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Research Article

Efficacy of a SIRT1 Aptamer as an Anti-Angiogenic and Antioxidant Agent in *in vitro*, *ex vivo*, and *in vivo* Models

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Abstract

Background: Angiogenesis and oxidative stress are closely related processes that facilitate pathological vascular remodeling and cancer progression. Given that sirtuin 1 (SIRT1) modulates endothelial function, redox balance, and angiogenic remodeling in a context-dependent manner. ***Objective***: To investigate the anti-angiogenic and antioxidant activity of a circular SIRT1 aptamer in multidesign settings. ***Methods***: The endothelial response was evaluated in HUVECs via the XTT assay; antioxidant activity was assessed using the DPPH radical scavenging assay; *ex vivo* angiogenesis was measured in the rat aortic ring model; and *in vivo* validation was conducted with the chick chorioallantoic membrane (CAM) assay. ***Results***: The impact of the aptamer on HUVEC was concentration- and time-dependent, with IC50 values of 3.910, 3.103, and 1.337 µM at 24, 48, and 72 hours, respectively. Furthermore, the aptamer exhibited substantial radical-scavenging activity in the DPPH assay, representing an IC50 of 0.0499 µM. It also inhibited microvessel sprouting in the rat aortic ring assay, with an IC50 of 0.613 µM, and suppressed CAM vascularization in a dose-dependent manner; the highest concentration tested produced an effect comparable to that of aspirin. These findings indicate that the circular SIRT1 aptamer possesses anti-angiogenic properties across *in vitro, ex vivo,* and *in vivo* models, in addition to its antioxidant capacity within cell-free systems. ***Conclusions***: This aptamer has potential to act as a bioactive oligonucleotide scaffold for angiogenic applications; however, further research is necessary to determine its target specificity and translational significance.

Keywords: Angiogenesis; Antioxidant activity; Chick chorioallantoic membrane; HUVEC; Rat aortic ring assay; SIRT1 aptamer.

فعالية الأبتامر SIRT1 كعامل مضاد لتكوين الأوعية الدموية ومضاد للأكسدة في النماذج المختبرية خارج وداخل الجسم الحي

الخلاصة

الخلفية: تكّون الأوعية الدموية والإجهاد التأكسدي عمليتين مترابطتين تسهمان في إعادة تشكيل الأوعية الدموية المرضية وتطور السرطان. أن إنزيم السيرتوين 1 (SIRT1)ينظّم وظيفة الخلايا البطانية، التوازن التأكسدي، وإعادة تشكيل الأوعية الدموية. **الهدف**: تقييم التأثيرات المضادة لتكوّن الأوعية والمضادة للأكسدة لأبتامر حلقي موجّه نحو SIRT1 عبر نماذج تجريبية متكاملة. **الطرائق**: تم تقييم استجابة الخلايا البطانية باستخدام خلايا الوريد السري البشري (HUVECs) من خلال اختبار XTT، بينما تم فحص النشاط المضاد للأكسدة باستخدام اختبار إزالة الجذور الحرة DPPH. كما تم تقييم تكوّن الأوعية خارج الجسم باستخدام نموذج حلقات الأبهر الجرذي، وتم إجراء التحقق داخل الكائن الحي باستخدام نموذج غشاء الكوريون (CAM)في بيض الدجاج. **النتائج**: أظهر الأبتامر تأثيرا على حيوية خلايا HUVEC يعتمد على التركيز والزمن، حيث بلغت قيم IC_{50} مقدار 3.910 و3.103 و1.337 ميكرومولار بعد 24 و48 و72 ساعة على التوالي. وأظهر نشاطا ملحوظا في إزالة الجذور الحرة خارج الخلايا، محققا قيمة IC_{50} مقدارها 0.0499 ميكرومولار. كما أدى إلى تثبيط نمو الأوعية الدقيقة في اختبار حلقات الأبهر بقيمة IC_{50} مقدارها 0.613 ميكرومولار، بالإضافة إلى تقليل التوعية الدموية في نموذج CAM بطريقة تعتمد على الجرعة، حيث كان التأثير عند أعلى تركيز مماثلا لتأثير الأسبرين. **الأستنتاجات**: أن الأبتامر الحلقي الموجه نحو SIRT1 يُظهر نشاطا مضادا لتكون الأوعية بشكل متناسق عبر النماذج المختبرية وخارج الجسم وداخل الكائن الحي، إلى جانب امتلاكه قدرة مضادة للأكسدة في الأنظمة الخالية من الخلايا. يمكن أن يمثل هذا الأبتامر دواء واعدا كجزيء قليل النوكليوتيد ذو نشاط حيوي لاستهداف تكون الأوعية، إلا أن هناك حاجة إلى دراسات إضافية للتحقق من الية العمل.

