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RESEARCH ARTICLE

A thiosemicarbazone derivative induces triple negative breast cancer cell apoptosis: possible role of miRNA-125a-5p and miRNA-181a-5p

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Abstract

Background Breast cancer, the most commonly diagnosed malignancy in women, accounts for the highest cancer-related deaths worldwide. Triple negative breast cancer (TNBC), lacking the expression of estrogen, progesterone and HER2 receptors, has an aggressive clinical phenotype and is susceptible to chemotherapy but not to hormonal or targeted immunotherapy. In an attempt to identify potent and selective anti-TNBC agents, a set of thiosemicarbazone derivatives were screened for their cytotoxic activity against MDA-MB 231 breast cancer cell line.

Methods MTT assay was used to examine cell viability. P53 phosphorylation status, poly (ADP-ribose) polymerase (PARP) cleavage as well as Bcl2 and Bax protein levels were assessed by Western blot. Quantitative Real Time-PCR was carried out to characterize miRNAs expression levels.

Results Combining Cisplatin + thiosemicarbazone compound **4** showed potent anti-TNBC potential. Cisplatin + compound **4** significantly enhanced p53 phosphorylation, induced Bax amount, reduced Bcl2 protein levels, enhanced PARP cleavage and modulated miRNAs expression profile in TNBCs, with a particular overexpression of miR-125a-5p and miR-181a-5p. Intriguingly, miR-125a-5p and miR-181a-5p could significantly downregulate *BCL2* expression by binding to their target sites in the 3′UTR.

Conclusions Collectively, our results demonstrate an anti-TNBC activity of Cisplatin + thiosemicarbazone compound **4** combination mediated via induction of apoptosis.

Keywords Thiosemicarbazone derivatives · Breast cancer · Apoptosis · miRNAs

Introduction

Breast cancer, representing the second leading cause of cancer death in women, is the most common cancer type that develops in women worldwide, and its incidence rate continues to rise (Kamangar et al. 2006). Breast cancer is a heterogeneous disease that includes different subtypes showing diverse clinical behaviors and sensitivities to treatment (Parker et al. 2009). Identifying breast cancer subgroups can be partly related to the expression of different breast-cancer-related genes. Among these are genes encoding estrogen receptor (ER) (Bartlett et al. 2011), progesterone receptor (PR) (Purdie et al. 2014) and human epidermal growth factor receptor 2 (HER2) (Rimawi et al. 2013). Patients with ER and/or PR positive status, which accounts for approximately 80% of hormone-sensitive breast cancers, have a better prognosis than those with negative status (Carey et al. 2006). Patients with upregulated HER2 expression are treated by trastuzumab, a monoclonal antibody which blocks HER2 growth factor receptor (Baselga et al. 1996; Hudis 2007). Triple negative breast cancer (TNBC), being characterized by the absence of ER, PR and HER2, represent about 10–20% of all breast cancers diagnosed and is considered a highly aggressive breast cancer subtype with poor prognosis (Podo et al. 2010; Carey et al. 2010; Boyle 2012). Due to the lack of known specific molecular therapeutic targets, the partially

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successful conventional chemotherapy, including doxorubicin and taxane, is basically used for treating TNBC patients (Liedtke et al. 2008; Oakman et al. 2011; Joensuu and Gligorov 2012). TNBC is being, nowadays, intensively investigated for identifying predictive biomarkers or designing novel efficient therapeutic agents. Although a large number of chemical compounds, with anti-cancer potential, have been identified, so far, their clinical use is limited by their high toxicity and adverse impact on normal cells (Buolamwini 1999). Thiosemicarbazones, belonging to a wide group of thiourea derivatives, exhibit various valuable biological properties and have therefore gained special pharmaceutical and medicinal interest (Pelosi 2010). Over the past 50 years, thiosemicarbazones have been well characterized for their antiviral, antibacterial, antifungal, and antimalarial activities (Pelosi 2010). In addition, thiosemicarbazones exhibit anticancer activity where different thiosemicarbazone derivatives and synthetic analogues, such as Triapine, are being used, today, for cancer treatment (Kalinowski and Richardson 2005; Hu et al. 2006; Yu et al. 2006; Vandresen et al. 2014). Thiosemicarbazones derivatives confer anticancer effects in free and in metal-complexed forms. In free forms, their biological activity is related to their ability to inhibit topoisomerase II α and ribonucleotide reductase enzymes (Bisceglie et al. 2018). The alternative anticancer mode of action is based on their coordination chemistry with functional proteins containing metal ions in their structure. This coordination complex is preferred to occur with transition metals, particularly Zinc (Zn), Copper (Cu), Iron (Fe), Cobalt (Co), Nickel (Ni), Platinum (Pt), and Palladium (Pd). It is note worthy that the metal-complexed forms confer better anticancer activity than their free counterparts (Bisceglie et al. 2018).

In this study, we screened, in vitro, the antitumor activity of different thiosemicarbazone derivatives against a TNBC cell line. Our results identified one thiosemicarbazone derivative, designated compound **4**, to intensify cisplatin-mediated cytotoxicity against TNBCs probably via upregulating the expression of certain tumor suppressor miRNAs, miR-125a-5p and miR-181a-5p, as well as enhancing cisplatin-dependent phosphorylation of p53 and activation of apoptosis.

Materials and methods

Cell culture

MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) (Rockville, USA). They were cultivated in DMEM and provided with 10% heat inactivated FBS, 2 mM L-glutamine, and 1% of penicillin and streptomycin. Cells were cultivated in 75 cm^2 flasks at 37 °C within humid environment in an incubator with 5% CO_2. When reaching 85% confluence, cells were harvested using 0.25% trypsin and then seeded in 96- or 6-well plates, depending on the performed experiment. Cells were at 70% confluent prior to treatment.

Peripheral blood mononuclear cells (PBMC) were obtained from the peripheral blood of healthy donors, and were immediately purified using gradient centrifugation in Ficoll-PaqueTM Plus (Amersham Biosciences, USA).

