

83001_Auto_Edited.docx

BZD9L1 benzimidazole analogue hampers colorectal tumor progression by impeding angiogenesis

Oon CE *et al.* BZD9L1 benzimidazole analogue hampers CRC angiogenesis

INTRODUCTION

Cancer may arise from genetic or epigenetic dysregulations. Many conventional therapies focus on removing and disrupting malignant cells but fail to target the tumor microenvironment, which could fuel tumor growth through pathway crosstalks and the secretion of cytokines. Epigenetic dysregulation has been shown to stimulate oncogenic transformation in many cancer types, including colorectal cancer (CRC). Furthermore, research has highlighted the importance of epigenetic regulation in angiogenesis, which is key to CRC growth and metastasis. Advancements in therapeutic care for CRC patients have identified various angiogenesis inhibitors targeting the tyrosine kinases and vascular endothelial growth factor (VEGF) proteins used in clinics. However, some patients are resilient or have become less susceptible to these anti-angiogenic drugs^[1], possibly due to dynamic host response factors or mutations within tumors that render drug insensitivity. Therefore, there is a need to uncover alternative targeted therapies. Sirtuins (SIRT 1-7) are nicotinamide adenine dinucleotide (NAD⁺) -dependent class III histone deacetylases. Their potential roles through epigenetic modulation in cancer have stimulated investigation to seek potent and selective SIRT inhibitors, potentially leading to new therapeutic breakthroughs.

Benzimidazole derivatives have been widely used in other areas, such as antiviral^[2] and anti-mycobacterial^[3] agents; hence their pharmacokinetics are well understood. The discovery of indole analogues as potent sirtuin inhibitors^[4] and the high similarities between the indole and benzimidazole core structures have led to the discovery of BZD9L1^[5] and its reported anticancer activities as a single agent^[6] or in combination with 5-fluorouracil in CRC^[7]. However, to our knowledge, no known sirtuin inhibitors have been studied concerning their application to modulate angiogenesis. Hence, this

study opens a new avenue for developing a prospective anti-angiogenic agent (BZD9L1) through sirtuin inhibition in CRC.

MATERIALS AND METHODS

Cells and cell culture

The Ea. hy926 endothelial cells (EC) and the HCT-116 CRC cell line ¹ were purchased from American Type Culture Collection (ATCC®). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI), respectively ⁴ (Thermo Scientific, United States), supplemented with 5% fetal bovine serum (Biowest, United States), 1% penicillin-streptomycin (Nacalai Tesque, United States). Cells were kept at 37°C in a humidified incubator with 5% CO₂ atmosphere.

Treatments

BZD9L1 was synthesized as previously described^[5]. BZD9L1 was prepared and dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, United States) for *in vitro* and *ex vivo* evaluations. Different concentrations of BZD9L1 and anti-angiogenic agents Sunitinib (AdooQ Bioscience, CA, United States)^[8] or activin A receptor like type 1 (ALK1) inhibitor (Axon MedChem)^[9] positive controls were used to treat the cells ²¹ according to assay requirements. For animal administration, BZD9L1 was prepared in 0.5% sodium carboxymethyl cellulose (CMC) solution as previously reported^[10] and administered to the rodents *via* intraperitoneal injection, in which 0.5% CMC was also employed as the vehicle control *in vivo*.

Animals for in vivo study

² The animal study was approved and conducted in strict accordance with Universiti Sains Malaysia Animal Ethical Committee [No. USM/IACUC/2017/(105)(872)]. Male and female athymic nude mice were procured from EMAN Biodiscoveries Sdn. Bhd. ² The mice were maintained in filter-top cages under controlled atmospheric conditions

at Natureceuticals Sdn Bhd, Kawasan Perindustrian Sungai Petani, Sungai Petani, Kedah. The mice were 4-6 wk of age with a body weight of 18-20 g. The mice were provided with autoclaved food and water. The bedding was refreshed every 48 h.

Animals for ex vivo study

Male and female C57BL/6J mice were sourced from Invivos (Singapore) and retained on a 12 h light-dark cycle. The mice were fed a standard rodent chow (NCD, 18% kcal from fat, Harlan). The *ex vivo* assays, which require the choroids from the mice, were performed in compliance with the National University of Singapore Institutional Animal Care and Use Committee guidelines (IACUC) (No. 2020/SHS/1597).