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INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for proper growth and tissue repair [1]. Blood vessels play essential roles in maintaining homeostasis, nutrient exchange, metabolism, and tissue growth [2]. Because of their central biological functions, targeting blood vessel formation has become an important therapeutic strategy for many diseases, including cancer [3,4]. Among vascular-targeted therapies, inhibition of tumor angiogenesis is one of the most validated approaches in cancer treatment, supported by more than two decades of clinical use of anti-angiogenic drugs [5]. Judah Folkman proposed in 1971 that tumor growth depends on angiogenesis, noting that tumors remain small without neovascularization [6]. This idea led to the creation of new ways to treat cancer [7]. Among the molecular regulators of endothelial biology, silent information regulator 1 (SIRT1) has attracted considerable interest [8]. SIRT1 is an NAD+-dependent deacetylase that integrates metabolic and redox signaling and influences endothelial survival, senescence, nitric oxide

signaling, and angiogenic remodeling [9]. Experimental studies have shown that SIRT1 modulates endothelial sprouting and vascular growth, while broader vascular evidence links SIRT1 to the regulation of oxidative stress and endothelial dysfunction [10]. Taken together, these reports suggest that the biological effects of SIRT1 modulation are context-dependent [11], making SIRT1-related molecules mechanistically interesting but in need of direct evaluation in relevant angiogenesis models. Aptamers are a type of single-stranded nucleic acid ligand. They can fold into exact three-dimensional shapes and attach to molecular targets with a high level of selectivity and affinity [12]. Aptamers provide a lot of practical benefits over standard protein-based binders. These benefits include a small size, the ability to be synthesized chemically, the fact that their structure can be easily modified, and a low overall immunogenicity [13]. This study investigated a circular single-stranded DNA aptamer that has established efficacy as a ligand for SIRT1. Al-Sudani *et al*. previously evaluated the mechanistic rationale of SIRT1 aptamer activity by applying complementary experimental approaches. This aptamer was developed by SELEX as a SIRT1-recognizing ligand. Cellular assays, including fluorescence microscopy and viability testing, demonstrated that the SIRT1 aptamer could enter cancer cells, localize intracellularly, and exert biological activity. Stability testing demonstrated the circular structure improves resistance to nuclease-mediated degradation in human plasma, while enzymatic assays demonstrated the SIRT1 aptamer can enhance SIRT1 catalytic activity [14]. The investigated aptamer differs from classical SIRT1 activators or inhibitors in two important aspects. First, it is not a small molecule; it is a circular single-stranded DNA ligand that recognizes SIRT1 through sequence-dependent three-dimensional folding. Second, unlike many small-molecule SIRT1 modulators, especially polyphenolic activators, which may interact with multiple intracellular targets, aptamers are selected for high-affinity target recognition [15,16]. The circular aptamer has been reported to bind SIRT1 and enhance its enzymatic activity, suggesting a target-directed modulatory mechanism that may involve allosteric regulation rather than a broad nonspecific small-molecule effect. This aptamer thus provides a unique oligonucleotide-based approach for SIRT1 modulation rather than another conventional SIRT1 inhibitor or activator [14]. It is well known that oxidative stress plays a vital role in regulating angiogenesis and controlling vascular stability. Oxidative stress happens when the body produces an increased amount of reactive oxygen species (ROS) and the antioxidant defense system in cells is malfunctioning. This could cause enhanced oxidative activity and produce harm to tissues [17]. Oxidative stress is linked to the initiation and worsening of various vascular diseases and is often considered a primary marker of vascular impairment [18]. Oxidative signaling can control processes such as tissue repair and angiogenesis [19]. Reactive oxygen species (ROS) can alter endothelial cell function by activating various signaling pathways [18]. Although SIRT1 modulation has been widely studied in angiogenesis and oxidative stress, the novelty of the present study lies in evaluating a circular SIRT1-binding DNA aptamer as a bioactive oligonucleotide scaffold across complementary *in vitro*, *ex vivo*, and *in vivo* angiogenesis-related models. Unlike conventional small-molecule SIRT1 modulators, this aptamer is a structurally defined nucleic acid ligand with reported SIRT1-binding activity, intracellular target engagement, and improved stability due to its circular configuration. Therefore, this study does not simply re-examine SIRT1 biology but investigates whether this circular SIRT1 aptamer can produce consistent anti-angiogenic activity across increasing levels of biological complexity while also exhibiting antioxidant activity in a cell-free redox assay.

METHODS

Study design

This study used complementary *in vitro*, *ex vivo*, and *in vivo* methods to test the biological activity of a circular single-stranded DNA SIRT1 aptamer. The study workflow included assessing endothelial cytotoxicity in human umbilical vein endothelial cells (HUVECs), evaluating antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, conducting *ex vivo* angiogenesis testing in rat aortic rings, and performing *in vivo* validation in the chick chorioallantoic membrane (CAM) model. The examined aptamer was the circular 40-nucleotide SIRT1-binding sequence 5′-CGAGTGGGTTACATCGAAACTGGATCTCAAC AGCGGTAAC-3′ [14]. Stock solutions were made in nuclease-free water and then diluted to the right working concentrations right before each experiment. The study was performed in the College of Pharmacy, Mustansiriyah University.

HUVEC culture with the XTT viability test

HUVECs were grown in Medium 199, which contains 20% fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated at 37°C, 95% humidity, and 5% CO_2 [20]. For viability experiments, cells were seeded into 96-well plates at a density of 5 × 10^3 cells per well and allowed to adhere overnight. After that, the culture medium was replaced with new medium that had the SIRT1 aptamer in it at final concentrations of 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µM. Cells were exposed to aptamer for 24, 48, or 72 hours. The XTT/PMS colorimetric test was used to check cell viability, using established tetrazolium-based methods. A microplate reader was used to measure absorbance at 450 nm with a reference wavelength of 630 nm. The viability of the cells was compared to that of untreated controls after subtracting the blank. GraphPad Prism 7 was used to find the IC50 values using four-parameter nonlinear regression [21].

