

Evaluation of the Cytotoxicity of a Circular SIRT1 Aptamer on MCF-7 and A549 Cell Lines and Its Impact on HIF-1 α and VEGFR2 Expression

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Abstract:

Background: Selective cytotoxicity against malignant cells with limited toxicity to normal cells remains an important goal in anticancer drug development.

Objectives: To evaluate the cell viability and preliminary relative selectivity of a circular single stranded DNA SIRT1 aptamer in A549 human lung adenocarcinoma cells and MCF-7 human breast adenocarcinoma cells compared with HUVECs human umbilical vein endothelial cells, and to study its effects on HIF-1 α and VEGFR2 protein expression.

Methods: A549 and MCF-7 cells were treated with SIRT1 aptamer concentrations ranging from 0.0195 to 10 μ M for 24, 48, and 72 h, while HUVECs were treated with concentrations ranging from 0.078 to 40 μ M for the same exposure times. Cell viability was measured by XTT assay. IC₅₀ and selectivity index values using HUVECs as the non-malignant comparator were calculated. HIF-1 α and VEGFR2 expression in A549 and MCF-7 cells were measured by Western blotting.

Results: The aptamer induced time- and concentration-dependent reduction in cell viability. The IC₅₀ values after 72 h were 0.742 μ M in A549, 0.473 μ M in MCF-7 and 4.018 μ M in HUVECs. The selectivity index values were 5.42 and 8.49 for A549 and MCF-7 respectively. HIF-1 α was significantly reduced in both cancer cell lines, while VEGFR2 was significantly reduced only in A549 cells.

Conclusion: The circular SIRT1 aptamer showed preliminary relative selectivity against A549 and MCF-7 cancer cell lines compared with HUVECs and was associated with reduced HIF-1 α



expression, with VEGFR2 reduction observed mainly in A549 cells. However, further studies are required to confirm SIRT1 involvement, define the mechanism of cell-death/viability reduction, and determine whether these molecular changes translate into functional antiangiogenic effects.

Keywords: SIRT1 aptamer; A549; MCF-7; HUVECs; cell viability; relative selectivity index; XTT assay.

Introduction

Cancer continues to be a major global health issue worldwide (1, 2). In 2022, over 20 million new cancer cases and 9.7 million cancer-related fatalities were reported globally (3). Lung cancer had the distressed distinction of being the most commonly diagnosed kind of cancer all over the world, in addition to being the leading cause of death from cancer throughout the world (4, 5). Female breast cancer also represented a considerable share of the global cancer burden (6, 7). Both tumor types continue to present major therapeutic challenges because of biological heterogeneity, variable treatment response, and the need for agents that can suppress malignant cells while minimizing injury to normal tissues (8, 9).

Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase that regulates multiple cellular processes, including genomic stability, metabolism, oxidative stress, apoptosis, and angiogenesis (10). In cancer biology, SIRT1 has a context-dependent role and may function either as a tumor suppressor or as a tumor promoter depending on the cell type, signaling environment, and metabolic state (11-13).

Aptamers, which are short and single-stranded nucleic acid molecules created using (SELEX) systematic evolution of ligands by exponential enrichment, can bind to target molecules with both strong attraction and high specificity (14). Unlike traditional small-molecule modulators, aptamers have several advantages. These include the ability to be made through chemical synthesis, the ability to be programmed structurally, less variation between different batches, and the

potential for improved selectivity (15). The circular SIRT1 aptamer employed in this investigation has been previously documented to bind and activate SIRT1, penetrate cancer cells, maintain stability in human plasma, and elicit direct viability-inhibitory effect in cancer cells while sparing a non-cancerous cell model, thus supporting its further preclinical investigation as a targeted anticancer ligand (16).

Because the therapeutic potential of an anticancer compound depends not only on potency against tumor cells but also on its relative safety toward non-malignant cells, early evaluation of cytotoxic selectivity is essential (17). HUVECs (Human umbilical vein endothelial cells) are widely used as an *in vitro* endothelial model and provide a relevant system for preliminary assessment of vascular tolerance, moreover, they serve as a useful platform for evaluating antiangiogenic-related molecular changes (18, 19).

Besides direct cytotoxicity towards tumor cells, the suppression of angiogenesis-related signaling constitutes a significant approach in cancer therapy. Hypoxia-inducible factor-1 α (HIF-1 α) is a major transcriptional regulator of tumor adaptation to hypoxia and contributes to angiogenesis by regulating VEGF-related pathways (20, 21). VEGFR2 is the principal signaling receptor for VEGF-A and mediates several angiogenic responses, including endothelial proliferation, migration, survival, and vascular permeability. Because SIRT1 has been linked to the regulation of hypoxia responses and angiogenic signaling, assessment of HIF-1 α and VEGFR2 protein expression may



provide preliminary mechanistic insight into whether the circular SIRT1 aptamer affects angiogenesis-associated pathways in addition to its effect on cell viability/metabolic activity (22). However, these markers are to be considered as molecular indications, and not as a clear indication of functional antiangiogenic efficacy.

Therefore, current study was designed to investigate the effect of a circular SIRT1 aptamer on cell viability in lung adenocarcinoma cells A549 and breast adenocarcinoma cells MCF-7 in comparison with HUVEC endothelial cells, with the aim of determining whether the aptamer shows preferential viability-inhibitory activity in malignant cells compared with HUVECs. In addition, the present study also evaluated the effect of the SIRT1 aptamer on HIF-1 α and VEGFR2 protein expression in A549 and MCF-7 cells using Western blot analysis.

Materials and method

Chemicals

The (XTT) reagent [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] and the electron coupling reagent phenazine methosulfate (PMS) were obtained from Hangzhou Hyper Chemicals Limited (China).

Study design

This *in vitro* study was carried out in higher studies/postgraduate laboratory at the Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, using three cell lines: A549 human lung adenocarcinoma cells, MCF-7 human breast adenocarcinoma cells, and HUVECs human umbilical vein endothelial cells. The study was designed in two main parts. First, the assessment of the cell viability/metabolic activity profile of a circular SIRT1 aptamer in malignant and non-malignant cells by subjecting them to the

comparable experimental settings. Second, to explore possible mechanistic effects related to angiogenesis-associated signaling.

Cell lines and culture conditions

High-glucose Dulbecco's Modified Eagle's Medium (DMEM) was used to grow A549 cells (23); RPMI-1640 medium was used for maintaining MCF-7 cells (24); and Medium 199 (M199) was used for culturing Human umbilical vein endothelial cells (HUVECs) (18). The culture media for A549 and MCF-7 cells were supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). While, the medium for HUVECs contained 20% fetal bovine serum in addition to 1% penicillin-streptomycin. All cell lines were kept at 37°C in a humidified incubator with 95% humidity and 5% CO₂. All three cell lines were subcultured at approximately 70–80% confluence using 0.05% trypsin-EDTA; Trypan blue exclusion method was used for viable cells counting before seeding (25).

Cell Cryopreservation and Thawing

All three cell lines were stored for an extended period in liquid nitrogen. The cells were immediately thawed in a water bath at 37 °C for 1–2 minutes. Subsequently, the cells were resuspended in 10 ml of fresh media and centrifuged at about 1000 rpm for 5 minutes. Following centrifugation, the cell pellets were carefully resuspended in complete media and transferred to a 75 cm² tissue culture flask containing 15 ml of complete growth medium (26).

