in vivo prediction has coincided with the results found by our in vivo model.

Once the computational predictions were performed, the experimental assays were carried out to validate it. The Ames test is an in vitro approach used to detect mutagenic effects of chemical compounds, especially in the drug discovery field. This test allows identifying if a compound produces a genetic injury in *Salmonella typhimurium* strains (Hsu et al. 2016). In this study, the strains TA98 and TA100 were employed to evaluate YSL-109 mutagenicity. Punctual mutations by frameshift or by base-pair substitution were detected with the strains TA98 and TA100, respectively (Ames et al. 1973).

For TA98, it was possible to detect a concentration-dependent mutagenic effect (Fig. 2A) from 30 to 100 µM with and without metabolic system (S9) (Fig. 2A), and in the case of TA100, this effect was not observed (Fig. 2B). Thus, YSL-109 produces a frame-shift mutation in *S. typhimurium* His-strain TA98 but did not produce mutation by base-pair substitution in TA100 strain. Mutagenic behavior in hydroxamic acids has been previously reported. Clastogenic and aneugenic activity of TSA on lymphoid cells were reported (Olaharski et al. 2006). Even though similar concentrations at what mutation was observed (from 30 µM) has been observed in other hydroxamates such as the O-benzoyl benzohydroxamate (Ching Yung 1977; Wang and Lee 1977; Lipczynska-Kochany et al. 1984). The possible mechanisms by which hydroxamate produces mutagenicity are because a Loosen rearrangement can occur, which transforms the

hydroxamate in their corresponding isocyanate derivative, which is unstable and react with nucleophiles compounds available in the environment or become hydrolyzed, these isocyanates react with imidazole groups of some enzymes forming urea type adducts (Shen and Kozikowski 2016). Carbamoylation of the hydroxamic acid has been suggested as an alternative mutagenic mechanism in which occurs the formation of carbamoyl group which is bound to DNA (Skipper et al. 1980). Moreover, this mutagenic activity may contribute to the antiproliferative activity of the hydroxamic acid under study (YSL-109) (Shen and Kozikowski 2016).

The oral acute toxicity was determined based on the guide TG 423 of OECD. With this study, it was possible to determine that YSL-109 has an oral LD_{50} higher than 2000 mg/kg on female CD-1 mice, with no signs or symptoms of toxicity for 14 days, neither affectations nor body weight gain in comparison to the control group (Table 3). Therefore, YSL-109 was classified as class 5, according to GHS (Nations U 2011), which indicates that the oral acute LD_{50} is between the range of 2000 and 5000 mg/kg.

After 14 days of the mice treatment, no macroscopic lesions in the organs or significant variations on weight were detected (Table 4); this parameter suggests that YSL-109 is a safe compound when it is orally administered. These results indicate that YSL-109 does not showed toxicity when it is orally administered to CD-1 female mice at doses of 2000 mg/kg; therefore, orally administration is safe at acute administration; additionally, other studies are required to determine the safety of chronic administration at 28, 60, or 90 days.

There are several assays used to investigate the genotoxic capacity of new compounds that may be used as drugs. According to ICH guideline S2 (R1) on genotoxicity testing for pharmaceuticals intended for human use, a test for gene mutation in bacteria and an in vivo assay (option 2) as a battery approach could be carried out for investigate the genotoxic potential of a compound (Food and Drug Administration H 2012).

Therefore, to asses in vivo genotoxicity we used mice for administering the compound YSL-109 and investigate its impact in micronucleus formation (Sommer et al. 2020). In the current work, a short term study with the top dose of 2000 mg/kg, as recommended by the ICH guideline S2 (R1), was IG administered to the test group (Food and Drug Administration H 2012). Micronucleus is a technique that allows to identify in a rapid way genotoxic compounds, which produce chromosomal instability, particularly due to DNA break down after being exposed to some substances, when cells are in division, leaving fragments, whole, acentric, or aberrant chromosomes that were not included in the main nucleus forming a micronucleus that allow to detect clastogens and aneugens compounds (Lenarczyk and Slowikowska 1995; OECD 2016). Genotoxicity of YSL-109 was assayed in female CD-1 mice peripheral blood. According to our finding, YSL-109 was not genotoxic neither cytotoxic in mice peripheral blood (Fig. 3, Table 5), since it did not induce a significant increase on %RET (Fig. 3A) that suggests cytotoxic activity neither an increase on %MN-RET (Fig. 3B) and % MN-CNE (Fig. 3C) that suggest genotoxicity effect. This results suggests that YSL-109 does not have the potential to be carcinogen in humans at higher doses (Morita et al. 1997), in comparison with others hydroxamic acids (Belinostat, Vorinostat or Panobinostat) that showed genotoxicity activity (Kerr et al. 2010; Shen and Kozikowski 2016; Al-Hamamah et al. 2019). TSA has also been reported as a genotoxic compound in different in vitro models, which also suggest that the genotoxicity in hydroxamic acids may contribute to its antiproliferative activity (Johnson and Walmsley 2013; Shen and Kozikowski 2016).

Conclusions

According to these results, YSL-109 shows antiproliferative activity in MDA-MB-231 (TNBC cell line) at μM range ($IC_{50} = 50.34 \pm 1.11$ μM). This activity could be due its inhibition HDAC6 selectively at nM range. On the other hand, YSL-109 shows an acceptable toxicological profile at preclinical level. It does not show in vivo genotoxicity activity and shows moderately mutagenic activity on the TA-98 *S. typhimurium* strain at 30 and 100 μM. This latter activity could also contribute to the TNBC antiproliferative activity observed. The oral acute toxicity study conducted on CD-1 female mice allows us to determine that there were no any toxicity signs at a dose of 2000 mg/kg. However, these are preliminary results and further studies to better characterize the acute and chronic toxicity are needed.

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Author contribution JCB was the grant holder. YSL, COP, and MCRH performed the biological experiments. YSL, JAGV, and JCB performed the chemical synthesis of the compound. All the authors have contributed to the analysis of the data. YSL drafted the manuscript. JCB revised the manuscript. All authors read and approved the published version of the manuscript.

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Data availability All data generated or analyzed during the present study are included in this published article.

Declarations

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Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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