DPPH radical scavenging assay

The antioxidant efficacy of the SIRT1 aptamer was evaluated via the DPPH radical scavenging assay, with vitamin C (L-ascorbic acid) serving as the reference antioxidant. Serial concentrations of aptamers (0.0195–10 µM) were combined with 0.1 mM DPPH in methanol and incubated for 30 minutes at ambient temperature in a dark place. Absorbance was measured at 517 nm. Radical scavenging activity was determined using the formula % inhibition = [(A0 − Ac)/A0] × 100, where A0 represents the absorbance of the control reaction and Ac denotes the absorbance of the sample. IC50 values were obtained by GraphPad Prism 7 from concentration-response curves [22-24].

Ex vivo rat aortic ring angiogenesis assay

Anti-angiogenic activity was evaluated using the rat aortic ring assay with minor modifications of established protocols [25]. All procedures involving animals were reviewed and approved by the Ethics Committee of the College of Pharmacy, Mustansiriyah University. Thoracic aortas were collected from 12–14-week-old male albino rats under anesthesia, cleaned of surrounding fibro-adipose tissue, and cut into 1-mm rings. Individual rings were embedded in a fibrin-based matrix in 48-well plates and incubated with M199 media supplemented with 20% fetal bovine serum, L-glutamine, aminocaproic acid, amphotericin B, and gentamicin. For the dose-response experiment, the aptamer was tested at 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 µM. Cultures were maintained at 37°C in 5% CO_2, and the overlay medium was renewed on day 4. Microvessel outgrowth was evaluated on day 5 by inverted microscopy. For confirmatory experiments, activity at the calculated IC50 and 2 × IC50 was compared with a matched vehicle control and aspirin (100 µg/mL) as the reference anti-angiogenic control. Vessel inhibition was quantified with ImageJ as the percentage reduction in sprouting density relative to control [26,27].

Chick chorioallantoic membrane (CAM) assay

The CAM assay was used as an in vivo-like screening model to validate the anti-angiogenic activity of the SIRT1 aptamer. Fertilized chicken eggs were disinfected with 70% ethanol and incubated at 37 °C and 60% relative humidity. After 72 h of incubation, 2 mL of albumin was removed through a small lateral opening to detach the developing CAM from the shell, and the eggs were returned to the incubator. On embryonic day 10, a 3–4 cm window was opened over the air sac under aseptic conditions. Sterile filter paper discs were loaded with 20 µL of aptamer solution (0.625, 1.25, 2.5, 5, or 10 µM). Vehicle-treated discs and aspirin (360 µg/20 µL) were used as negative and positive controls, respectively. One disc was placed on an avascular region adjacent to small branching vessels, the shell window was resealed, and eggs were incubated for a further 72 h without turning. Images were acquired around the disc and quantified using ImageJ. Anti-angiogenic activity was expressed as the percentage inhibition of vessel growth relative to the vehicle-treated control [28,29].

Ethical considerations

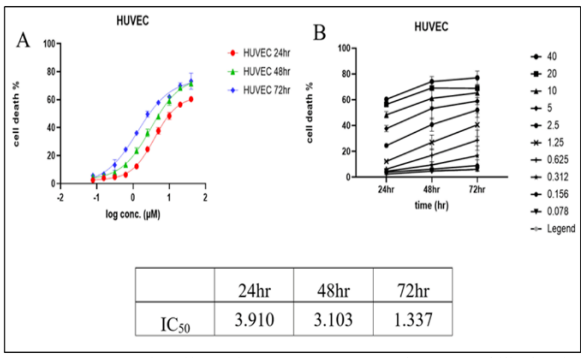
The study protocol was approved by the Research Ethics Committee of the College of Pharmacy, Mustansiriyah University (ID 63 in 2025).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc.). All values are presented as the mean ± SD of 3 independent experiments. Concentration-response curves and IC50 values were obtained by nonlinear regression (log[inhibitor] versus response, variable-slope/four-parameter model). Group comparisons were performed by one-way analysis of variance (ANOVA). Statistical significance was set at $p< 0.05$.

RESULTS

To evaluate the cytotoxic profile of the SIRT1 aptamer in a non-malignant endothelial model, HUVECs (cultured in M199 supplemented with 20% FBS) were exposed to increasing aptamer concentrations (0.078–40 µM) for 24, 48, and 72 hours. In Figure 1, colorimetric XTT assay was used to quantify cell viability. The XTT assay estimates the metabolic activity of cells through the reduction of XTT to a soluble formazan product.



	24hr	48hr	72hr
IC_{50}	3.910	3.103	1.337

Figure 1: Cytotoxic effect of SIRT1 aptamer on human umbilical vain endothelium. Cells were treated with increasing concentrations of SIRT1 aptamer (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078µM) for 24, 48 and 72 hours. The cell viability was determined using a colorimetric XTT assay. The absorbance was measured at 450 nm using a microplate reader. The data represent the mean ± SD of three experiments. GraphPad Prism software was utilized to analyze the data and generate graphics. **A)** Dose response curves of SIRT1 aptamer on HUVEC. **B)** Concentration-dependent cytotoxic effect of SIRT1 aptamer on HUVEC.