SIRT1 Aptamer and Cell

Viability/Metabolic Activity Assessment

The compound under investigation was a circular single-stranded DNA SIRT1 aptamer, characterized by the sequence 5'-CGAGTGGGTTACATCGAACTGGATC TCAACAGCGGTAAC-3' (16). The manufacturer (BioNeer, Korea) performed



desalting and lyophilization of the aptamer. A ligation splint, 5'-TCTCTTCCGAATTCCATCAGGA-3', was used as a linker sequence for the circularization of the aptamer, (Sigma-Aldrich Ltd., USA). To enhance the purification of the aptamer, polyacrylamide gel electrophoresis (PAGE) was directed by applying 10% denaturing gel containing 8 M urea (27).

To evaluate cell viability/metabolic activity, all three cell lines were plated in 96-well plates at a density of 5×10^3 cells per well, using 100 μ L of complete medium. Then, cells were permitted to adhere overnight. On the subsequent day, the medium was replaced with fresh medium containing final aptamer concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039 and 0.0195 μ M for A549 and MCF-7, while HUVECs received slightly higher concentrations 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 μ M. Untreated cells of the three lines receiving vehicle-containing medium were used as the negative control, whereas wells containing

medium without cells were used as blanks. Plates were incubated for 24, 48, and 72 h under standard culture conditions. Each concentration was evaluated in triplicate, and the entire experiment was independently repeated three times (28).

At the end of the treatment period, XTT reagent was prepared freshly. A solution of phenazine methosulfate (PMS) was prepared by dissolving 3 mg of PMS in 1 mL of phosphate-buffered saline (PBS). To prepare the working XTT solution, 4 mg of XTT was dissolved in 4 mL of the culture medium. The detection mixture was prepared by combining 10 μ L PMS solution with 4 mL XTT solution. Following this, 50 μ L of the detection mixture was introduced into each well, and the plates were incubated at 37°C for a duration of 4 hours. Absorbance readings were subsequently acquired at 450 nm, employing a reference wavelength of 630 nm, utilizing a microplate reader (29). Cell viability was calculated according to the following equations:

$$\text{Cell viability (\%)} = \frac{A_0 - A_1}{A_2 - A_1} * 100$$

Where: A0: Absorbance of sample, A1: Absorbance of blank, A2: Absorbance of control

$$\text{Cell viability inhibition (\%)} = 100 - \text{Cell viability (\%)}$$

Selectivity index (SI)

To assess preliminary relative selectivity, SI was calculated using HUVECs as the non-malignant endothelial comparator, a SI was computed, employing HUVECs as the

normal-cell reference. The SI was derived from the ratio of the half-maximal inhibitory concentration IC_{50} in HUVECs to the IC_{50} in the corresponding cancer cell line, as expressed by the equation:

$$\text{Selectivity index} = \frac{IC_{50} \text{ in HUVEC}}{IC_{50} \text{ in cancer cells}}$$

A higher SI value suggests a greater preferential cell viability/metabolic activity inhibition effect on malignant cells and lower sensitivity of HUVECs compared with the tested cancer cell lines under the present XTT conditions. In this investigation, the relative

selectivity index was calculated independently for A549 and MCF-7 cells, utilizing the 72-h IC_{50} values obtained from the XTT assay (30).



Protein extraction and western blotting

Protein lysates were prepared from A549 and MCF-7 cells treated for 48 h with the SIRT1 aptamer at one-half of the calculated 48 h IC_{50} concentration, 0.480 μ M for A549 and 0.467 μ M for MCF-7, while control cells were treated with nuclease-free water for the same duration. Cells were lysed using RIPA lysis buffer supplemented with protease inhibitors. The lysates were then centrifuged at 12,000 rpm for 15 min at 4°C to remove cellular debris, and the resulting supernatants were collected for protein analysis. Total protein concentration was determined using the bicinchoninic acid (BCA) assay. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST to reduce nonspecific binding. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against HIF-1 α , VEGFR2, and GAPDH, which was used as the loading control. After incubation with the appropriate secondary antibodies, protein bands were visualized using enhanced chemiluminescence (ECL). Band intensities were quantified by densitometric analysis using ImageJ software (31).

Statistical analysis

To estimate IC_{50} values, the concentrations of the SIRT1 aptamer were logarithmically transformed and plotted against percentage of inhibitory effect on cell viability using Microsoft Excel. A linear regression equation and coefficient of determination (R^2) were obtained for each cell line. The IC_{50} value was calculated by substituting $y = 50$ into the regression equation, which then allowed for the determination of the corresponding concentration through antilogarithmic functions (32). For the cell viability/metabolic activity assay, the effects

of aptamer concentration and exposure time on cell viability were assessed using two-way analysis of variance (two-way ANOVA). Comparisons between groups in western blot were performed using independent-samples t-test. The findings are presented as mean $\pm$ standard deviation (SD) from three replicates ($n = 3$). Statistical analyses were performed using Minitab software, version 19. Statistical significance was considered at p -value < 0.05 .

Results

Cell viability assessment

The A549 cells were incubated with different concentrations ranging from 0.0195 to 10 μ M of SIRT1 aptamer. Cell viability assessment was conducted to determine the inhibitory effect of the investigated aptamer on A549 cell line. The half-maximal inhibitory concentrations (IC_{50}) of SIRT1 aptamer in A549 cells was obtained after plotting the concentrations following log transformation against cell viability inhibition percentage. The IC_{50} values of the investigated aptamer were 2.464, 0.960 and 0.742 μ M, at 24, 48, 72 h respectively as shown in **Figure 1**. The regression equations obtained by Excel were $y = 40.641x + 34.082$, $y = 35.254x + 50.624$, $y = 36.08x + 54.668$, with $R^2 = 0.9164$, 0.9314, 0.9392 at 24, 48, 72 h respectively. In MCF-7 cell line, the IC_{50} value was obtained from the same concentration range of SIRT1 aptamer. The result showed that the IC_{50} of the investigated aptamer were 1.638, 0.934 and 0.473 μ M at 24, 48, 72 h in MCF-7 cells, as shown in **Figure 2**. The regression equations were $y = 32.046x + 43.133$, $y = 33.622x + 50.994$ and $y = 31.117x + 60.122$, with $R^2 = 0.937$, 0.9584 and 0.9154 at 24, 48, and 72 h respectively. These findings suggest that MCF-7 cells were more sensitive to SIRT1 aptamer than A549 cells under the same experimental conditions. In HUVEC cell line, concentrations range from 0.078 to



40 μM of SIRT1 aptamer were applied, the IC_{50} values of SIRT1 aptamer were 17.443, 6.53 and 4.018 μM at 24, 48, and 72 h respectively as shown in **Figure 3**. The regression equations obtained by Excel were $y = 24.434x + 19.662$, $y = 28.447x + 26.818$ and $y = 28.333x + 32.887$, with $R^2 = 0.9264$, 0.9606 and 0.9759 at 24, 48, and 72 h. The higher IC_{50} value in HUVEC cells indicates

that a higher concentration of SIRT1 aptamer was required to produce 50% inhibition in endothelial cells, suggesting that HUVECs were less sensitive than the tested cancer cell lines under the same experimental conditions. IC_{50} values of SIRT1 aptamer in tested cells line at different time showed in **Table 1**.