The mixture was centrifuged for 40 min at 18–20 °C (400×*g*) without a break. Buffy coat was aspirated and washed twice with balanced salt solution (PBS) and then resuspended in RPMI 1640 medium provided with 10% fetal bovine serum, HEPES (20 nM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were activated upon being treated with Mitogen phytohemagglutinin-P (PHA-P) (5 µg/ml; sigma) and human interleukin (IL-2) (10 ng/ml; R&D Systems).

MTT assay

PBMC and MDA-MB-231 cells were first seeded in 96-well plates (8×10^3 cells/well). Cells were treated with the different indicated products at concentrations ranging from 100 to 1 µM for 48 h. Cell viability was examined using Cell Proliferation Assay, MTT (Thiozolyl Blue Tetrazoluim Bromide; Sigma; USA; M-5655-1G).

Western blotting

Protein samples were charged into stacking gel wells and then run until bromophenol blue reached the bottom of the gel. Gels were then allowed to transfer on nitrocellulose membranes (1 h at 4 °C; 80 V). The membranes were then incubated (2 h at room temperature) in the presence of blocking solution: 3% BSA (Bovine Serum Albumin, Sigma A2153), prepared in Tween PBS. The used primary antibodies were: monoclonal antibodies for p53 (Santa Cruz Biotechnology Inc., Texas, USA; *sc-126*), Bcl2 (Santa Cruz Biotechnology Inc., Texas, USA; sc-7382), Bax (Cell signaling; USA; D2E11), PARP (abcam; ab191217) and GAPDH (abcam; USA; ab9485)—the latter was used to ensure equal loading of samples.

MiRNA profiling and data analysis

Trizol total RNA isolation reagent (Roche Diagnostics, vilvoorde, Belgium) was used to exact total RNA from cells. NanoDrop Spectrophotometer was used to quantify the concentration. In a first step, and in order to synthesize cDNA from miRNAs, a TaqMan miRNA Reverse Transcription Kit (#4366596; Applied Biosystems, Gent, Belgium) and Megaplex RT primers (Human Pool A, #4399966; Applied Biosystems, Gent, Belgium) were used following

the manufacturer's protocol. Using 500 ng of total template RNA, this enabled simultaneous reverse transcription of 380 mature human miRNAs. RT was carried out using Mastercycler Epgradient thermocycler (VWR International, Leuven, Belgium) with the following parameters: 40 cycles of 16 °C for 2 min, 42 °C for 1 min, 50 °C for 1 s, and a final step of 80 °C for 5 min to inactivate the reverse transcriptase. The products were then diluted with RNase-free water, mixed with TaqMan gene expression Master Mix and then charged into TaqMan Human miRNA Array A (#4398965; Applied Biosystems, Gent, Belgium), corresponding to a 384-well formatted plate and real-time PCR-based microfluidic card containing embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs. RNU48 transcript was used as a normalization control. Quantitative PCR (qPCR) was carried out following the manufacturer's instructions. Real-time PCR was carried out using ABI PRISM 7900HT sequence detection system (Applied Biosystems, Gent, Belgium) with the following parameters: 50 °C for 2 min, 94.5 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 59.7 °C for 1 min. RNU48 embedded in the TaqMan Human miRNA Arrays was considered as an endogenous control. The relative expression levels of miRNAs were calculated following the comparative $\Delta\Delta C$t method as described before (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The fold changes in miRNAs expression levels were calculated by the equation $2^{-\Delta\Delta Ct}$.

Taqman miRNA assay for individual miRNAs

For each miRNA, reverse transcription was carried out using 10 ng of purified total RNA, 100 mM dNTPs, 50 U MutliScribe reverse transcriptase, 20 U RNase inhibitor, and 50 nM of RT primer samples using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium). Reactions (15 µl) were incubated: 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. Real Time-PCR reactions (5 µl of RT product, 10 µl TaqMan 2×Universal PCR master Mix (Applied Biosystems, Gent, Belgium), and 1 µl TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes) were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Gent, Belgium) at the following parameters: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The expression levels $2^{-\Delta\Delta Ct}$ of miRNAs were calculated as previously described (Schmittgen and Livak, 2008).

Bioinformatics

MicroRNA Data Integration Portal (mirDIP) (http://ophid.utoronto.ca/mirDIP/) was searched for potential miRNA target sites. BCL2 was identified as having putative miR-125a-5p and miR-181a-5p target sites.

Plasmid constructions

A 710-bp fragment of BCL2 3′UTR containing the miR-125a-5p and miR-181a-5p potential target sites was cloned downstream of the Renilla luciferase gene (EcoRI/XhoI sites) in the psiCHECK-1 plasmid (Promega, Mannheim, Germany) and designated as psiCHECK 3′UTR WT. PCR primers used for amplification of the BCL2 3′ UTR were:

BCL2:

Forward primer: 5′-TCTGTATTAACTTTGGAATGTACTCTG-3′

Reverse primer: 5′-CAAAGGTCTGATCATTCTGTTCC-3′.

The constructs were verified by sequencing.

Site directed mutagenesis

QuikChange site-directed mutagenesis mutation/deletion of the miR-125a-5p and miR-181a-5p potential target sites respectively was carried out following the manufacturer's guidelines (Stratagene, La Jolla, CA, USA) and designated as psiCHECK-UTRdel. QuikChange site-directed mutagenesis was carried out using the following primers:

The following primers were used to carry out the deletion:

BCL2 (miR-125a-5p deleted):

Forward: 5′-CCTCCCCGGCGGGCAACAGAATG-3′

Reverse: 5′-CATTCTGTTGCCCGCCGGGGAGG-3′.

BCL2 (miR-181a-5p deleted):

Forward primer: 5′-GTATTAACTTTGACTCTGTTCAATG-3′

Reverse primer: 5′-CATTGAACAGAGTCAAAGTTAATAC-3′.

The constructs were verified by sequencing.

Cell culture

Hela cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Verviers, Belgium) provided with 10% heat inactivated fetal bovine serum (Invitrogen Europe, Paisley, UK), 2 mM L-glutamine, 50 IU/ml Penicillin, and 50 µg/ml Streptomycin (all from Lonza, Verviers, Belgium).