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) cell viability assay was performed to determine half inhibitory concentration (IC₅₀) and suitable dosage of BZD9L1. MTT reagent diluted with phosphate-buffered saline (PBS) at 5 mg/mL was used for the MTT assay. Cells (2.5×10^3) were seeded in 96-well plates with 100 μ L of compatible media per well and incubated in 5% CO₂ at 37 °C for 24 h. Different doses of BZD9L1 (0 μ M, 1.560 μ M, 3.125 μ M, 6.250 μ M, 12.500 μ M, 25.000 μ M, 50.000 μ M, and 100.000 μ M) were tested on Ea.hy926 cells to select the best cytotoxic concentrations and then incubated in 5% CO₂ at 37 °C. After 72 h, 20 μ L of MTT reagent per well was added and then incubated in 5% CO₂ at 37 °C for 4 h. Next, the supernatant was carefully aspirated and 200 μ L of DMSO added. After gently shaking the plate, the mixture's optical density (OD) was measured using a microplate reader (TECAN, Switzerland) at 570 nm primary and 620 nm reference wavelength. The percentage of viable cells at each treatment concentration was calculated using the following equation: Cell viability (%) = (absorbance of samples/absorbance of vehicle control) \times 100%.

xCELLigence cell adhesion assay

The effect of BZD9L1 towards cell adhesion was determined using the xCELLigence Real-Time Cell Analysis (RTCA) instrument (Agilent Technologies, United States) according to the standard manufacturer's protocol. Briefly, the cells were resuspended in treatment media conditions: vehicle control, 2.5 μ M, 5.0 μ M, and 10.0 μ M of BZD9L1. Then, the cells and treatment media were seeded into the E96 xCELLigence plate at 1×10^4 cells per well. The E-96 xCELLigence plate was incubated for 30 min at room temperature and placed on the xCELLigence station in the cell culture incubator. The cellular impedance was continuously monitored every 30 min for 5 h. Impedance recordings from each well in the E96 xCELLigence plate were automatically converted to cell index (CI) values by the xCELLigence RTCA software.

xCELLigence cell proliferation assay

Real-time analysis of EA.hy926 cell proliferation was evaluated using the xCELLigence RTCA instrument (Agilent Technologies, United States). Firstly, 100 μ L of growth media was added to each well of an E96 xCELLigence plate. The plate was then inserted into the xCELLigence station. Next, baseline impedance measurement was performed to obtain background readings. EA.hy926 cells were harvested and adjusted to 1×10^4 cells per well. The cells were resuspended in 100 μ L of media and seeded into the E96 xCELLigence plate. E-96 xCELLigence plate containing cells was incubated for 30 min at room temperature and placed on the xCELLigence station in the cell culture incubator. After 24 h, treatment comprising of vehicle control, BZD9L1 at 2.5 μ M, 5.0 μ M, and 10.0 μ M were introduced to the cells in the plate, then returned to the xCELLigence station in an incubator for continuous impedance recording. Cell proliferation was monitored every 30 min for 72 h. Measured impedance recordings from cells in each well on the E96 xCELLigence plate were automatically converted to Cell Index (CI) values by the xCELLigence RTCA software.

Cell cycle analysis

The cell cycle analysis was performed to differentiate different cell cycle phases that were arrested after the treatments above. On day 0, Ea.hy926 cells were seeded in a T25 culture flask at a concentration of 5×10^5 per flask. Each treatment group was assigned a flask. The cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ to promote cell attachment. The next day, the medium was meticulously removed and replaced with fresh medium containing treatments and then incubated for 72 h at 37 °C in a humidified atmosphere of 5% (v/v) CO₂. After 72 h, the cells were collected, centrifuged, and fixed with 70% ethanol (molecular grade) at 4 °C. Finally, the fixed cells were stained with 500 µL of warm propidium iodide (PI) solution plus 50 µL of RNase A stock solution (1 mg/mL). The cells were incubated in the dark for 30 min. The stained cells were kept on ice until the scheduled flow cytometry analysis using BD FACSCalibur (BD Biosciences, United States).

Apoptosis detection

Apoptotic cells were detected by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Elabscience, China). On day 0, Ea. hy926 cells were seeded in a T25 culture flask at a concentration of 5×10^5 per flask. Each treatment group was assigned a flask. The cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ to promote cell attachment. The next day, the medium was meticulously removed and replaced with fresh medium containing treatments before incubation for 72 h at 37 °C in a humidified atmosphere of 5% (v/v) CO₂. After 72 h, the cells were harvested and centrifuged, and the cell concentration was adjusted to 1×10^6 for each tube. The Annexin V-FITC Apoptosis Detection Kit was used to stain the cells, and cell apoptosis was determined by flow cytometry, according to the manufacturer's instruction. The tubes were incubated at room temperature for 15 min in the dark, after which the cells were subjected to flow cytometry analysis immediately using BD FACSCalibur (BD Biosciences, United States) using 488 nm excitation and 525 nm emission for FITC and 655-730 nm emission for PI.