A dose–response curves by applying nonlinear regression analysis. The results established that the effect of the SIRT1 aptamer on HUVEC viability was both concentration- and time-dependent (Figure 1 and Table 1), with IC_{50} values lowering with time: 3.91 µM (24 h), 3.103 µM (48 h), and 1.337 µM (72 h). The slope of the curve remained roughly the same throughout time (HillSlope: 1.346, 1.057, and 0.955 for 24, 48, and 72 hours, respectively), and the regression fits were quite good ($R^2 = 0.997$, 0.996, and

0.996). The readings were reasonably accurate, with 95% confidence intervals of 3.590–4.286 µM (24 h), 2.755–3.538 µM (48 h), and 1.086–1.627 µM (72 h). In general, these data suggest that the SIRT1 aptamer causes time- and concentration-dependent cytotoxicity in HUVECs under this experimental condition. The DPPH free radical scavenging test was used to assess the ability of the SIRT1 aptamer to neutralize free radicals. L-ascorbic acid (vitamin C) was used as the standard reference.

Table 1: The average IC_{50} values, 95% confidence Intervals and R^2 for HUVEC cell line after 24, 48 and 72 hours of aptamer SIRT1 activator treatment

Incubation Time (hr)	IC_{50} (µM) [#]	Hill Slope	CI at 95 % (µM)	R^2
24	3.910	1.346	3.590 to 4.286	0.9967
48	3.103	1.057	2.755 to 3.538	0.9957
72	1.337	0.9553	1.086 to 1.627	0.9957

[#]The log (inhibitor) *vs*. response-variable slope (four parameters) non-linear regression analysis was used to calculate IC_{50}. The equation of the line is given as Y = Bottom + (Top-Bottom)/(1 + 10^((LogIC50 − X)*Hillslope)).

The experiment involved incubating samples with different concentrations of vitamin C and SIRT1 aptamer and then measuring the decrease in DPPH radicals' absorbance at 517 nm **(**Table 2 and Figure 2**)**.

Table 2: DPPH Radical scavenging activity (%) of vitamin C and SIRT1 aptamer at different concentrations

Vitamin C		SIRT1 aptamer	
Concentration (µg/ml)	DPPH scavenging activity (%)	Concentration (µM)	DPPH scavenging activity (%)
1000	97.1±3.43	10	85.74±3.22
500	99.72±1.78	5	83.93±5.69
250	91.46±2.79	2.5	89.12±7.81
125.00	91.82±2.29	1.25	85.98±6.92
62.50	88.96±2.96	0.625	84.29±4.58
31.25	84.17±1.04	0.3125	86.39±0.37
15.63	82.96±2.03	0.15625	79.5±3.91
7.81	84.61±3.45	0.078125	68.79±3.05
3.91	61.18±3.84	0.039063	56.91±3.81
1.95	46.68±1.51	0.019531	44.11±3.09

Values are presented as mean±SD.

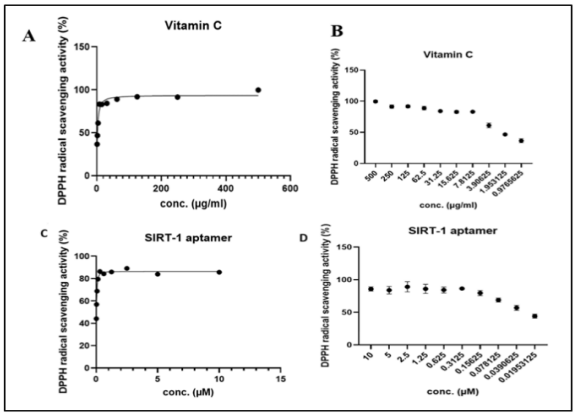


Figure 2: DPPH radical scavenging activity of Vitamin C (L-ascorbic acid) and SIRT1 aptamer. Serial concentrations of tested compounds were mixed with DPPH solution; the decrease in DPPH absorbance at 517 nm was used to calculate the radical scavenging activity (%). Data are presented as mean ± SD of three independent experiments. GraphPad Prism was used for curve fitting and statistical analysis**. A)** Dose response curve for vitamin C in DPPH radical scavenging activity. **B)** Concentration–dependent plot of the DPPH radical scavenging activity of Vitamin C. **C)** Dose response curve for SIRT1 aptamer in DPPH radical scavenging activity. **D)** Concentration–dependent plot of the DPPH radical scavenging activity of SIRT1 aptamer.

It was established that vitamin C's efficacy in eliminating DPPH radicals was concentration-dependent. The decline in DPPH absorbance correlated with increasing concentration, indicating effective neutralization of free radicals. At elevated doses, vitamin C exhibited a pronounced scavenging effect approaching maximal inhibition. Through linear regression analysis, the IC_{50} value of vitamin C in the DPPH assay was determined; it was approximately 2.578 µg/mL, and R^2 was 0.9658, indicating that the curve fitting was dependable. One-way ANOVA indicates that vitamin C produces concentration-dependent scavenging activity with $p<$ 0.0001. The DPPH assay results indicated that the SIRT1 aptamer exhibited free radical scavenging action. Through regression analysis in GraphPad Prism, the IC_{50} value of the SIRT1 aptamer was 0.0499 µM (R^2= 0.988), indicating it is a potent antioxidant. The SIRT1 aptamer exhibited a reduced IC_{50} value compared to the reference standard, vitamin C. This indicates superior potency in scavenging free radicals and enhanced antioxidant capabilities within the same experiment. The result of one-way ANOVA indicates that the SIRT1 aptamer produces concentration-dependent scavenging activity across the tested concentration range with $p<$ 0.0001. A series of concentration ranges from 0.01 to 10 µM of SIRT1 aptamer were examined to determine the dose-response curve in rat aortic rings. Figure 3 shows the effect of different concentrations of SIRT1 aptamer on blood vessel growth and the dose-response curve of SIRT1 aptamer in rat aortic rings.