Luciferase assays

Assays were performed in a 24-well format. Hela cells were con-transfected, using Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) according to the manufacturer's guidelines, with reporter plasmids (psiCHECK/psiCHECK 3′UTR WT/psiCHECK 3′UTR deleted) (100 ng) along with miR-125a-5p and miR-181a-5p-mimic/miR-negative control-mimic at final concentration of 10 µM (miRIDIAN mimic, Dharmacon, Geel, Belgium) and control firefly plasmid pGL3-CMV (100 ng). The cells were first assessed for their expression levels of

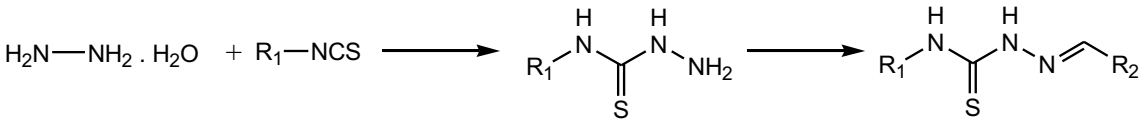
miRNAs of interest using quantitative RT-PCR, as described below. After 40 h post transfection, cells were collected and luciferase levels were determined using the Dual luciferase reporter assay system (Promega, Mannheim, Germany) following the manufacturer's guidelines. Relative protein levels corresponded to Renilla/Firefly luciferase ratios.

Quantitative PCR for BCL2 expression

Quantitative mRNA expression was determined by real-time PCR, with PRISM 7900 sequence detection system (Applied Biosystems, Gent, Belgium), and the TaqMan Master mix kit with EF1- α mRNA was used as an internal control. Human Taqman gene expression assays for BCL2 (**Hs04986394_s1**) and EF1-α (Hs00951278_m1) were purchased from Applied Biosystems.

Chemistry

The thirteen thiosemicarbazone derivatives (Table 1) were prepared starting from the suitable isothiocyanate (commercially available or prepared from the corresponding amine) (Wong and Dolman 2007). Treatment of isothiocyanate with hydrazine hydrate gave the thiosemicarbazide derivative which was condensed with the appropriate aldehyde to give the corresponding thiosemicarbazone.



Statistical analysis

Presented data corresponds to mean ± SEM of at least three independent experiments and was analyzed using Student's t-test. P-values < 0.05 (*) and < 0.01(**) were considered significant.

Results

Antiproliferative activity of different thiosemicarbazone derivatives against MDA-MB-231 breast cancer cell line

All the synthesized thiosemicarbazone compounds (**1**–**13**) were screened for their in vitro anticancer activity against the triple negative breast cancer cell line MDA-MB-231 (ER^-, PR^- and $HER2^-$), using MTT assay. The inhibitory activities (IC_{50}) are summarized in Table 2. Compounds **2**, **3**, **4**, **5**, and **6** showed a cytotoxic effect against MDA-MB 231 cells at concentrations close to those exhibited by Cisplatin (known anticancer agent) (Table 2), and lower than other compounds.

Interestingly, combining compound **4** with Cisplatin further enhanced the anticancer activity of Cisplatin (Table 2). On the other hand, no significant improvement of Cisplatin cytotoxic effect was detected upon combining it with either compound **2**, **3**, **5,** or **6** (data not shown).

For a chemical compound to be beneficial as an anticancer drug, it must exhibit preferential anti-proliferative activity against tumor cells over normal and mortal cell-types. Hence, the ability of **4** versus that of cisplatin, to selectively target cancer cells, was evaluated by examining its cytotoxic effect against a mortal cell-type, namely peripheral blood mononuclear cells (PBMC). Noteworthy, PBMCs have been widely used as control cells to assess the specificity of many potential anticancer agents (Varamini et al. 2007; Pinto et al. 2011; Bendale et al. 2017). Interestingly, none of the indicated compounds showed significant cytotoxicity towards PBMCs (Table 2). Moreover, the combination of Cisplatin + compound **4** exerted no cytototoxic effect against PBMCs (Table 2).

Effect of Cisplatin + compound 4 combination on apoptotic components

Since our above results revealed that the combination of Cisplatin + compound **4** has a striking cytotoxic effect against TNBCs and in order to get further insight into the molecular mechanisms underlying the observed cell death, we assessed the effect of this treatment on different components regulating cellular apoptosis. The p53 protein is a tumor suppressor that becomes active once phosphorylated. In order to check whether the observed potent anticancer potential of Cisplatin + compound **4** was associated with p53 activation, we assessed the phosphorylation level of p53 in untreated cells versus Cisplatin, compound **4** and Cisplatin + compound **4** treated cells. A slight increase in p53 phosphorylation was observed in Cisplatin or compound **4** treated cells. Interestingly, p53 phosphorylation level was more pronounced in Cisplatin + compound **4** treated cells (Fig. 1a, b).

Table 1 The 13 thiosemicarbazone derivatives used in this study

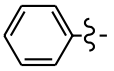
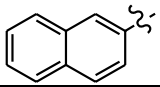
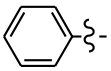
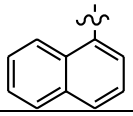
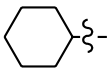
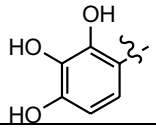
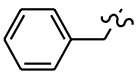
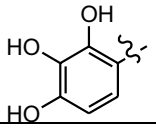
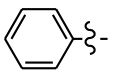
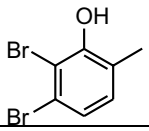
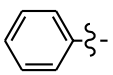
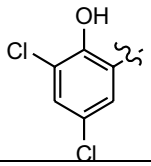
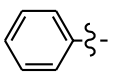
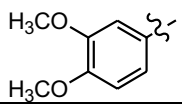
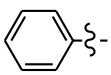
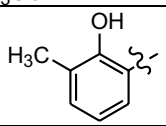
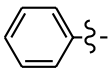
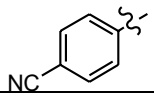
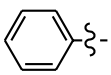
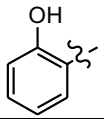
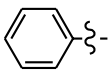
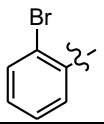
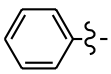
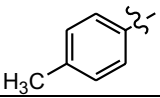
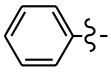
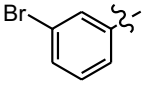
Compound	R_1	R_2
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		