Table 1. IC_{50} values of the SIRT1 aptamer in A549, MCF-7 and HUVEC cells line at different time points

<i>Cell lines</i>	<i>IC_{50} values (μM)</i>		
	24h	48h	72h
A549	2.464	0.960	0.742
MCF-7	1.638	0.934	0.473
HUVEC	17.443	6.530	4.018

Two-way ANOVA result in **Figure 4**, showed significant effects of concentration, exposure time, and concentration $\times$ time interaction on cell viability inhibition in A549, MCF-7, and HUVECs cells. In all three cell lines, concentration, time, and their interaction were highly significant, $p < 0.0001$. These results indicate that the effect of the SIRT1 aptamer was both concentration- and time-dependent.

Comparison of percentage cell viability between A549, MCF-7, and HUVEC cell lines treated with SIRT1 aptamer at 72 h is shown in **Figure 5**. The results demonstrated a concentration-dependent reduction in cell viability in all tested cell lines. At the highest comparable concentration (10 μM), the percentage of cell viability was 18.83% in A549, 21.76% in MCF-7, and 37.96% in HUVEC cells. At the lowest comparable concentration (0.078 μM), the percentage of cell viability was 99.35%, 79.65%, and 94.25% in A549, MCF-7, and HUVEC cells, respectively. Overall, MCF-7 cells showed the greatest sensitivity to SIRT1 aptamer,

followed by A549 cells, whereas HUVEC cells showed the lowest sensitivity.

Using HUVECs as the non-malignant comparator, the selectivity index of the SIRT1 aptamer at 72 h was 5.42 for A549 cells and 8.49 for MCF-7 cells. These values were calculated from the IC_{50} ratios 4.018/0.742 for A549 and 4.018/0.473 for MCF-7. The findings indicate that the concentration required to inhibit HUVEC viability by 50% was approximately 5.4-fold higher than that required for A549 cells and 8.5-fold higher than that required for MCF-7 cells. Therefore, the results suggest a preliminary relative selectivity profile, as the tested cancer cell lines showed greater sensitivity than HUVECs under the present experimental conditions. However, because HUVECs are endothelial cells and do not represent normal lung or breast epithelial tissues, the selectivity index should be interpreted as a preliminary screening estimate rather than definitive evidence of tumor-specific selectivity.



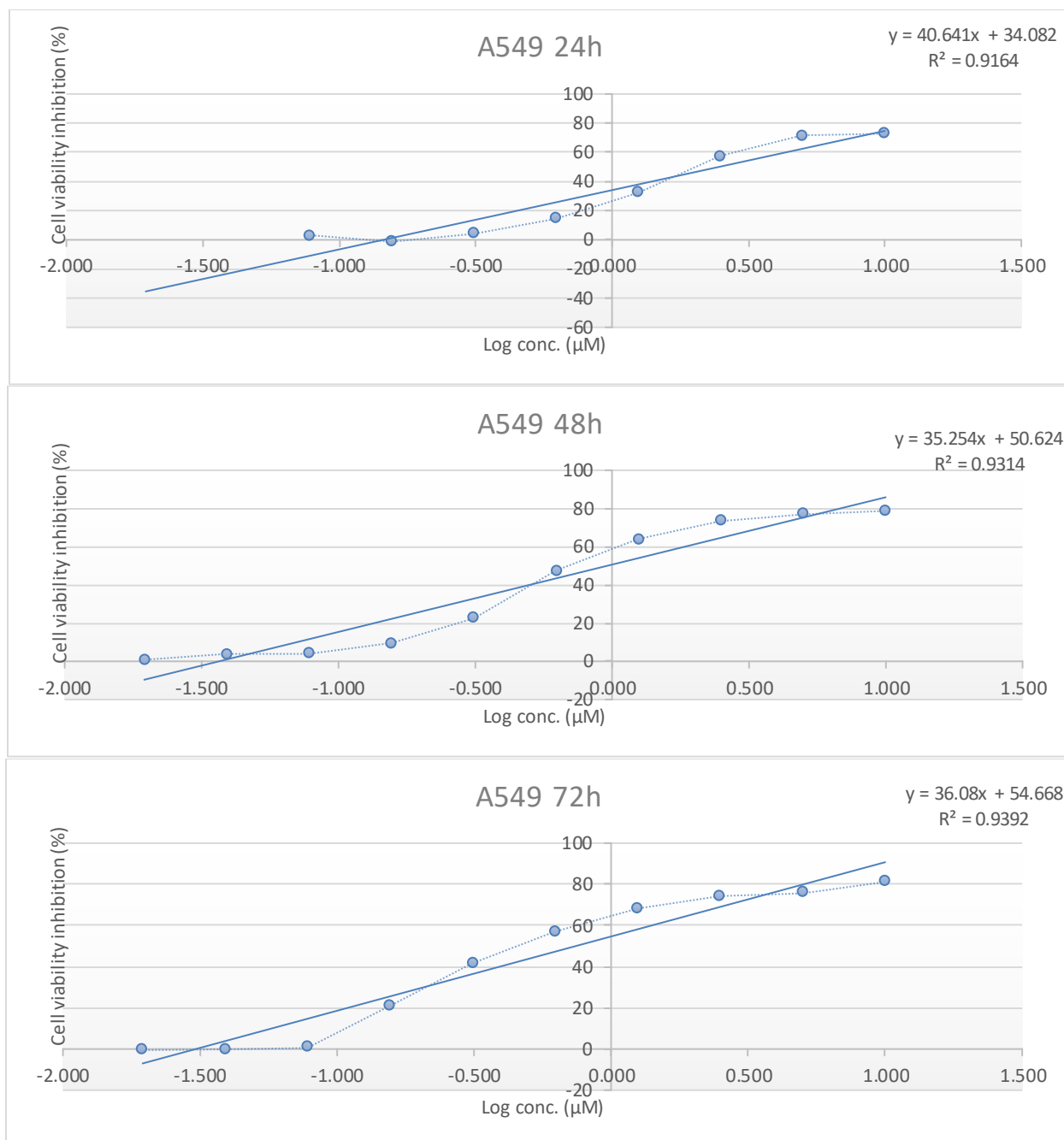


Figure (1) IC_{50} of SIRT1 aptamer in A549 cell line. A549 human lung adenocarcinoma cells were treated with increasing concentrations of SIRT1 aptamer (0.0195–10 μ M) for 24, 48, and 72 h. Cell viability was determined using the XTT assay. The absorbance was measured at 450 nm and the reference wavelength was 630 nm using a microplate reader. The dose–response curve was constructed by plotting the logarithm of concentrations against percentage cell viability inhibition (%) using Microsoft Excel.