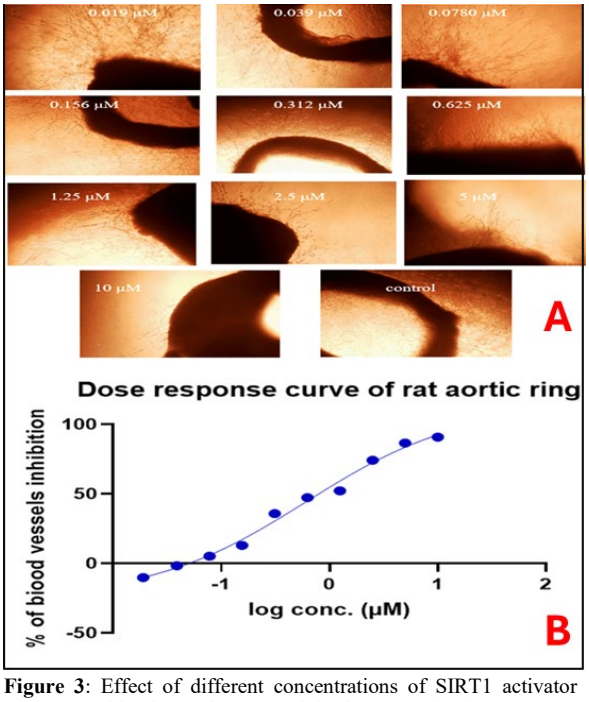


Figure 3: Effect of different concentrations of SIRT1 activator aptamer on blood vessel outgrowth in the rat aortic ring assay (*ex vivo*). Rat thoracic aortic rings were embedded in fibrin matrix and treated with serial concentrations of the SIRT1 activator aptamer. After 5 days microvessel sprouting was examined under an inverted microscope (10× magnification), images were taken by specialized microscope camera. **A)** Microscopic images of blood vessel inhibition at different concentrations of aptamer in rat aortic ring assay **B)** Dose response curve of SIRT1 aptamer in rat aortic ring.

Table 3 represents the percentage of inhibition, shown as mean ± SD as measured by ImageJ software. This result was analyzed by GraphPad Prism by applying nonlinear regression to calculate half-maximal inhibitory concentration (IC50). The fitted curve showed a strong goodness of fit ($R^2 = 0.95$), yielded an IC50 equal to 0.613 µM, and had a hillslope of 0.06.

Table 3: Serial concentrations of aptamer SIRT1 activator and their respective blood vessel inhibition percentage in rate aortic ring assay

Concentration (µM)	Blood vessel inhibition (%)
10	90.77±4.79
5	86.62±2.96
2.5	74.14±7.4
1.25	52.19±9.31
0.625	47.26±10.1
0.312	35.76±13.01
0.156	12.96±4.93
0.078	5.16±7.17
0.039	-1.73±9.74
0.019	-10.23±3.02

Values are presented as mean±SD.

Figure 4 shows a microscopic and graphical presentation of the effect of the SIRT1 aptamer at concentrations IC_{50} and $2IC_{50}$ compared to positive and negative controls. These results indicate a significant change in the percentage of blood vessel inhibition in both the IC50 and 2IC50 groups at a *p*-value < 0.0001 compared to the DMSO group.

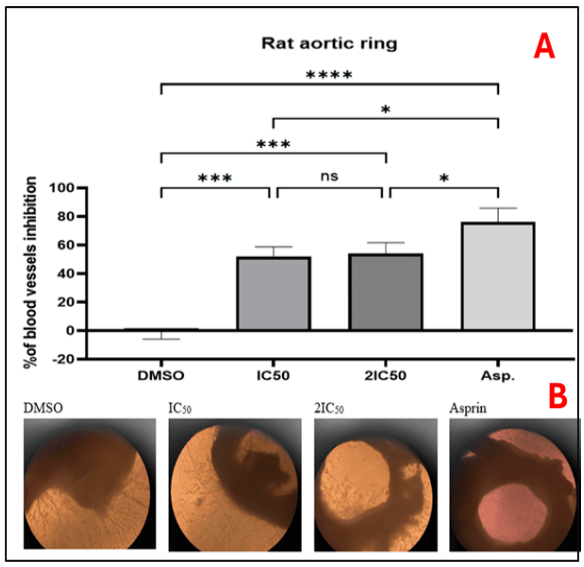


Figure 4: Microscopic images and graphical presentation of effect of SIRT1 aptamer on blood vessels growth inhibition depending on rat aorta ring assay (ex vivo assay) at concentrations of IC_{50}, $2IC_{50}$ of SIRT1 aptamer, aspirin 100 µg/ml and DMSO. The pictures from RAR assay were quantified by ImageJ software and analyzed by GraphPad prism; ns = not significant (p> 0.05), * = p< 0.05, *** = p< 0.001, **** = p< 0.0001.