Table 2 In vitro antiproliferative activity of thiosemicarbazone derivatives

Compounds (C)	IC50 (μM)	
	MDA-MB 231	PBMC
C1	> 100	–
C2	70 ± 7.8	> 100
C3	60 ± 5.2	> 100
C4	60 ± 1.5	> 100
C5	60 ± 1.7	> 100
C6	70 ± 2.4	> 100
C7	> 100	–
C8	> 100	–
C9	> 100	–
C10	> 100	–
C11	> 100	–
C12	> 100	–
C13	> 100	–
Cisplatin	44.8 ± 3.1	> 100
Cisplatin + C4	16 ± 2.9	> 100

PBMC (Peripheral Blood Mononuclear Cells) and MDA-MB-231 cells were treated with different concentrations (5, 25, 50, 75 and 100 μM) of indicated compounds for 48 h. IC50 value corresponds to the concentration of compound required to inhibit cell growth by 50% in comparison to untreated cells. Each value represents a mean ± SD ($n = 3$)

Once activated, p53 can activate the expression of *BAX*, a *BCL2* family member that triggers apoptosis. In parallel with p53 phosphorylation status, Bax protein levels increased in Cisplatin + compound **4** treated cells versus untreated cells (Fig. 1a, c). Moreover, an increase in Bax levels was observed after treating cells with either Cisplatin or compound **4** alone.

Further, we assessed the effect these products on the protein levels of Bcl2, an anti-apoptotic component. Differently from Cisplatin that caused only a slight decrease in Bcl2 levels, compound **4** triggered a more pronounced reduction in Bcl2 protein amount (Fig. 1a, d). Bcl2 amount was strikingly lowered in Cisplatin + compound **4** treated cells.

Cleavage of poly(ADP-ribose) polymerase (PARP) by caspases is a key event taking place during apoptosis. Here, we checked the ability of our products to trigger PARP cleavage. In contrast to untreated conditions where PARP was present in its full length uncleaved form, the cleaved form appeared in response to Cisplatin. Interestigly, the amount of cleaved PARP was further enhanced in cells treated with Cisplatin + compound **4** (Fig. 2a, b).

Altogether, these observations indicated that Cisplatin + compound **4** combination can interfere with the signalling pathways regulating MDA-MB-231 cells apopotosis.

Effect of Cisplatin + compound 4 combination on MDA-MB-231 cells' miRNA expression profile

Abnormal miRNA expression has been associated with the development of breast cancer and other tumors (Iorio et al. 2005; Kumar et al. 2007) and miRNAs are considered important molecular biomarkers and therapeutic targets in many diseases especially cancer (Raisch et al. 2013). In order to check whether miRNAs are involved in regulating the molecular mechanisms involved in triggering the cytotoxic effect of Cisplatin + compound **4** combination against TNBCs, we assessed the difference in miRNA expression profile in Cisplatin + compound **4** treated versus untreated MDA-MB-231 cells. RNA was first prepared from those cells and TaqMan Low Density Array (TLDA) was then applied to determine the miRNA expression profiles. 15 miRNAs appeared to be differentially expressed in treated versus untreated control cells (Table 3). In a second step, quantitative Real Time-PCR (qRT-PCR) was carried out to validate the differential expression of these miRNAs. The effect of Cisplatin + compound **4** on MDA-MB-231 cells miRNA expression profile was confirmed for 10 miRNAs exhibiting significantly altered expression levels where miR-23a, miR-125a-5p, miR-181a-5p, and miR-330 were upregulated whilst miR-133b, miR-324-5p, miR-521, miR-372, miR-509-5p, and miR-636 were downregulated in treated *versus* control cells (Fig. 3).

miR-125a-5p and miR-181a-5p negatively regulate BCL2 expression upon targeting the 3′UTR

Using the computational microRNA Data Integration Portal (mirDIP), *BCL2* was identified as a potential target for miR-125a-5p and miR-181a-5p. We, therefore, checked whether *BCL2* expression could be directly regulated by miR-125a-5p and/or miR-181a-5p. A *Renilla* luciferase reporter gene-based vector was designed in which 200 bp fragment of the 3′-UTR of *BCL2* containing the miR-125a-5p and the miR-181a-5p target sequences was cloned downstream of the reporter gene. This vector was referred to as psiCHECK-UTRwt. In parallel, the same procedure was applied to clone this *BCL2* 3′-UTR fragment with a deleted miR-125a-5p (psiCHECK-UTRdel1) or miR-181a-5p (psiCHECK-UTRdel2) target site. Transient transfection of psiCHECK-UTRwt in Hela cells led to ~ 20 to 30% decrease in reporter luciferase activity, when compared with the psiCHECK control vector (Fig. 4a, b). However, no decrease in reporter luciferase activity was observed in cells transfected with either psiCHECK-UTRdel1 or psiCHECK-UTRdel2 (Fig. 4a, b). Notably, a more substantial reduction in reporter luciferase activity was observed in Hela cells being co-transfected with either miR-125a-5p and psiCHECK-UTRwt (~ 65%) or miR-181a-5p and psiCHECK-UTRwt (~ 55%)