Hanging drop spheroid formation and sprouting assay

The 3-dimensional spheroid sprouting assay mimics the *in vivo* microenvironment of tissue or tumors. Hanging drop spheroid formation and sprouting assay was conducted to provide a three-dimensional aspect to both architecture and share the limited drug penetration properties since drugs are primarily confined to the outer cell layers. Culturing cells in three dimensions is much more representative of the *in vivo* environment than traditional two-dimensional cultures. In this present study, Ea.hy 926 was used to generate micro-spheroids. After trypsinization of cultured cells, the cells were harvested and resuspended in DMEM medium containing 0.25% methylcellulose. Drops (20 μ L) of medium containing 2×10^3 cells were seeded onto the lids of 100 mm petri dishes, and the dishes were supplied with 5 mL of PBS to maintain humidity. The drops were incubated for 72 h to encourage the occurrence formation of spheroids. Treatments were prepared at different concentrations of BZD9L1 (2.5 μ M and 5.0 μ M) and sunitinib (18 μ M) as a positive control in media and then added to each well. The resulting aggregate cells (spheroids) were harvested carefully using 200 μ L pipettes and then introduced into a 96-well plate precoated with 50 μ L matrigel per well. The plate was left to incubate in the incubator at 37 °C, and pictures were taken using a phase contrast microscope (Zeiss, Germany) on days 0 and 3. The length of the sprouts was measured and analyzed as previously described^[11] using Image J (Fiji).

Angiogenesis array

The Quantibody Human Angiogenesis Array (RayBiotech, Inc, Norcross, GA) was used to determine the concentration of ten proangiogenic cytokines [angiogenin, angiopoietin-2, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), heparin-binding EGF, hepatocyte growth factor, leptin, platelet-derived growth factor (PDGF-BB), placental growth factor (PIGF), and VEGF-A] secreted by the EC. Each cytokine was arrayed in quadruplicate, together with positive and negative controls. In addition, one standard glass slide was divided into 16 wells of identical cytokine antibody array. Approximately 2.5×10^3 of Ea. hy926 cells were seeded in 96-well plates

with 100 μ L of compatible media per well and incubated in 5% CO₂ at 37 °C for 24 h. The next day, vehicle control, 2.5 μ M, and 5.0 μ M of BZD9L1 treatment were prepared using 1% serum media and introduced to the cells. After 48 h, the media was discarded, and the cells were washed with 1 \times PBS twice before reintroducing serum-free media. After another 48 h, the conditioned media was collected. The conditioned media was used on the angiogenesis antibody array kit (Quantibody Human Angiogenesis Array 1) as per manufacturer's instructions. The slides were mailed to RayBiotech testing services (Singapore) for laser scanning analysis. Data were extracted and analyzed using the RayBio Q Analyzer software (RayBiotech, Inc).

RNA extraction

Total cellular RNA was extracted from EA.hy926 cells treated with vehicle control (DMSO) or different concentrations of BZD9L1 at 4 h time point, or HCT116 xenograft tumors post-treatment, using GENEZOL (Geneaid, New Taipei City, Taiwan) according to the manufacturer's instructions. Firstly, the spent medium was discharged from each well and the cells were rinsed with 1 \times PBS twice. Next, 1 mL of GENEZOL reagent solution was added to each well. The cells were lysed via repeated pipetting and followed by 10 min of incubation at room temperature for sample homogenization. The lysed cells were then transferred into 2 mL microcentrifuge tubes. An accurate amount of 200 μ L chloroform was added to each tube. Each tube was mixed thoroughly by vortexing for 15 s. The lysates were then centrifuged at 16000 g for 15 min at 4 °C. The colourless upper aqueous phase was carefully transferred into a new 1.5 mL microcentrifuge tube without drawing any of the interphase or organic phase layer that appears white and red in the three-layers mixture respectively. Next, 1 to 1 volume of ice-cold isopropanol was added to the aqueous phase. The 1.5 mL tubes were inverted several times for proper mixing, followed by 10 min of incubation at room temperature. The tubes were centrifuged at 16000 \times g for 10 min at 4 °C to form a tight RNA pellet. The supernatant was removed completely, and the pellet was resuspended with 1 mL 75% ethanol. The mixture was vortexed briefly and centrifuged again at 16000 g at 4 °C