Additionally, as expected, aspirin 100 mg shows a significant change in the inhibition percentage compared to the DMSO group. The inhibition percentages were significant for IC_{50} and $2IC_{50}$ compared to aspirin at $p< 0.0205$ and 0.0322, respectively. The other important note is that there was no significant change between IC_{50} and $2IC_{50}$ in percentage of blood vessel inhibition, and this consists with the result in the dose-response curve. Following the investigation of SIRT1 aptamer ability to inhibit angiogenesis in both *in vitro* and *ex vivo* settings, its anti-angiogenic activity was further evaluated in an *in vivo* setting. The chick chorioallantoic membrane (CAM) assay was employed to assess the ability of the SIRT1 aptamer to inhibit angiogenesis *in vivo*. A series of aptamer concentrations were employed to determine the concentration-dependent anti-angiogenic response. Figure 5 displays images of the CAM assay after treatment with varying concentrations of the SIRT1 aptamer. Aspirin (360 µg/20 µl) served as the positive control, while DMSO (0.1%) functioned as the negative control. Table 4 Shows quantification of these results as mean ± SD.

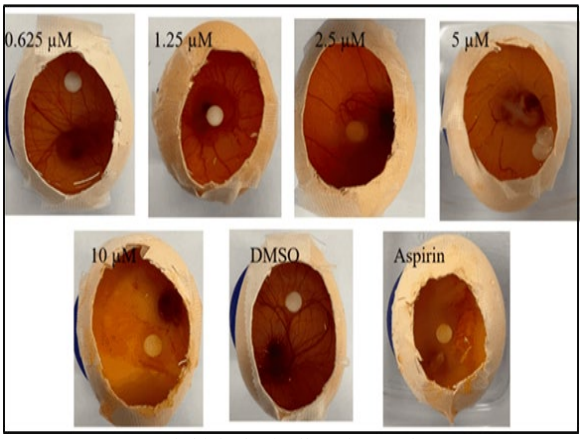


Figure 5: Images of chick chorioallantoic membrane (CAM) assay illustrating the effect of different concentrations of SIRT1 activator aptamer on blood vessel growth. Fertilized eggs were treated with aptamer SIRT1 activator (0.625, 1.25, 2.5, 5, and 10 µM/20 µL), aspirin (360 µg/20 µL) as a positive control, and DMSO (0.1%) as a negative control. Images were captured after 72 hr, images were taken by iPhone 13.

Table 4: Effect of SIRT1 aptamer on blood vessel inhibition in CAM assay

Treatment	Blood vessel inhibition (%)
DMSO 1%	0.0±4.54
Aptamer 0.625 µM/20 µL	6.94±3.49
Aptamer 1.25 µM/20 µL	31.31±14.01
Aptamer 2.5 µM/20 µL	54.32±12.64
Aptamer 5 µM/20 µL	81.80±9.72
Aptamer 10 µM/20 µL	86.59±2.03
Aspirin 360 µg/20 µL	87.29±9.22

Values are presented as mean±SD.

Statistical analysis using one-way ANOVA demonstrated a significant, concentration-dependent inhibition of blood vessel growth compared with the DMSO control (Figure 6). The lowest tested concentration (0.625 µM/20 µl) did not induce a significant reduction in angiogenesis (ns), whereas higher concentrations (2.5 and 10 µM/20 µl) produced a marked and statistically significant inhibition of blood vessel growth ($p< 0.0001$). Multiple comparisons confirmed that angiogenesis inhibition increased significantly with increased SIRT1 aptamer concentrations. Importantly, no significant difference was observed between the 10 µM SIRT1 aptamer and aspirin (360 µg/20 µl), indicating that the anti-angiogenic efficacy of the SIRT1 aptamer at higher concentrations is comparable to a standard reference anti-angiogenic agent.

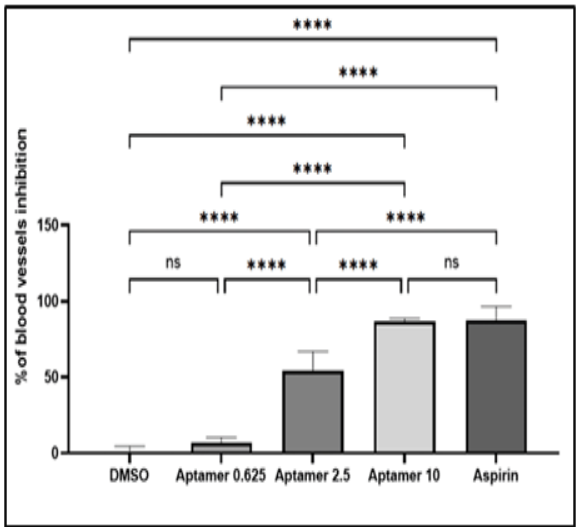


Figure 6: Graphical presentation of percentage of inhibition of blood vessel assessed by chick chorioallantoic membrane assay (*in-vivo* assay) at different concentration of aptamer SIRT1 activator (0.625, 2.5 and 10 µM/20µl**);** the picture of CAM assay was quantified by ImageJ software and analyzed by GraphPad prism; ns = not significant ($p > 0.05$), **** = $p < 0.0001$.