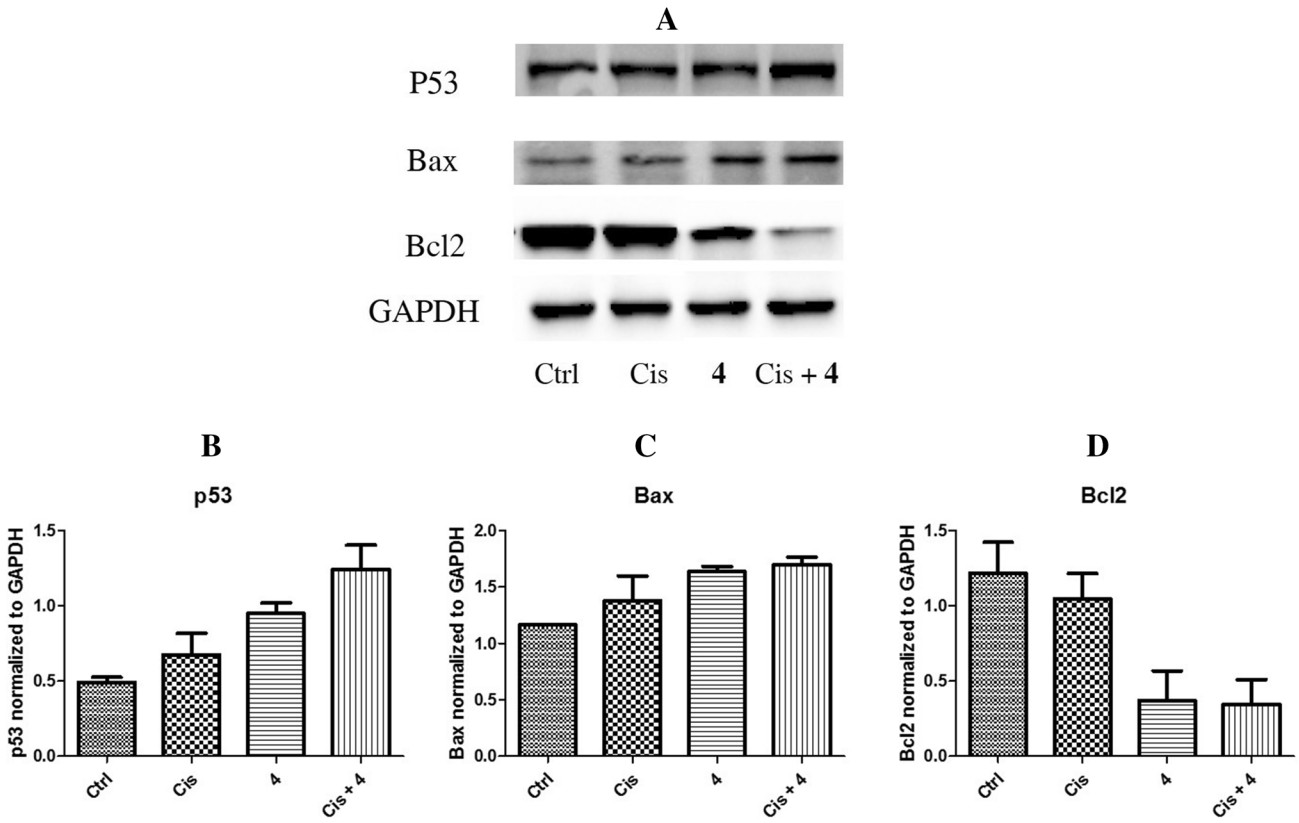


Fig. 1 Effect of thiosemicarbazone derivative compound 4 on Bax and BCL2 expression and p53 phosphorylation. **a** MDA-MB-231 cells were treated, for 48 h, with Cisplatin (45 µM), C4 (60 µM) or Cisplatin + compound 4 (16 µM). Total protein extracts were prepared and p53, Bax, BCL2 and GAPDH were detected as described in materials and methods. Representative western blot was shown. Levels of phosphorylated p53 (**b**), Bax protein (**c**) and BCL2 protein (**d**) were quantified by densitometer and normalized to GAPDH

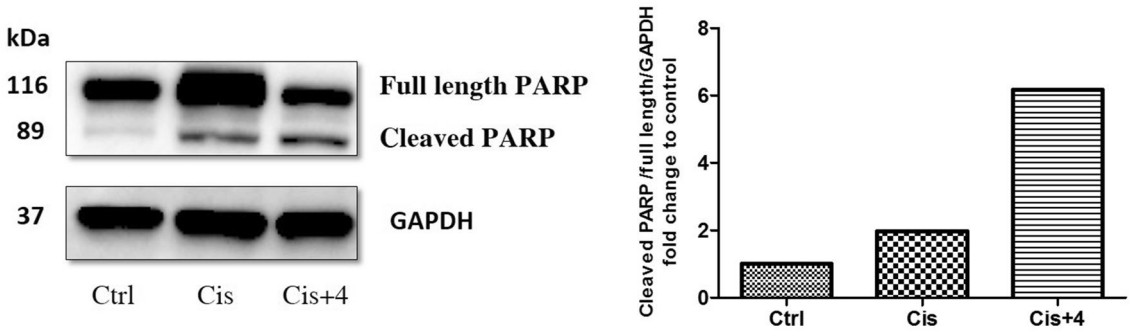


Fig. 2 Effect of thiosemicarbazone derivative compound 4 and cisplatin on PARP cleavage. **a** MDA-MB-231 cells were treated, for 48 h, with Cisplatin (45 µM) or Cisplatin + compound 4 (16 µM). Total protein extracts were prepared and PARP and GAPDH levels were detected. Representative western blot was shown. Levels of cleaved PARP form (**b**) were quantified by densitometer and normalized to full PARP form and GAPDH

in comparison to cells being transfected with psiCHECK-UTRwt alone (Fig. 4a, b). Further, the negative effect of either miR-125a-5p or miR-181a-5p was bypassed in cells co-transfected with either miR-125a-5p and psiCHECK-UTRdel1 or miR-181a-5p and psiCHECK-UTRdel2 (Fig. 4a, b). Remarkably, co-transfection of Hela cells with psiCHECK-UTRwt and both miR-125a-5p and miR-181a-5p resulted in more striking negative effect (~ 75%) in comparison to cells co-transfected with psiCHECK-UTRwt and either miR-125a-5p or miR-181a-5p (Fig. 4c). Altogether, these data indicate that *BCL2* expression could be negatively regulated by miR-125a-5p and miR-181a-5p.