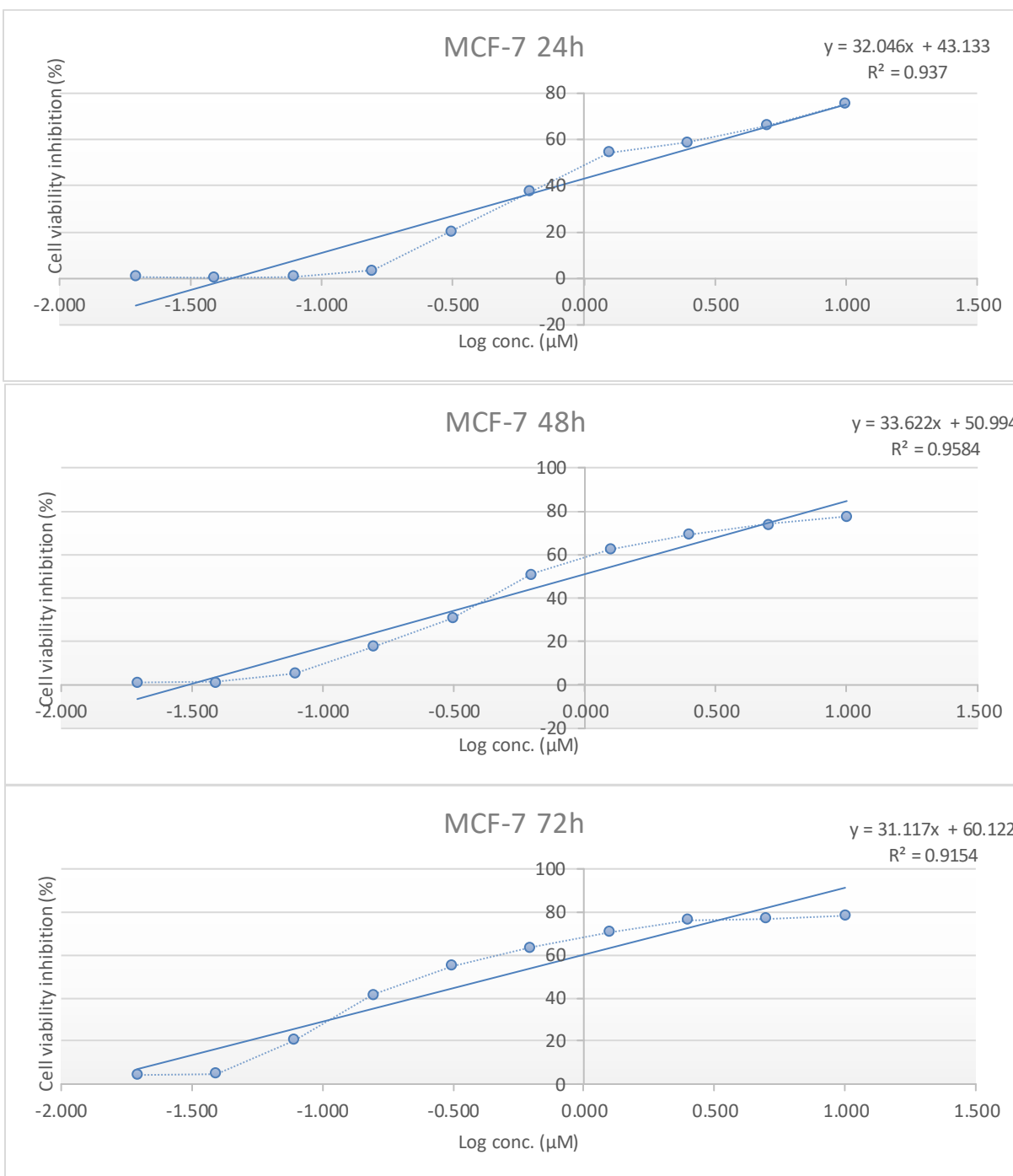


Figure (2) IC_{50} of SIRT1 aptamer in MCF-7 cell line. MCF-7 human breast adenocarcinoma cells were treated with increasing concentrations of SIRT1 aptamer (0.0195–10 μ M) for 24, 48, and 72 h. Cell viability was determined using the XTT assay. The absorbance was measured at 450 nm and the reference wavelength was 630 nm using a microplate reader. The dose–response curve was constructed by plotting the logarithm of concentrations against percentage cell viability inhibition (%) using Microsoft Excel.