for 5 min to remove all traces of ethanol. The supernatant was carefully removed by using a pipette tip, and the pellet was allowed to air dry for 15 min. After 15 min of air drying, the pellet was resuspended with 20 μ L DEPC-treated water and incubated at 60 °C for 15 min to dissolve the RNA pellet. RNA was then converted to cDNA or immediately stored at -80 °C freezer until further use. The purity and concentration of the isolated RNA were determined by measuring the optical density at 260 nm and 260/280 nm ratio using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Pennsylvania, United States). A preset of 40 ng/ μ L per OD was used with a baseline correction of 340 nm.

Quantitative polymerase chain reaction

The RNA was reverse transcribed into cDNA using Tetro cDNA Synthesis Kit (Bioline, United States) according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (qPCR) was performed using the KAPA SYBR FAST qPCR Kit Master Mix (2 \times) Universal (Biosystems, United States) with the following conditions: denaturation, 95 °C for 20 s; annealing, 58 °C, 20 s and extension, 68 °C, 30 s. All samples were tested in triplicate PCR reactions, and the mean of the reactions was used for calculating the expression levels. All the data were collected from the linear range of each amplification. Two housekeeping genes (HKGs) were used to normalize the expression of genes of interest (GOIs). Expression levels of GOIs were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and human 18S ribosomal RNA (18S rRNA) gene for human targets, and mouse 18S ribosomal RNA (m18S rRNA) and mouse Peptidylprolyl isomerase A (mPpia) genes for mouse targets. The data were analyzed using the comparative CT ($\Delta\Delta$ CT) method, where the expression value of the GOI was normalized to the respective HKG. The change of gene expression was determined by setting the expression value of vehicle control sample to 1, and expression values of GOI were compared where values < 1 represented downregulation and values > 1 represented upregulation of that particular GOI. The final results were

reported as mean \pm SEM to reflect the triplicate measurements. The list of primers used for qPCR analysis is listed in Table 1

Ex vivo mouse choroid microvascular assay

Organotypic cultures are *in vitro* growth of 3D biological tissues that closely emulate part of their natural function and physiology. Choroid sprouting assay was performed as described^[12]. C57BL/6J mice were killed by cervical dislocation. The choroid explants from post-natal day 3 C57BL/6J mice were isolated through a very tedious process under a stereo microscope. Briefly, the cornea and lens were removed from the anterior of the eye, followed by the separation of the peripheral choroid-sclera complex from the retina. The choroid was then cut into 1mm \times 1mm segments. The choroid explants were then incubated in reduced growth factor Matrigel (BD Biosciences). Vascular outgrowth, including cells from the microenvironment from the choroidal tissues treated with DMSO vehicle control, BZD9L1 at 10 μ M and 20 μ M or ALK1 inhibitor (Axon MedChem) at 100 nM. After 96 h, the effect of BZD9L1 on the morphological changes of vessel sprouts was noted. The regression study performed as previously reported^[13], in which treatment media containing either DMSO (negative control), BZD9L1 at concentrations of 10 μ M and 20 μ M or ALK1 inhibitor at 50 nM were added to the explants on day 2 when the embedded choroids had sprouted. Media were changed every other day. Images were taken 24 h post-treatment. Imaging and quantifying the sprouting area were performed under the Eclipse Ti-E Inverted Research Microscope (Nikon, Tokyo, Japan). and TRI2 software (version: 3.0.1.2, TRI2, Oxford, United Kingdom).

Indirect co-culture assay

The co-culture assay was conducted to study the interaction between ECs and cancer cells. The application of a co-culture model promotes a better understanding of the dynamic tumor-EC relationship that occurs *in vivo* in an inexpensive and *in vitro* laboratory setting. In this study, the co-culture was conducted indirectly. Ea. hy926 and

HCT116 cell lines were used in this experiment. Steps to collect conditioned media were repeated as per described for the angiogenesis array. The conditioned media can be used fresh or stored in a -80 °C freezer for later use. Treatments were prepared at different concentrations of BZD9L1 (2.5 µM and 5.0 µM) and sunitinib (18 µM) as a positive control. HCT 116 spheroids were prepared as described in the hanging drop spheroid formation assay and introduced into the rat tail collagen mixed to a concentration of 1.6 mg/mL before mixing it with spheroid suspension. The mixture was dispensed at 400 µL in each well in a 24-well plate. The plate was incubated for 30 min before 500 µL of the conditioned media was introduced (Liu *et al* 2020). The plate was left in the incubator at 37 °C, and pictures were taken using a phase contrast microscope (Zeiss, Germany) on days 0 and 3.