Table 3 miRNA signature identified by TLDA technique

MicroRNA	Cisplatin	Cisplatin + C4	P value
hsa-miR-23a	2.5	5.5	0.021
hsa-miR-330	3.8	7.2	0.015
hsa-miR-125a-5p	5.5	11.5	0.001
hsa-miR-181a-5p	6.1	13.5	0.003
hsa-miR-489	3.3	7.1	0.03
miR-133b	0.45	0.12	0.013
miR-324-5p	0.144	0.02	0.015
miR-372	0.32	0.1	0.023
miR-509-5p	0.33	0.012	0.014
miR-521	0.14	0.01	0.023
miR-636	0.31	0.018	0.011
miR-616	0.41	0.28	0.041
miR-655	0.43	0.21	0.044
miR-708	0.48	0.27	0.039
miR-758	0.51	0.29	0.04

TLDA analysis unravelled 15 miRNAs to be differentially expressed in treated versus non-treated cells with a p value < 0.05

Lentiviral transduction of MDA-MB-231 cells and the negative effect of miR-125a-5p and miR-181a-5p on *BCL2* mRNA levels

Lentiviral based systems have been developed to generate replication incompetent lentiviral vectors that were efficiently used in the transduction of both dividing and non-dividing mammalian cells and provided stable, long term expression of RNA of interest. To investigate the effects of miR-125a-5p and miR-181a-5p on MDA-MB-231 cells and more specifically on *BCL2* expression, lentiviral vectors (lenti-miR-125a-5p, lenti-miR-181a-5p) containing copGFP as a reporter gene were produced and transduced into MDA-MB-231 cells. A scrambled lenti-miR-ctrl was used as a negative control. After optimization of transduction conditions, we defined an MOI (Multiplicity of infection) of six as the one providing the highest transduction rate. This MOI was then chosen to transduce MDA-MB-231 cells. The efficacy of transduction was measured 1 week after transduction. Measurement of GFP expression by flow cytometry showed 87–92% transduction efficiency. Subsequent flow cytometry sorting of

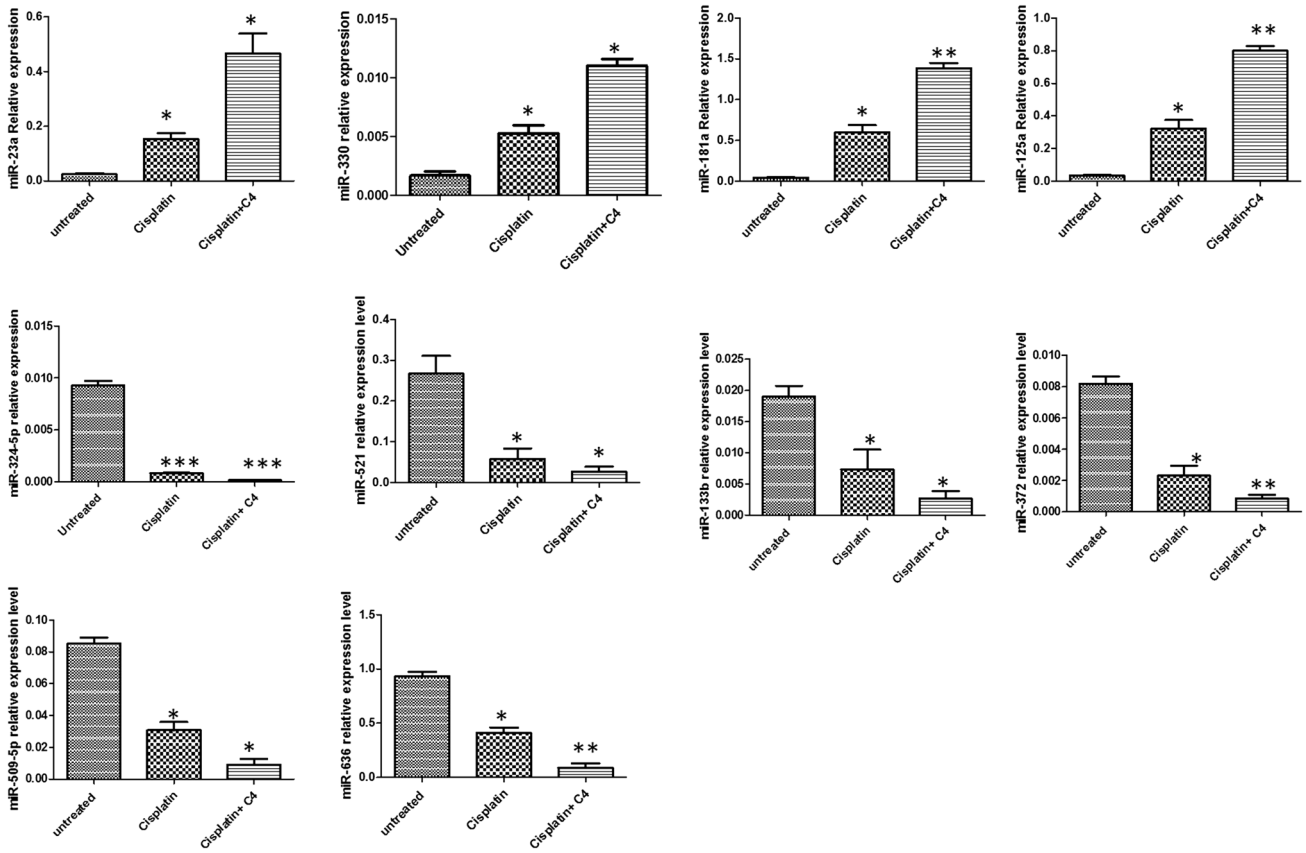


Fig. 3 Ten miRNAs are differentially expressed after Cisplatin + compound 4 treatment. MDA-MB-231 cells were cultivated in the absence or presence of Cisplatin + compound 4. *RNU48*-normalized miRNA levels were quantified by qRT-PCR. The statistical significance was determined using unpaired Student's t test (*p < 0.05, **p < 0.01, ***p < 0.001 v.s. untreated control cells)