DISCUSSION

This study shows that the circular SIRT1 aptamer exhibits anti-angiogenic effects *in vitro*, *ex vivo*, and *in vivo* models in addition to antioxidant effects. In HUVECs, the aptamer diminished viability in a concentration- and time-dependent manner. The aptamer exhibited significant DPPH radical scavenging activity, diminished microvessel outgrowth in the rat aortic ring assay, and suppressed vascularization in the CAM model. The maximum evaluated dosage exhibited effects analogous to those of aspirin. These findings collectively suggest that the SIRT1 aptamer has potential as a bioactive anti-angiogenic oligonucleotide scaffold. A notable feature of the present dataset is the time-dependent increase in potency in HUVECs, where the IC50 declined, representing an almost threefold reduction over time. This progressive leftward shift is compatible with the concept that the SIRT1 aptamer has a cumulative biologic effect on HUVECs. The fall in endothelial IC_{50} overtime may be produced by sustained target engagement. The circular structure may enhance functional longevity by diminishing nuclease susceptibility and extending physiologically beneficial exposure. This interpretation is consistent with previous characterization of the circular SIRT1 aptamer as a SIRT1-binding ligand that modulates enzymatic activity, recognizes its target with high affinity and specificity, can localize intracellularly, and remains stable in human plasma. Even though such qualities were shown in cancer cells instead of endothelial cells, they offer a reasonable explanation for why the antiangiogenic effect in this study grew stronger with time [14]. The rat aortic ring test served as a significant *ex vivo* link between simpler endothelial cell research and a more structured vascular environment. The aortic ring keeps the structure of the artery wall and keeps endothelial cells, perivascular cells, and fibroblast-like cells together in a supportive matrix, unlike monolayer cultures. Therefore, the inhibitory pattern observed in this model suggests that the circular SIRT1 aptamer can suppress angiogenic remodeling within a multicellular vascular system rather than acting only on isolated endothelial cells. This increases the biological relevance of the anti-angiogenic signal and supports the interpretation that the response is not merely a cell-culture artifact [30]. The CAM assay extends this interpretation into a living, highly vascularized environment. It is widely used as an *in vivo*-like model for evaluating anti-angiogenic compounds before mammalian studies. The observed inhibitory effect on the CAM in this study suggests that the SIRT1 aptamer's ability to block blood vessel formation is maintained in a more complex biological environment compared to tests done outside of a living organism. This consistent finding across different experimental models supports the idea that the observed effect is biologically relevant rather than limited to a specific laboratory setting [31,32]. The main strength of the present work lies in the harmony of the anti-angiogenic signal across *in vitro*, *ex vivo*, and *in vivo* systems rather than in any single assay taken in isolation. HUVEC-based testing is valuable for detecting direct endothelial susceptibility, but isolated-cell systems capture only a limited part of the angiogenic cascade. By contrast, the aortic ring model preserves vessel-wall architecture and multicellular crosstalk, including endothelial cells, pericytes, smooth muscle, fibroblast-like stromal cells, and macrophage-associated components, thereby bridging simplified *in vitro* cell assays and more complex biological *in vivo* settings [30,33]. The CAM assay extends that evaluation to a rapidly vascularizing membrane in a living environment [34]. Agreement across these complementary platforms therefore supports the interpretation that the SIRT1 aptamer exerts a true anti-angiogenic effect rather than an assay-specific bias. At the same time, because none of these systems fully reproduce the tumor microenvironment, the present findings are best framed as evidence of anti-angiogenic potential rather than definitive proof of mechanism or therapeutic equivalence to established anti-angiogenic agents. When the three models are compared qualitatively, the rat aortic ring IC50 after 5 days is particularly important because sprouting was inhibited at a concentration lower than the 72-h HUVEC viability IC_{50}. This supports the idea that the aptamer may suppress angiogenic behavior at doses that are not explained solely by an overt endothelial toxicity. In the CAM assay, the result indicates that activity was retained in the low-micromolar range even in a more complex biologic setting. Because these assays measure different endpoints over different durations, their IC50 values are not directly interchangeable; however, together they define a coherent antiangiogenic potency profile from isolated endothelial cells to higher-order vascular models. The anti-angiogenic characteristics observed in this study can be explained by the complex biology of SIRT1, which is context-dependent [8]. SIRT1 has been shown to suppress Hypoxia-Inducible Factor 1-alpha (HIF-1α) transcriptional activity and attenuates