Tumor xenograft model

Xenograft models are based on the implantation of human tumor cells into genetically modified mice models that are immunocompromised to avoid graft *vs* host reaction of the mouse against the human tumor tissue. Male/female athymic nude mice (procured from EMAN Biodiscoveries Sdn. Bhd.) were maintained in filter-top cages under controlled atmospheric conditions. The mice were 4-6 wk of age with a body weight of 18-20 g. Mice were provided autoclaved food and water, and the bedding was changed every 48 h. HCT-116 cells at 90% confluency were harvested and injected subcutaneously onto the right dorsal flank of the animal at a density of 1×10^6 cells per 200 µL of media mixed with matrigel at a ratio of 1:1. The mice were then randomly divided into four groups ($n = 6$) and given different treatments, consisting of vehicle control, BZD9L1 at 50 mg/kg or 250 mg/kg and sunitinib at 40 mg/kg as the positive control^[14], when at least three tumors reached 100 mm³. The treatments were diluted in 0.5% carboxymethylcellulose (CMC) solution. The control group was treated with 0.5% CMC only. The treatments were injected intraperitoneally at a maximum volume of 250 µL once every three days, alongside the measurement of weight and tumor size using

the standard formula (Zhou *et al* 2017): Volume of tumor = $\pi/6 \times (\text{length} \times \text{width} \times \text{height})$.

The animals were euthanized when the tumor reached the maximum size of 1000 mm³. The tumors were harvested and fixed in a 10% buffered formaldehyde solution and then processed by an automated tissue processing machine for histological examination. The HCT116 xenograft tumor samples were cut into small pieces using a sterile surgical blade for gene expression analyses. GENEZOL reagent was added to the tumor tissue sample and placed on ice. The tissue was then homogenized using a handheld homogenizer. The mixture was centrifuged at 13000 × g for 15 min at 4 °C to separate the phases. The upper aqueous phase was transferred to a new 2.0 mL microcentrifuge tube, followed by the RNA extraction protocol described above.

Hematoxylin & Eosin staining

The impact of BZD9L1 treatment on tumor necrosis affecting tumor growth was determined by establishing a necrotic score. First, the slides were deparaffinized by heating the slides in the oven at 60 °C for 10 min. The slides were then immersed in the following order: Xylene (twice), absolute ethanol (twice), 95% ethanol (twice), 70% ethanol (once) and lastly, in distilled water, for 5 min each. Next, the slides were stained with hematoxylin for 5 min and then washed with distilled water for another 5 min. The slides were later counterstained with eosin for 15 s. Finally, the slides were washed for 5 min and air-dried before being mounted.

Immunohistochemistry

The effect of BZD9L1 on proliferation was ascertained through Ki67 protein expression analysis on the xenograft tumor sections. The formalin fixed paraffin embedded slides were deparaffinized by heating the slides in an oven at 60 °C for 10 min. The slides were then immersed in the following order: absolute ethanol (twice), 95% ethanol (twice), 70% ethanol (once), followed lastly in distilled water for 5 min each. This procedure was carried out using the Dako Envision FLEX Kit. The sections were incubated in High pH

antigen retrieval buffer (Dako) for 30 min. Next, the slides were washed with the wash buffer three times, for 5 min each. A few drops of Envision FLEX Peroxidase solution were introduced to the tissue for 10 min. The sections were then incubated with primary antibody Ki67 (Dako, Clone MIB-1, Cat#M7240, mouse monoclonal) and incubated at 4 °C overnight at 1:50 dilution. On the next day, the sections were rinsed in TBST and incubated with goat anti-mouse IgG secondary antibody for 1 h at 1:500 dilution, rinsed three times in TBST and followed by incubation with Dako® DAB solution. The slides were counterstained with hematoxylin for 5 min and then rinsed off before being mounted with glycerol (Sigma) and examined under a light microscope (CX41, Olympus). The Ki-67 score was established as the percentage of tumor cells positive for brown nuclear staining over the total number of nuclei from five random fields per tumor section.