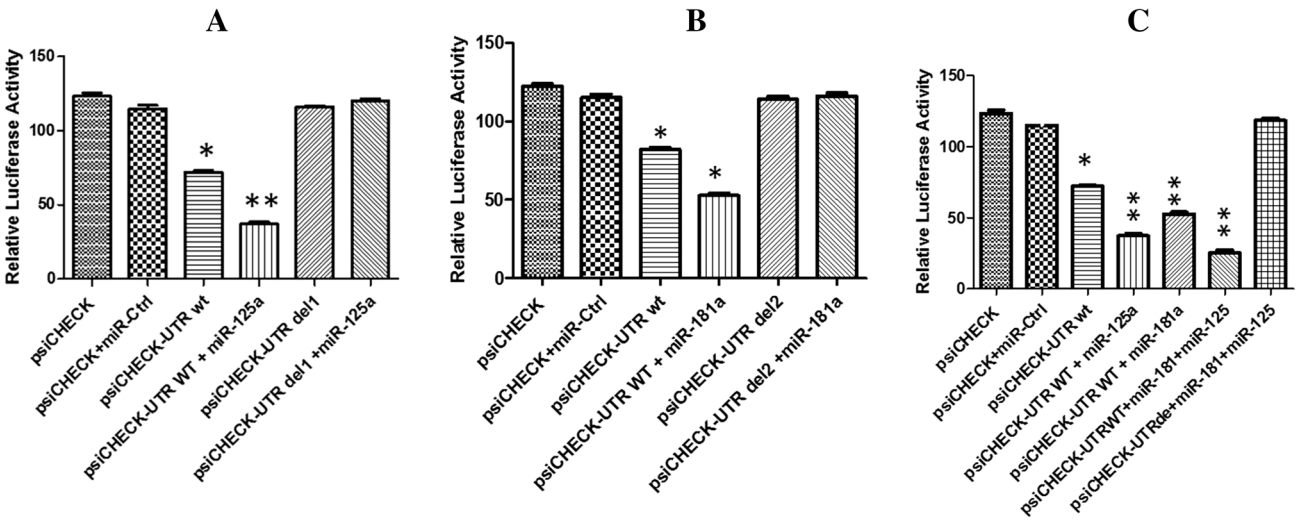


Fig. 4 A repressive effect of miR-125a-5p and miR-181a-5p on BCL2 expression via targeting BCL2 3′-UTR. Hela cells were transiently co-transfected with psiCHECK-UTRwt (bearing 200 bp of the BCL2 3′-UTR), psiCHECK-UTRdel1 or del2 (miR-125a-5p or miR-181a-5p target site was deleted, respectively) with miRNA control mimic, miR-125a-5p or mR-181a-5p mimics. Transfection with empty psiCHECK was used to assess the background promoter activity of the vector. Data represent mean ± SEM of three independent experiments, each performed in triplicate. **a** The effect of miR-125a-5p. **b** The effect of miR-181a-5p. **c** The synergistic effect of both miR-125a-5p and miR-181a-5p. $*p < 0.05$; $**p < 0.01$ vs. psiCHECK transfected cells; Student's t-test

the GFP-positive cells resulted in a 99% pure population. Quantitative real-time RT-PCR revealed an approximate twofold decrease of the mRNA level of *BCL2* in the lenti-miR-125a-5p, and lenti-miR-181a-5p transduced MDA-MB-231 cells compared to the Lenti-miR-ctrl transduced cells (Fig. 5) indicating that *BCL2* expression is negatively regulated by miR-125a-5p and miR-181a-5p.

Discussion

TNBCs, being characterized by absence of ER, PR and HER2, are among the most aggressive subtypes of epithelial breast malignancies. For patients with TNBC, endocrine therapies including Tamoxifen and aromatase inhibitors as well as HER2 targeted therapies including Trastazumab and Lapatinib are not efficient. On the other hand, TNBCs are sensitive to chemotherapy where anthracyclines and taxanes are commonly used for high risk patients (Chacón and Costanzo 2010). Despite this chemo-susceptibility, patients with TNBCs are at higher relapse risk than those presenting hormone positive breast cancer (Hudis and Gianni 2011). Designing an optimal chemotherapy which will ensure a longer overall survival of TNBC patients remains a challenge. Thiosemicarbazones correspond to a group of molecules with promising therapeutic potential, particularly cancer treatment (Pelosi 2010). For instance, Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone) is now used in clinical trials due to its potent cytotoxicity against various cancer cell lines, including pancreas, lung, ovary, kidney, and prostate (Murren et al. 2003). In this study, we examined the anti-cancer potential of 13 thiosemicarbazone derivatives against TNBC cells. Among these, different compounds showed moderate cytotoxic potential against TNBC cells. Intriguingly, combining compound **4** with Cisplatin resulted in a striking cytotoxic effect. Notably, this cytotoxicity was selective against cancer cells without harming the normal PBMCs. Such observations highlight an advantageous effect of compound **4** by reinforcing the selective cytotoxic potential of Cisplatin against TNBC cells.

The tumor suppressor p53 plays an essential role in controlling genomic integrity, cell cycle, and apoptosis in response to various stress signals (Hu et al. 2012). P53 is activated following phosphorylation in its N-terminal domain by protein kinases of the MAPK family (Eliaš et al. 2014). Activated p53 can then translocate to the nucleus, bind to its target sequences, and activate the transcription of genes involved in cell cycle arrest and apoptosis induction (such as *BAX*, *BAD* and *BIM*) (Yao et al. 2014; Mollereau and Ma 2014). On the other hand, accumulation of phosphorylated p53 in the mitochondria leads to apoptosis induction upon interacting with pro- and anti-apoptotic *BCL2* family members. In fact, binding of phosphorylated p53 to the pro-apoptotic proteins Bax and Bak leads to conformational changes alleviating the antagonism mediated by anti-apoptotic components (Westphal et al. 2011). Another major event taking place during apoptosis is the cleavage of PARP by various caspases, especially caspase-3 (Kaufmann et al. 1993). Interestingly, our results