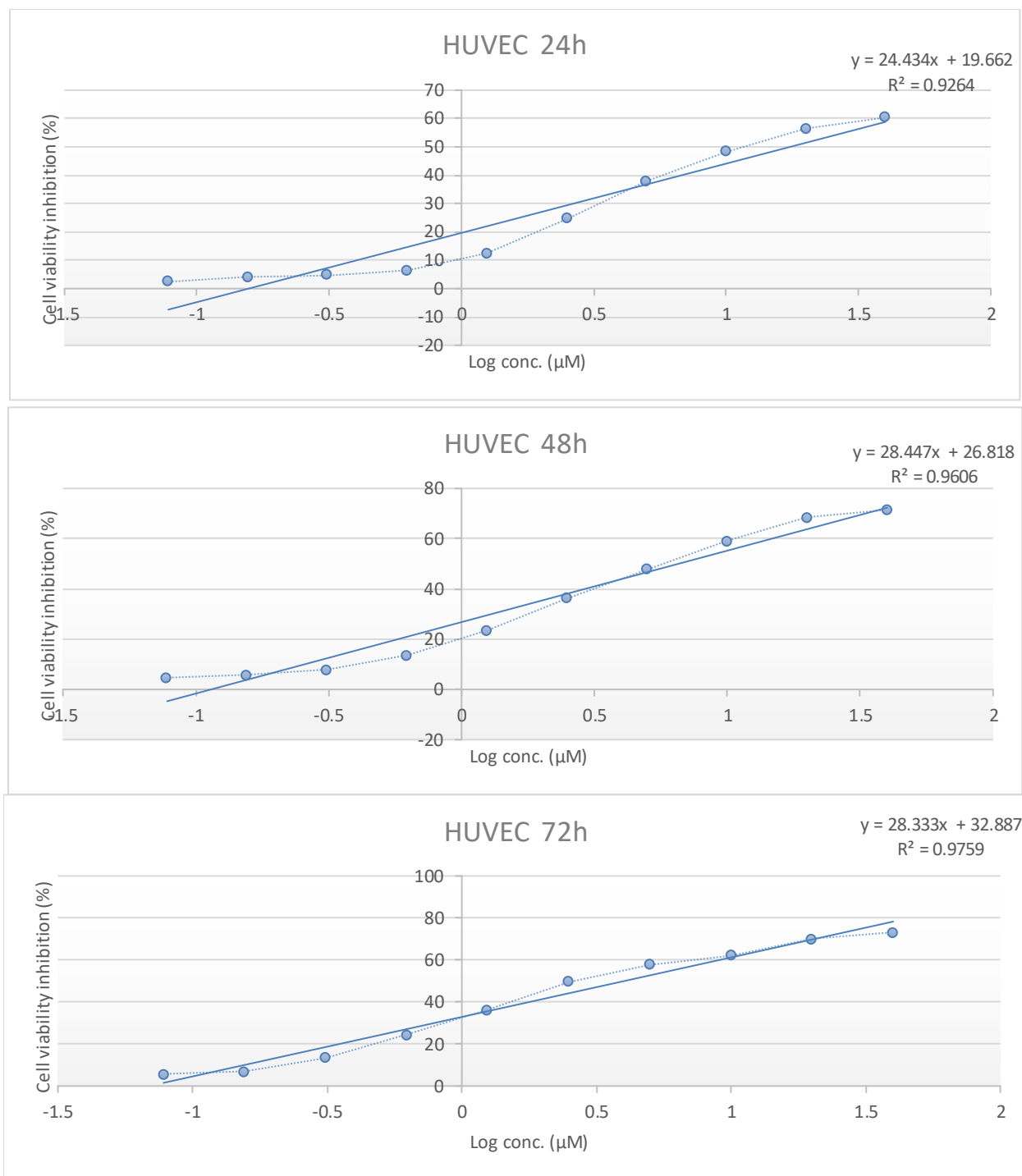


Figure (3) IC₅₀ of SIRT1 aptamer in HUVEC cell line. Human umbilical vein endothelial cells (HUVECs) were treated with increasing concentrations of SIRT1 aptamer (0.078–40 μM) for 24, 48, and 72 h. Cell viability was determined using the XTT assay. The absorbance was measured at 450 nm and the reference wavelength was 630 nm using a microplate reader. The dose–response curve was constructed by plotting the logarithm of concentrations against percentage cell viability inhibition (%) using Microsoft Excel.

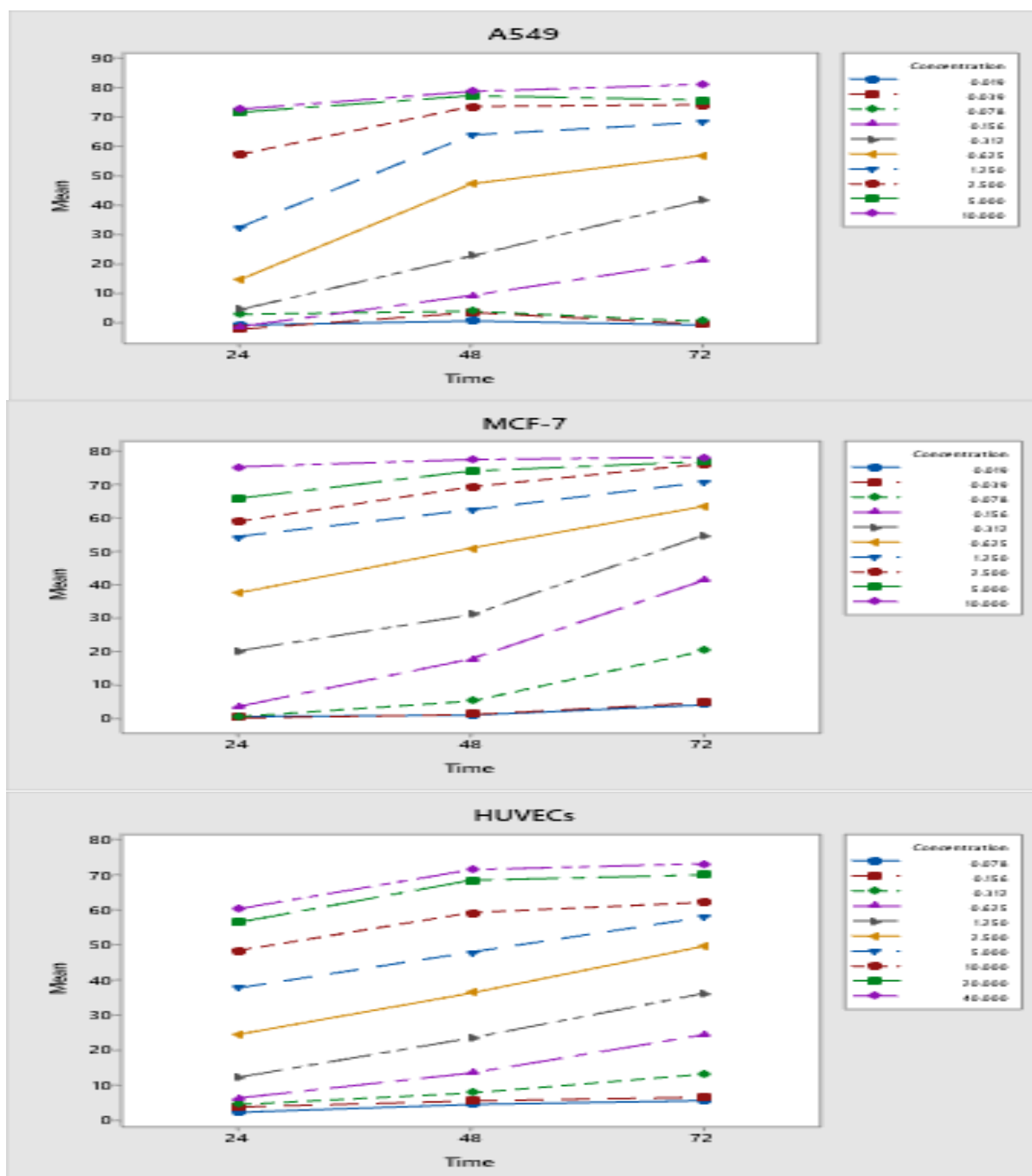


Figure (4). Interaction plots showing the effects of SIRT1 aptamer concentration and exposure time on cell viability inhibition in A549, MCF-7, and HUVEC cells. A549 and MCF-7 cells were treated with 0.0195–10 μ M SIRT1 aptamer, whereas HUVECs were treated with 0.078–40 μ M for 24, 48, and 72 h. Cell viability was assessed using the XTT assay, and each point represents the mean cell viability inhibition from three independent experiments. Each line represents one aptamer concentration across the three exposure times.