Statistical analysis

GraphPad Prism 9.4.0 (GraphPad, United States) and Microsoft Excel (Microsoft, United States) were used for statistical analysis. The analysis of variance (One-way ANOVA) test was employed to compare mean values among three or more data sets, and Bonferroni post-test was employed to compare any two data sets among the three or more sets. Statistical significance was indicated in the figures, where ^a $P < 0.05$; ^b $P < 0.01$; and ^c $P < 0.001$, compared to the negative control or vehicle control. The non-regression curve was used to track the change in the tumor size and mouse body weight over time. Error bars represent the SEM.

RESULTS

BZDL1 reduced the viability and adhesion of Ea.HY926 cells

The effects of BZD9L1 on cell viability were assessed with MTT assay at 72 h. The half-IC₅₀ of BZD9L1 in Ea HY926 was established to be at 5.2 μM (Figure 1A). BZD9L1 at 10 μM reduced the ability of the EC in suspension to adhere to the microtiter plates (E-Plates®) at 5 h (Figure 1B). BZD9L1 at 2.5 μM and 5.0 μM had no significant impact on

cell proliferation at 24 h and 48 h (Figure 1C). Hence the aforementioned IC₅₀ value (5.0 µM) and half its IC₅₀ (2.5 µM) were selected for downstream experiments.

BZD9L1 reduced SIRT1, SIRT2, intercellular adhesion molecule 1, integrin-alpha V and vascular endothelial cadherin genes in Ea.HY926 cells

As BZD9L1 is a small molecule inhibitor with SIRT1 and 2 inhibitory activities, Ea.HY926 cells were treated with non-killing doses of BZD9L1 at 2.5 µM and 5.0 µM to investigate its repressive effects on SIRT 1 and SIRT 2. SIRT 1 and 2 gene expression were significantly reduced in cells treated with BZD9L1 at 2.5 µM and 5.0 µM, compared to the negative control (Figure 2A). BZD9L1 also significantly down-regulated intercellular adhesion molecule 1 (ICAM-1), integrin-alpha V (ITGA5) and vascular endothelial cadherin (VE-cadherin) cell adhesion molecules (Figure 2B) compared to the negative control.

BZD9L1 induced apoptotic cell death and arrested Ea HY926 cells at the G1 phase

The live, necrotic, early apoptotic, and late apoptotic fractions and cell cycle distribution of cells were detected by Annexin V and PI staining at 72 h post-treatment. Cells treated with BZD9L1 at 2.5 µM and 5.0 µM showed a significant increase in early and late apoptosis compared to the negative control (Figure 3A). BZD9L1 at 2.5 µM and 5.0 µM induced cell cycle arrest at the G1 phase compared to the negative control group (Figure 3B).

BZD9L1 hampered Ea.HY926 EC spheroid and mouse choroid sprouting

BZD9L1 at 2.5 µM and 5.0 µM significantly reduced Ea.HY926 EC spheroid sprouting compared to negative control 72 h post-treatment (Figure 3C). Considering the native tissue and microenvironmental factor, BZD9L1 employed at higher concentrations of 10 µM, and 20 µM decreased the sprouting area in mouse choroids compared to the negative control 96 h post-treatment (Figure 4A). Interestingly, BZD9L1 at both concentrations also regressed sproutings of mouse choroids 24 h post-treatment of

sprouted choroids (Figure 4B). ALK1 inhibited both the sprouting and regression processes in mouse choroids.

Conditioned media from BZD9L1-treated ECs reduced CRC spheroid invasion

Co-culture models are widely utilized to examine how physical contact between cells and autocrine and/or paracrine interactions affect cell activity. An indirect co-culture was performed to determine the effects of BZD9L1 on EC and CRC spheroids. The 48-h post-treatment conditioned media from Ea.HY926 cells were used to treat CRC spheroids to study the tumor-EC relationship. Analysis of the EC-conditioned media revealed that BZD9L1 at 5 μ M diminished Angiogenin (A), bFGF (B), PDGF-BB (C), and PIGF (D) levels compared to the negative control group (Figure 5A). The other six cytokines were either not expressed at basal levels or below the detection limit. The conditioned media from all treatment groups significantly reduced the invasion of the CRC spheroids relative to the negative control group (Figure 5B).