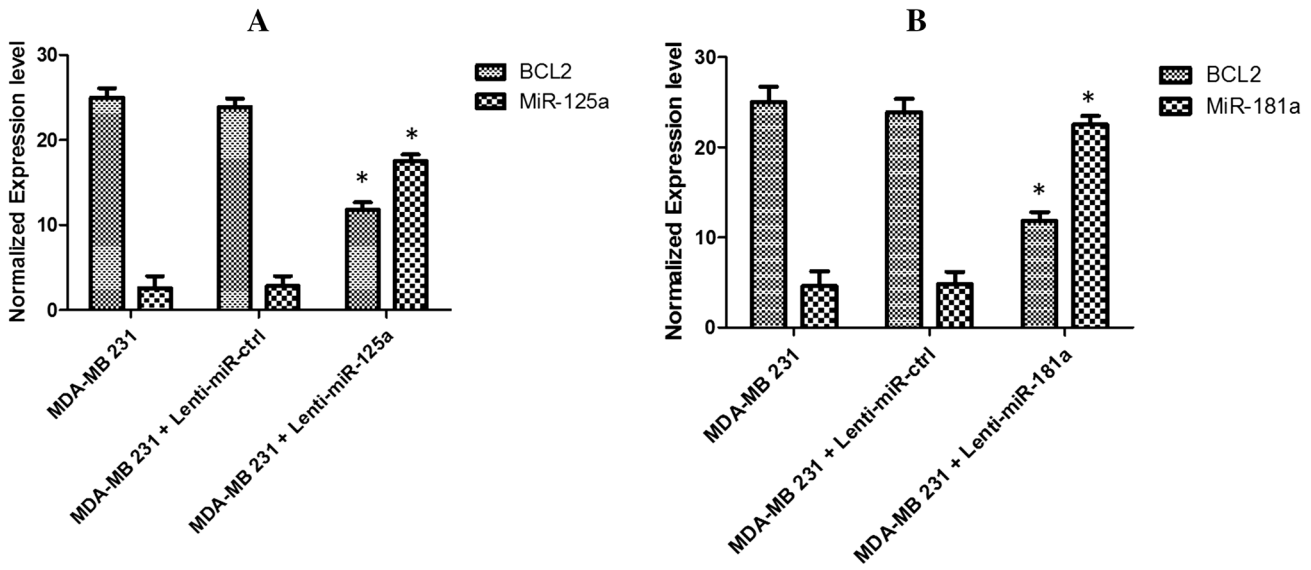


Fig. 5 Lentiviral-mediated miR-125a-5p and miR-181a-5p expression and their effect on BCL2 expression in MDA-MB-231 cells. miR-125a-5p, miR-181a-5p, and BCL2 expression were determined by qRT-PCR in MDA-MB-231 cells, miR-125a-5p, miR-181a-5p, and miR-Ctrl transduced MDA-MB-231cells. Mean of three independent experiments are shown. $*p < 0.05$ (lenti-miR-125a-5p, lenti-miR-181a-5p vs. lenti-miR-Ctrl transduced MDA-MB-231cells; Student's *t*-test)

showed that Cisplatin + compound **4** combination induced p53 phosphorylation, and this was accompanied with elavated Bax levels, reduced Bcl2 amount and enhanced PARP cleavage, thus indicating that Cisplatin + compound **4** anticancer effect could be attributed, at least in part, to their ability to trigger cell apoptosis. So far, the exact mechanism underlying the ability of compound 4, specifically, to enhance the anticancer potential of cisplatin is still unclear. The biological activities of thiosemicarbazone derivatives are often related to chelation of metal ions. They can coordinate to the metal center in N,S-bidentate mode, but when additional coordinating groups exist, more binding modes become possible. Particularly, the presence of three hydroxyl groups on compound 4 might provide a more favorable simultaneous coordination for one or more metal ions (such as Fe^{2+} or Cu^{2+}) which in turn could lead to downregulation of key enzymatic and regulatory functions regulating cell survival such as Ribonucleotide Reductase (iron-dependent enzyme) activity. Moreover, it could also be possible that the three hydroxyl groups present in compound 4 might render it soluble in aqueous medium and therefore can affect its ionization and lipid solubility.

Epigenetic modifications, including miRNAs expression, have been associated with cancer initiation and progression (Peña-Chilet et al. 2014). MiRNAs are regulatory elements capable of modulating a wide array of physiological processes, including proliferation, differentiation and apoptosis, upon modulating gene expression via binding the 3′UTR in target mRNA. Numerous miRNAs have been identified to be deregulated between normal and tumor breast tissues (Iorio et al. 2009; Leivonen et al. 2014). Besides, miRNAs can be involved in the tumorgenesis process via modulating the expression of certain oncogenes or tumor suppressor genes (Zhang et al. 2007; Cho 2007; Hummel et al. 2010). Intriguingly, drug-sensitive and drug resistant cancer cell lines exhibit distinct miRNA signatures (Chen et al. 2010; Zhou et al. 2010; Pogribny et al. 2010) and thus can serve not only as biomarkers but also as anticancer therapeutic targets. In this study, we examined changes in miRNA signature after the treatment of TNBC cells with Cisplatin + compound **4** combination. Importantly, this treatment affected the expression of 10 miRNAs, among which 4 were upregulated and 6 were downregulated. Upon screening for targets of the upregulated miRNAs, we identified the antiapoptotic *BCL2* gene as a putative target for miR-125a-5p and miR-181a-5p. The direct interaction between miR-125a-5p, miR-181a-5p, and *BCL2* was confirmed using dual-luciferase reporter assay. Indeed, we showed that miR-125a-5p and miR-181a-5p, via targeting their potential binding sites in *BCL2* gene, can negatively regulate gene expression. In fact, a repressive effect of miR-125a-5p on *BCL2* expression has already been established. For instance, miR-125a-5p was reported to be involved in repressing *BCL2* expression in response to CD40 ligand in human Leukemic B-cells (Willimott and Wagner 2012). Moreover, miR-125a-5p was shown to inhibit cell proliferation and trigger apoptosis in colon cancer upon targeting *BCL2* (Tong et al. 2015). Further, miR-125a-5p was described to enhance chemotherapy sensitivity to Cisplatin via downregulating *BCL2* expression in gallbladder cancer (Yang et al. 2017). Mir-181a-5p has also been described to enhance drug sensitivity in lymphocytic

leukemia cells upon targeting multiple anti-apoptotic genes including *BCL2* (Zhu et al. 2012).

Conclusion

In conclusion, we report here a potent anticancer effect for a new drug combination, Cisplatin + thiosemicarbazone compound **4** against TNBC cells upon modulating the activity and expression of different cellular elements (p53, Bax, Bcl2, PARP and miRNAs) and triggering cellular apoptosis.

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Compliance with ethical standards

Conflict of interest Rania El Majzoub, Mohammad Fayyad-kazan, Assaad Nasr El Dine, Rawan Makki, Eva Hamade, René Grée, Ali Hachem, Rabih Talhouk, Hussein Fayyad-Kazan and Bassam Badran declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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