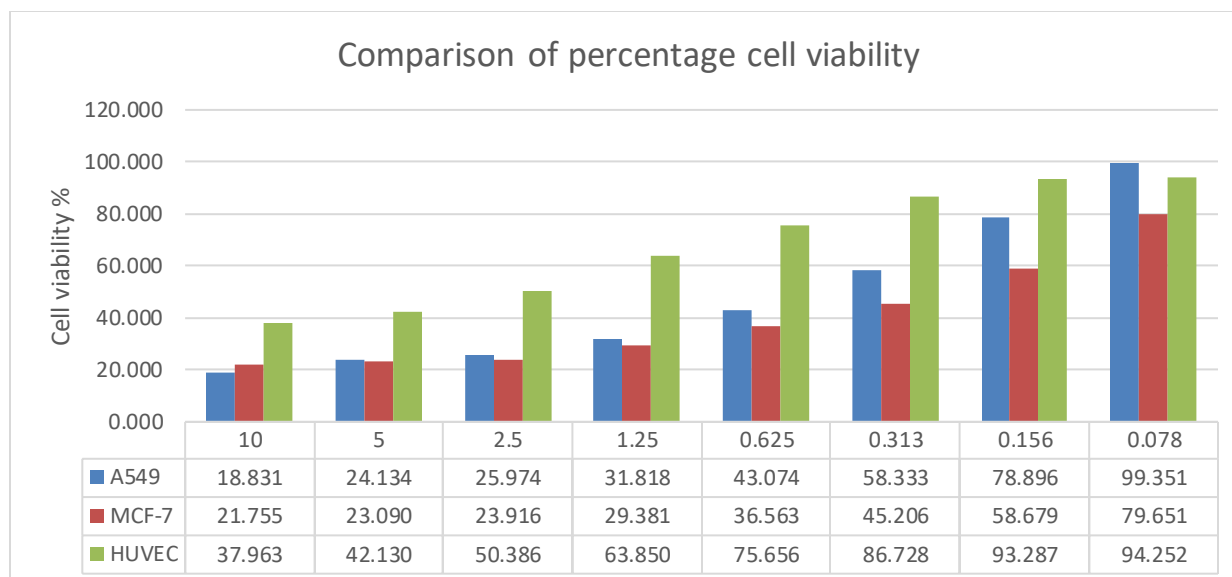


Figure (5) Comparison of percentage cell viability between A549, MCF-7, and HUVEC cell lines after treatment with SIRT1 aptamer. A549, MCF-7, and HUVEC cell lines. This figure presents only the common comparable concentration range of 0.078–10 μ M shared by the three cell lines for 72 h. Cell viability was assessed using the XTT assay. Absorbance values were measured at 450 nm and the reference wavelength was 630 nm and normalized to untreated control cells.

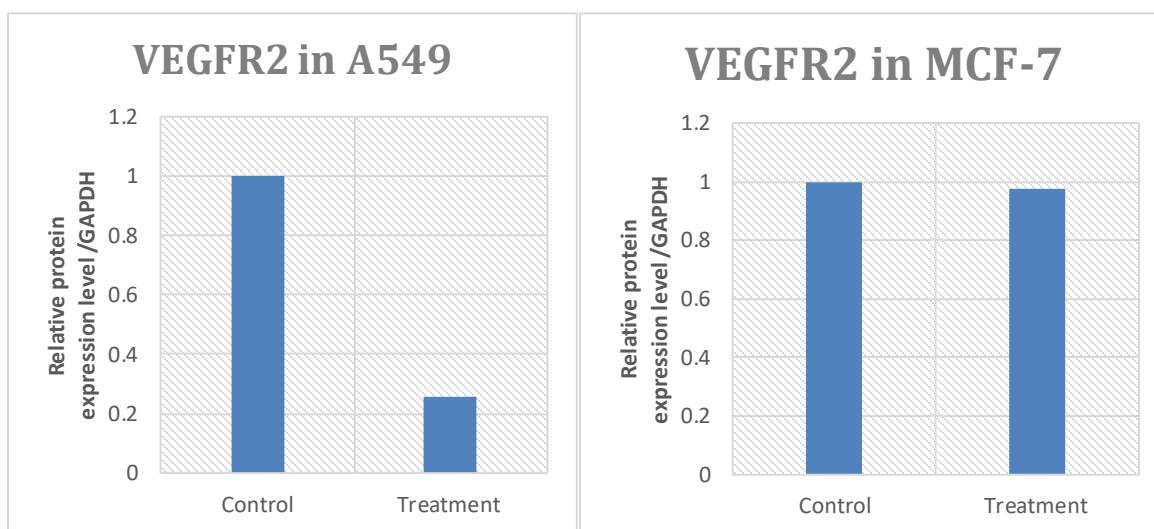
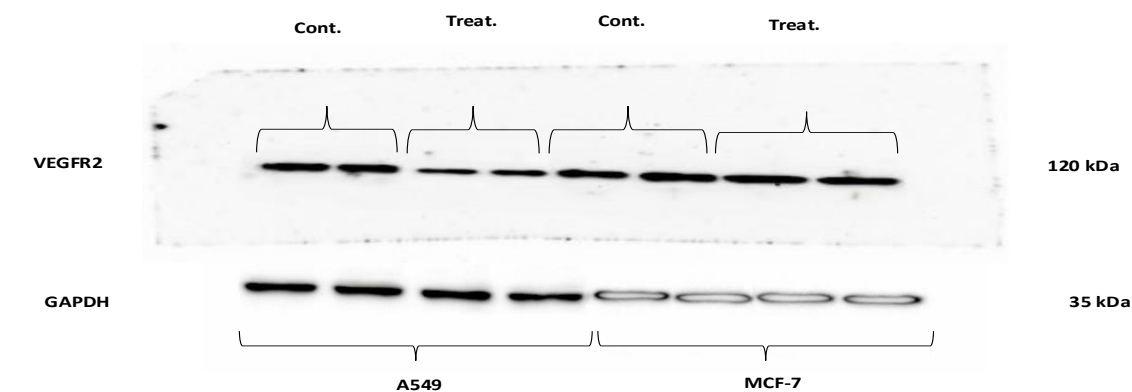
The effect of aptamer on expression of HIF-1 α and VEGFR2 proteins

To assess the ability of aptamer to modulate angiogenic parameters, HIF-1 α and VEGFR2 were assessed at protein level. VEGFR2 serves as the principal receptor for VEGF-A and is an essential element in angiogenic processes, encompassing endothelial proliferation, migration, survival, and enhanced vascular permeability. Evaluating VEGFR2 protein levels after SIRT1 aptamer treatment may provide preliminary molecular insight into whether the aptamer influences the VEGF/VEGFR2-related signaling pathway (33). The protein blot results as shown in **Figure 6**. demonstrated a decrease in VEGFR-2 band intensity in treated A549 cells relative to the control group. Densitometric analysis using Minitab software, employing independent-samples t-test, indicated a significant decrease in VEGFR2 expression in A549 cells following treatment with the SIRT1 aptamer (p-value =

0.0324). In MCF-7 cells, the intensity of the VEGFR2 band appeared similar in both control and treated samples, and densitometric analysis revealed no significant difference between the two groups following aptamer SIRT1 treatment (p-value = 0.6960).