BZD9L1 inhibited colorectal tumor growth and angiogenesis in the xenograft model

Sunitinib at 40 mg/kg and BZD9L1 inhibited tumor growth in low- (50 mg/kg) and high- (250 mg/kg) dose groups compared to the vehicle group (Figure 6A). Notably, the tumor growth in high-dose-treated mice was significantly impeded compared to the low-dose-treated mice group. The 250 mg/kg BZD9L1-treated xenograft tumor growth was equally inhibited to the same level as the 40 mg/kg Sunitinib positive control-treated mice. The weight of the tumor also decreased with the increasing doses of BZD9L1 (Figure 6B). There was no change in the body weight in all treatment groups compared to the vehicle control group except for the Sunitinib-treated group (Figure 6C). BZD9L1 (50 mg/kg) did not significantly reduce tumor necrosis compared to vehicle control (Figure 6D). However, only BZD9L1 (250 mg/kg) and Sunitinib positive control significantly inhibited tumor necrosis compared to the vehicle control group. In addition, BZD9L1 at low and high doses significantly reduced Ki67 protein expression in tumor tissue compared to vehicle control (Figure 6E). qPCR analyses of the xenograft

tumors revealed no significant change in murine SIRT1 (mSIRT1) but a down-regulation of mSIRT2 in BZD9L1-treated groups at both 50 mg/kg and 250 mg/kg, compared to the vehicle control group (Figure 7A). In addition, BZD9L1 at 250 mg/kg reduced hSIRT1 and hSIRT2 gene expression in mice, compared to the vehicle control group. Although BZD9L1 at the low dose did not significantly reduce cluster of differentiation 31 (CD31) gene expression compared to the vehicle control, BZD9L1 at high doses decreased both CD31 and CD34 gene expression compared to the vehicle control (Figure 7B).

DISCUSSION

The fundamental stages of sprouting angiogenesis comprise enzymatic degradation of the EC basement membrane, followed by EC proliferation, migration, tube formation in response to growth factors gradient and finally, mural cell stabilization. Despite the emergence of novel targeted therapies targeting cancer cells or the tumor vasculature, the development of small molecule inhibitors to treat CRC to arrest the two main features of CRC, namely uncontrollable malignant cell proliferation and angiogenesis simultaneously, remain a potential avenue to be explored. The anticancer and SIRT 1 and 2 inhibitory activities of BZD9L1 have previously been established^[15]. The human EC line Ea.HY926 was chosen for this study because it is continuous, exhibits various characteristics common to vascular ECs, and is frequently employed as an *in vitro* model for angiogenesis^[16,17]. Despite the reduction of EC adhesive capability at double the IC₅₀ of BZD9L1 (10 μ M) compared to the negative control at 5 h (Figure 1B), the implicated dose was non-toxic, as revealed by the mean cell index of the EC at early point (Figure 1C). The downregulation of ICAM-1, VE-cadherin and ITGA5 cell adhesion molecules post BZD9L1 treatment in Ea HY926 highlights its potential to inhibit the early steps of angiogenesis. The effect of BZD9L1 on a panel of cell adhesion molecules and their post-translational modifications may be crucial to determine the specific mechanisms that may impact EC adhesion stability and function, thus warrants further investigations.

Cell viability is influenced by cell proliferation and cell death. During angiogenesis, ECs exhibit an increased proliferation rate. Cell proliferation is important in the elongation and maturation of new blood vessels. BZD9L1 at all doses reduced the mean cell index (indicative of cell viability or proliferation) of EC at 72 h when compared to the negative control. Nevertheless, a significant decrease in cell index was only noted in EC treated with 10 μ M BZD9L1 at 48 h, which served as the basis for proceeding with downstream molecular assays using half the IC₅₀ (2.5 μ M) and IC₅₀ (5.0 μ M) BZD9L1. BZD9L1 at the tested doses significantly induced apoptosis and arrested EC at the G1 phase (Figure 3A and B), which suggests that BZD9L1 reduced EC viability by triggering apoptotic cell death and inhibiting cell cycle progression. The G1 phase is where cells prepare to divide. If cells cannot carry out DNA repair at G1 cell cycle arrest, they will enter the apoptosis stage. Cells commit suicide during the process of cell apoptosis as a result of signals that start programmed cell death. Apoptosis is crucial for blood vessel regression during angiogenesis^[18,19]. Correspondingly, BZD9L1 increased the percentage of apoptotic EC compared with the negative control group, which is corroborated by our previous report in CRC^[6].