downstream hypoxia-responsive genes, whereas prolonged hypoxic stress has also been reported to permit SIRT1-dependent stabilization of HIF-1α and enhancement of Vascular Endothelial Growth Factor (VEGF)-linked signaling [35,36]. These apparently divergent observations indicate that SIRT1 is not uniformly pro-angiogenic or anti-angiogenic; rather, its vascular output depends on oxygen tension, redox state, metabolic context, and cell type. Within that framework, the current study suggests that aptamer-mediated SIRT1 modulation shifted the balance toward suppression of new vessel formation under the experimental conditions used here. The present interpretation is in agreement with studies showing that SIRT1-linked signaling can suppress hypoxia-responsive angiogenic pathways. Lim *et al*. reported that SIRT1 deacetylates HIF-1α and modulates cellular responses to hypoxia [34], and Zhang *et al*. found that SIRT1-dependent signaling can inhibit the HIF-1α/VEGF/VEGFR2 axis [36]. These reports support the view that the aptamer could restrain neovascularization. In contrast, other studies reached the opposite conclusion. Potente *et al*. showed that SIRT1 is required for endothelial angiogenic activity during vascular growth [37]. A reasonable explanation for these inconsistencies is that direct aptamer-mediated modulation of SIRT1 is not necessarily equivalent to pharmacologic activation by pleiotropic small molecules and that cancer-associated angiogenesis, physiological angiogenesis, and hypoxia-adapted tumor behavior are not biologically interchangeable. Acknowledging this agreement-disagreement pattern strengthens the discussion by showing that the current findings were interpreted within the real complexity of the field rather than against a selectively supportive background. An important interpretive point is that vascular sprouting in the higher-order models appears to be controlled in a range compatible with interference in angiogenic programming, not only with indiscriminate endothelial damage [38]. This distinction matters because many compounds suppress angiogenesis *in vitro* simply by reducing cell survival. The present study does not fully separate cytotoxic, cytostatic, and signaling-mediated effects, but the overall cross-model pattern favors a biologically directed anti-vascular response. Further evidence will need investigations of endothelial tube development [39] or spheroid sprouting at subcytotoxic concentrations, together with studies of apoptosis, proliferation, and the analysis of cell cycles [40,41]. Additionally, analyzing associated angiogenic pathway markers at the protein or mRNA level may clarify the underlying mechanism of action. Such experiments would clarify whether the aptamer primarily interrupts angiogenic signaling or whether endothelial growth suppression remains the dominant mechanism. The antioxidant component of the study improves our knowledge of aptamer mechanisms, but it should be interpreted with care. The antioxidant effect observed in this study may represent more than a mere secondary attribute of the analyzed aptamer; it may potentially augment the antiangiogenic profile by inhibiting redox-sensitive pathways that facilitate pathological neovascularization [42]. Oxidative stress is closely linked to angiogenesis, since an excess of reactive oxygen species can increase HIF-1α stability, enhance VEGF signaling, and facilitate endothelial migration and vascular permeability [43]. SIRT1 simultaneously modulates endothelial antioxidant defense through Forkhead box O3 (FoxO3)/Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α)-mediated regulation of genes such as Manganese Superoxide Dismutase (MnSOD), catalase, peroxiredoxins, and thioredoxin-related enzymes [44,45]. Additionally, SIRT1 is broadly connected to redox regulation through inflammatory, nitric oxide, and mitochondrial pathways [8,46]. In this light, the antioxidant signal in the present study is best seen as a secondary mechanism that may help create a less favorable environment for abnormal vessel formation. From that perspective, an oligonucleotide scaffold that combines antiangiogenic activity with radical scavenging capacity could be attractive in diseases where reactive oxygen species amplify vascular dysfunction. However, the DPPH assay is a cell-free chemical test. It demonstrates that the aptamer can reduce a stable radical in solution, but it does not by itself prove intracellular antioxidant activity, endothelial redox reprogramming, or SIRT1-dependent antioxidant signaling [47,48]. In other words, the antiangiogenic and DPPH findings may be complementary, but they should not be mechanistically conflated. The DPPH signal most directly supports a direct chemical antioxidant property of the aptamer scaffold, whereas the biologic vascular assays support antiangiogenic activity. To make a stronger mechanistic link between these two parts of the study, quantifying ROS in cells, investigating the redox status of glutathione, profiling antioxidant enzymes, and employing redox-sensitive readouts will be required [46-48]. The aptamer was previously identified as a SIRT1-binding ligand, but the present study did not directly assess SIRT1 activation, SIRT1 expression, or downstream SIRT1 substrate deacetylation in HUVECs, aortic rings, or CAM tissues. Therefore, the anti-angiogenic effects observed here should not be interpreted as unequivocal evidence for direct SIRT1 activation signaling. Rather, the results demonstrate that the circular SIRT1 aptamer evokes consistent anti-angiogenic activity in a variety of biological models. These effects may include direct SIRT1-mediated pathways, secondary redox-related mechanisms, inhibition of endothelial viability, and/or combined modulation of angiogenic signaling. The main novelty of this work is the cross-model demonstration that a circular SIRT1 aptamer suppresses angiogenesis-related endpoints in HUVECs, rat aortic rings, and CAM tissue, together with antioxidant activity in the DPPH assay. This distinguishes the current work from studies that only describe SIRT1 as a regulator of endothelial biology or oxidative stress. The present findings position the circular aptamer as a potential oligonucleotide-based scaffold for angiogenesis-focused applications, although further mechanistic validation is still required.

Study Limitations

It is important to openly admit a number of limitations. First, because endothelial viability was affected, part of the antiangiogenic effect may reflect suppression of endothelial growth or survival rather than selective inhibition of sprouting pathways alone. Further investigations should be required to distinguish endothelium cytotoxicity from real manipulation in the angiogenic signaling program [32,33]. Second, SIRT1 target engagement was inferred from prior characterization of the aptamer rather than directly measured in the endothelial, aortic ring, or CAM systems used here [14]. Confirming SIRT1 activation in these models would substantially strengthen the mechanistic claim. Third, the aortic ring model preserves multicellular vascular interactions but uses non-neoplastic tissue, whereas the CAM assay is an efficient preclinical screen but does not reproduce full mammalian pharmacokinetic, immune, and tumor-microenvironment complexity [32,33]. These limitations do not diminish the value of the current work; instead, they define a logical path toward mammalian tumor models in which antiangiogenic efficacy, vascular function, redox modulation, and normal tissue tolerance can be assessed together.

Conclusion

This study showed the circular SIRT1 aptamer as a promising anti-angiogenic oligonucleotide with associated antioxidant capabilities. In complementing *in vitro, ex vivo,* and *in vivo* models, the aptamer consistently inhibited vascular development, indicating a biologically probable antiangiogenic activity. Collectively, these findings expand the potential significance of SIRT1-targeting aptamers and underscore circular SIRT1 aptamers as promising options for angiogenesis-focused intervention. Although the existing evidence is more accurately regarded as an initial proof of concept rather than conclusive mechanism-based validation. Further studies could confirm the circular SIRT1 aptamer as a stable and informative tool for future research on anti-angiogenic therapies.

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Conflict of interests

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Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request.

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