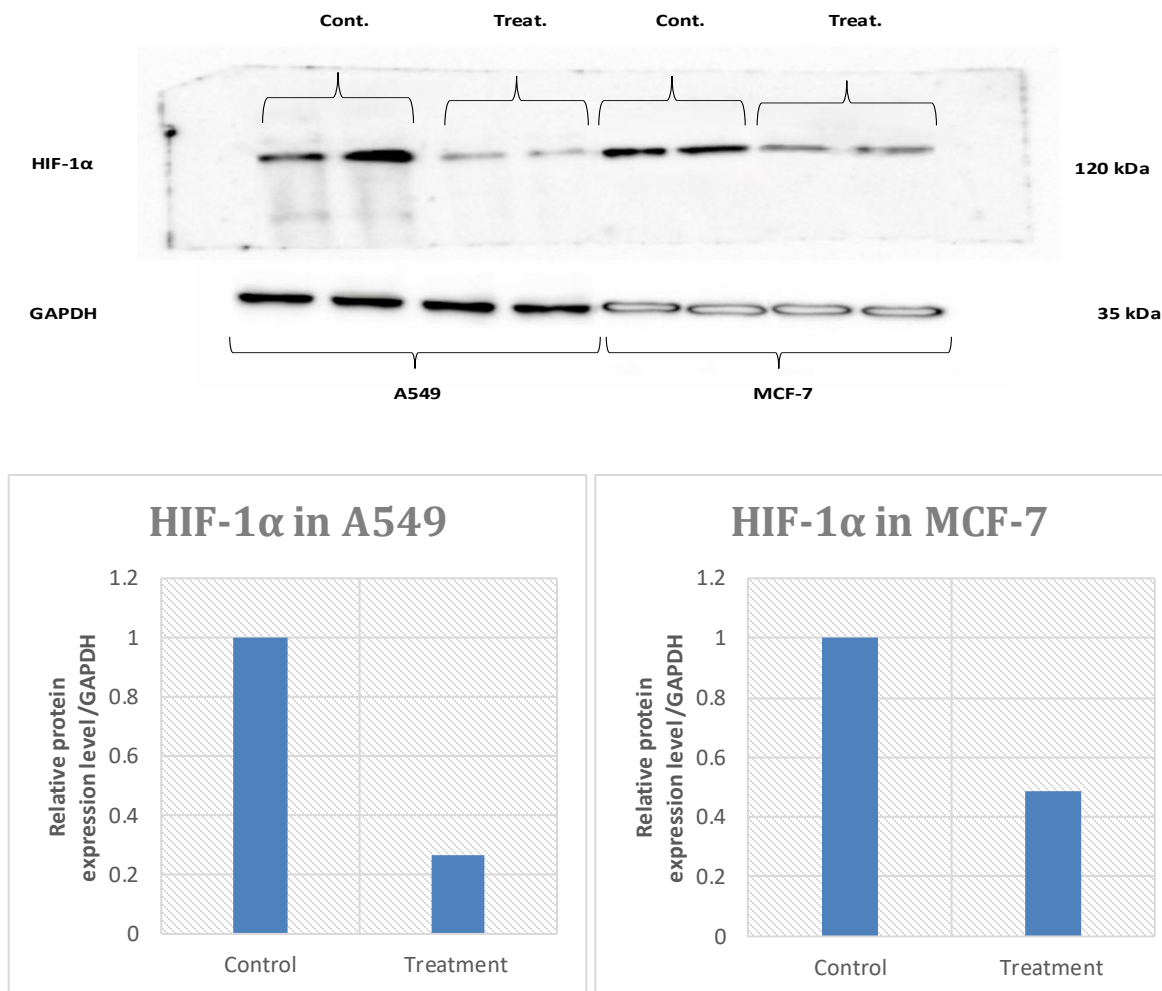
HIF-1 α is an oxygen-sensitive transcription factor that accumulates under hypoxic conditions and activates genes associated with angiogenesis, such as VEGF-A and other vascular regulators (34). The result of western blotting, as illustrated in **Figure 7**. demonstrated a decrease in HIF-1 α band intensity in treated A549 cells relative to the control group. Also, densitometric analysis, employing independent-samples t-test in Minitab, indicated a substantial decrease in HIF-1 α expression in A549 cells following treatment with the aptamer SIRT1 activator (p-value = 0.0117). Likewise, HIF-1 α expression was markedly diminished in MCF-7 after treatment (p-value = 0.008).





VEGFR2		
Cell line	A549	MCF-7
Control (mean ± SD)	1 ± 0.35	1 ± 0.04
Treatment (mean ± SD)	0.26 ± 0.19	0.98 ± 0.07

Figure (6) The impact of the SIRT1 activator aptamer on VEGFR2 protein expression in A549 and MCF-7. Cells were treated for 48 h with one-half of the calculated 48 h IC_{50} concentration of the SIRT1 aptamer, corresponding to 0.480 μ M for A549 cells and 0.467 μ M for MCF-7 cells. Cell lysates were examined by Western Blot analysis. GAPDH is identified as the internal control.



HIF-1 α		
Cell line	A549	MCF-7
Control (mean ± SD)	1 ± 0.26	1 ± 0.17
Treatment (mean ± SD)	0.27 ± 0.12	0.49 ± 0.06

Figure (7) The impact of the SIRT1 activator aptamer on HIF-1α protein expression in A549 and MCF-7. Cells were treated for 48 h with one-half of the calculated 48 h IC₅₀ concentration of the SIRT1 aptamer, corresponding to 0.480 μM for A549 cells and 0.467 μM for MCF-7 cells. Cell lysates were examined by Western Blot analysis. GAPDH is identified as the internal control.

Discussion

The present work demonstrated that the circular SIRT1 aptamer reduced the viability of A549 and MCF-7 cells, suggesting a preliminary viability-inhibitory effect in cancer cells under the evaluated in vitro

circumstances. Cell viability was also generally lower at higher aptamer concentration and longer exposure duration, indicating a dose- and time-dependent response under the experimental conditions. The results show that the aptamer decreased

the viability of the A549 and MCF-7 cancer cell lines under the experimental settings, however XTT data alone cannot differentiate whether the effects were cytotoxic, cytostatic or metabolic inhibition. Nevertheless, given HUVECs were the only non-malignant comparator employed in this investigation, the data should be considered an initial indication of preliminary relative selectivity, rather than conclusive evidence of safety or lack of toxicity (35, 36).

The current findings are in agreement with the original description of this circular SIRT1 aptamer, in which the molecule penetrated cancer cells, enhance SIRT1 activity and induced viability-inhibitory effect in several cancer models, while sparing a non-cancerous comparator cell line. Therefore, the present findings support further preclinical evaluation of this aptamer as a candidate experimental anticancer agent. (16).

The greater sensitivity of MCF-7 cells compared with A549 cells may reflect differences in the degree to which each tumor type depends on effects possibly associated with SIRT1-related pathways, for example: SIRT1-regulated survival, stress-response, and angiogenesis-related pathways. This is biologically plausible because SIRT1 is a NAD⁺-dependent class III histone deacetylase with a dual, context-dependent role in cancer (37). Through histone and non-histone deacetylation, SIRT1 may promote tumor progression by epigenetically silencing tumor-suppressor genes and by deacetylating p53, thereby weakening tumor-suppressor activity; however, in other settings it may exert antitumor effects by deacetylating and suppressing oncogene-driven transcriptional programs (13, 38). In addition, SIRT1 has been shown to deacetylate HIF-1 α and attenuate hypoxia-driven signaling in some cancer models (22). Therefore, the biological consequences of SIRT1 modulation are highly dependent on

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cell type and microenvironment, and the greater response of MCF-7 cells observed here may be related to this dual behavior of SIRT1 in cancer. Nevertheless, the overall phenotype observed here remains favorable from an anticancer perspective.

The calculated SI values of 5.42 for A549 and 8.49 for MCF-7 indicate that, under the present experimental conditions, cancer cells were more sensitive to the circular SIRT1 aptamer than HUVECs, suggesting preliminary evidence of relative selectivity. Nevertheless, SI remains a screening parameter rather than a definitive measure of safety; therefore, the present findings should be interpreted as preliminary indication of relative cytotoxic selectivity, which requires confirmation in additional non-malignant human cell models and more advanced preclinical systems (30).

An important observation is that HUVECs were less sensitive than cancer cells, but they were not completely spared. At the concentration 0.078 μ M, the viability of HUVECs was 94.25% at 72 h. This suggests a small but measurable decrease, even at a very low dose. Moreover, the endothelial response was still dependent on the concentration across the entire range tested. Although this finding alone does not indicate a direct antiangiogenic mechanism, it raises the possibility that the aptamer may also have antiangiogenic-related molecular changes in addition to its effect on cancer cell viability. Because HUVECs are widely used as an endothelial model, this low-dose endothelial response deserves attention. Nevertheless, measuring HUVEC viability by XTT alone is not sufficient to confirm antiangiogenic-related molecular changes, since reduced metabolic activity may simply reflect general toxicity (39).

The SIRT1 aptamer treatment significantly decreased VEGFR2 expression in A549 cells suggesting a possible inhibition of VEGF/VEGFR2 axis in lung cancer cells.



This result supports the idea that the aptamer might have an additional antiangiogenic-related action in A549 cells. However, the expression of VEGFR2 in MCF-7 cells was not significantly altered despite the large reduction in HIF-1 α . This variation may be because of cell line-specific regulation of VEGFR2, variances in baseline VEGFR2 expression, or participation of different angiogenic pathways in MCF-7 cells (33). Thus, the action of SIRT1 aptamer on VEGFR2 should be seen as a cell-type specificity and not a universal response of all cancer cells.

The absence of significant VEGFR2 suppression in MCF-7 cells should be interpreted with caution, because MCF-7 is an epithelial breast cancer cell line rather than an endothelial cell model. Therefore, VEGFR2 expression may be relatively low or less functionally dominant in these cells compared with vascular endothelial cells. Accordingly, the higher sensitivity of MCF-7 cells to the SIRT1 aptamer is unlikely to be explained by VEGFR2 downregulation. Instead, this response may reflect VEGFR2-independent mechanisms, including modulation of HIF-1 α and other SIRT1-related survival pathways. Other SIRT1-regulated pathways may instead be implicated in the cell viability/metabolic activity response. SIRT1 can affect cancer cell survival by regulating p53, FOXO proteins, oxidative stress responses, DNA repair, metabolism, and hypoxia-related signaling (11). Furthermore, SIRT1 has been reported to interact with HIF-1 α and to regulate the expression of hypoxia-responsive genes, which supports the biological relevance of the observed HIF-1 α reduction (22). Although there was no detectable downregulation of VEGFR2, the increased sensitivity of MCF-7 cells to the aptamer might be associated with HIF-1 α inhibition and disruption of SIRT1-dependent survival pathways. This suggests

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that the effect of the circular SIRT1 aptamer is not limited to VEGFR2 inhibition and may involve broader cell-line-specific molecular responses.

A prominent reduction of HIF-1 α in both A549 and MCF-7 cells may represent a probable molecular association between reduced cancer cell viability and possible angiogenesis-related signaling changes of the circular SIRT1 aptamer. HIF-1 α is not only an angiogenesis related transcription factor but also a survival regulator that facilitates tumor cells adaptation to hypoxia, metabolic stress, oxidative stress and nutrient limitation. Thus, HIF-1 α suppression may lead to the reduction of the expression of proangiogenic mediators such as VEGF and impair the capacity of cancer cells to sustain angiogenesis supporting signals. In addition, HIF-1 α inhibition may contribute to inhibit viability by reducing hypoxia-adaptive survival pathways, disturbing metabolic adaptation, and increasing cellular vulnerability to treatment-induced stress (20, 40). In MCF-7 cells, the absence of significant VEGFR2 reduction does not exclude an important role for HIF-1 α suppression, because HIF-1 α can regulate tumor-cell survival and angiogenesis-related signaling through VEGFR2-independent mechanisms. Thus, the reduction of HIF-1 α may help explain why MCF-7 cells remained highly sensitive to the aptamer despite unchanged VEGFR2 expression (41). However, these findings should be interpreted as preliminary molecular evidence, and further studies measuring VEGF secretion, HIF-1 α transcriptional targets, VEGFR2 phosphorylation, and endothelial functional assays are required to confirm whether HIF-1 α suppression translates into true antiangiogenic-related molecular changes.

The present study has several limitations that should be considered when interpreting the findings. First, HUVECs were the only non-



malignant comparator; therefore, the calculated selectivity index represents preliminary relative selectivity and does not establish tumor-specific safety in normal lung or breast epithelial cells. Future studies should include additional non-malignant epithelial cell models, particularly BEAS-2B normal bronchial epithelial cells for lung cancer comparison and MCF-10A non-tumorigenic breast epithelial cells for breast cancer comparison. Also, the approach applied for IC_{50} calculation was used as a preliminary method for IC_{50} estimation. Future analyses should employ nonlinear dose–response curve fitting to report IC_{50} values with confidence intervals. Second, the XTT assay alone cannot distinguish true cytotoxicity from cytostatic effects or changes in cellular metabolic activity. Accordingly, future studies should include additional viability and cell-death assays, such as apoptosis/necrosis markers, caspase activation, or mitochondrial membrane-potential assessment. Another important limitation is absence of a mechanism-matched or standard anticancer positive control. Future studies were recommended to include doxorubicin, cisplatin, paclitaxel or a suitable SIRT1-related comparator, depending on the experimental context.

Mechanistically, the present study did not directly measure SIRT1 expression or activity, aptamer cellular uptake, or downstream SIRT1-related targets. Therefore, the proposed involvement of SIRT1 remains inferential and is based mainly on previously published evidence showing that this circular aptamer can bind and activate SIRT1 and enter cancer cells (16). Accordingly, direct validation in the present experimental model is still required using SIRT1 activity assays, fluorescent aptamer uptake studies, and rescue or knockdown approaches. In addition, HIF-1 α and VEGFR2 protein expression were

assessed only in A549 and MCF-7 cancer cells, but not in HUVECs; therefore, the present data cannot define the baseline expression of these markers in normal endothelial cells or determine whether the aptamer alters endothelial HIF-1 α /VEGFR2 under normoxic or hypoxic conditions. Finally, because no functional angiogenesis assay was performed, the observed changes in HIF-1 α and VEGFR2 should be interpreted as preliminary angiogenesis-related molecular findings rather than definitive proof of antiangiogenic activity. Future studies should include VEGF secretion, VEGFR2 phosphorylation, endothelial tube formation, migration/invasion assays, *ex vivo* and *in vivo* validation.

Conclusion

Under the current *in vitro* conditions, the circular SIRT1 aptamer decreased the viability of A549 and MCF-7 cancer cell lines in a concentration- and time-dependent way. The greater IC_{50} in HUVEC compared to the cancer cell lines suggests a preliminary relative selectivity profile, but should not be taken as final safety or tumor specific selectivity data. The aptamer also lowered the expression of HIF-1 α in both cancer cell lines and the expression of VEGFR2 in A549 cells and had no significant effect on the expression of VEGFR2 in MCF-7 cells. These findings are suggestive of the fact that the aptamer might influence hypoxia and angiogenesis associated molecular markers in a cell-line dependent manner. Further investigations employing other non-malignant cell models, direct measurement of SIRT1 activity, tests of cell-death mechanisms and functional angiogenesis assays are needed to confirm the biology and therapeutic relevance of these findings.



Conflict of interests

No potential conflicts of interest were disclosed by the authors.

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Data sharing statement

Supplementary data may be provided to the associated author upon a justified request.

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