In a meta-analysis, elevated expression of SIRT1 in CRC is correlated with vascular invasion and inferior outcomes^[20]. Meanwhile, increased SIRT2 expression was associated with poor prognosis in CRC patients and the inhibition of SIRT2 Limited CRC tumor angiogenesis *via* inactivation of the STAT3/VEGFA signalling pathway^[21]. BZD9L1 downregulated the gene expression of SIRT1 and 2 in Ea.HY926 cells *in vitro* (Figure 2A). Endothelial cell proliferation, migration, and the assembling of vascular networks were reduced when SIRT 1 and SIRT 2 activities were blocked *in vitro*^[21-23]. BZD9L1 at 250 mg/kg reduced the expression of hSIRT1 and hSIRT2 genes, highlighting the ability of BZD9L1 to modulate human SIRTs in HCT116 xenograft tumours which collectively led to the inhibition of tumor growth. However, mSIRT2 but not mSIRT1 was significantly downregulated by BZD9L1 *in vivo* (Figure 7A). BZD9L1 was previously reported to display a higher affinity for SIRT2 than SIRT1^[15], which may explain this observation. Furthermore, the tumor microenvironment may play a role in

therapeutic response. SIRT1 and SIRT2 are also expressed in other stromal cells, and their interplay may regulate tumor immune responses that impact CRC progression^[24,25].

SIRT 1 and SIRT 2 inhibition have been shown to suppress EC proliferation, migration and angiogenesis^[26,27]. In human retinal microvascular ECs, SIRT1 downregulation prevented EC migration and tube formation, whereas SIRT1 overexpression had the opposite effects^[27]. The SIRT2 inhibitor, AK-1, dramatically reduced the ability of human umbilical vein ECs (HUVECs) to form tubes^[28]. EC spheroid (Figure 3C) and choroidal sprouting (Figure 4A) were shown to be negatively affected by BZD9L1 in an increasing dose. In addition, the compound also regressed sprouting choroids compared to the negative control (Figure 4B). A recent study proved that when sprouting angiogenesis occurs, the vascular endothelium expresses SIRT1 in high levels, and inhibiting SIRT1 function prevents the development and migration of endothelial sprouts as well as the *in vitro* construction of a primitive vascular network^[22]. Furthermore, a study on primary murine lung ECs exhibited that when the chemotactic response was muted, tube development was attenuated, and the length of the EC spheroid sprout was shorter in cells lacking SIRT1^[29]. Furthermore, SIRT2 knockdown in HUVECs diminished its angiogenic potential, while overexpression of SIRT2 Led to contrasting outcomes^[30]. Altogether, these findings highlighted that BZD9L1 negatively regulated SIRT 1 and SIRT 2 to reduce EC viability, adhesion and sprouting.

The EC-conditioned media treated with BZD9L1 at 5 μ M portrayed angiogenin, bFGF, PDGF-BB, and PIGF to be significantly reduced compared to the negative control (Figure 5A). Several studies have previously reported angiogenin being essential in EC proliferation that can promote the development of new blood vessels^[31,32]. Angiogenin was also shown to stimulate progenitor cell proliferation and protect the stemness of primitive hematopoietic stem/progenitor cells, which suggests that its downregulation may negatively regulate angiogenesis *via* BZD9L1 inhibitory effects. bFGF can impact the upregulation of VEGF directly and promote EC proliferation^[33]. The ECs secrete

PDGF-BB that binds to PDGFR- β on the mural cells, which then affects the formation and maturation of new capillaries. PlGF also positively regulates angiogenesis; thus, its inhibition reduces EC growth, migration, and survival^[34]. Therefore, the downregulation of these cytokines in BZD9L1-treated EC at 5 μ M may collectively impede the steps of angiogenesis and HCT116 CRC invasion through paracrine signalling in the co-culture model, while at 2.5 μ M, BZD9L1 may potentially reduce CRC invasion through the regulation of other growth factors that were not captured by the protein array (Figure 5B).

BZD9L1 was previously reported to have anti-tumor effects in CRC xenograft models when used in adjunct with 5-Fluorouracil^[7]. BZD9L1 did not lead to any acute or repeated dose toxicity symptoms, and neither were there any cellular or molecular changes that would be considered significantly and biologically toxic^[10]. In this study, BZD9L1 significantly inhibited the progression of CRC tumor xenograft models. Although the body weight of mice treated with BZD9L1 remained unaffected compared to the vehicle control group, the weight of the mice treated with sunitinib declined treatment, as also confirmed by other studies supporting the weight loss potential of sunitinib in mice by targeting fat cells, leading to appetite loss^[35,36]. The mechanisms of weight loss due to sunitinib treatment are poorly understood.

Tumor necrosis is linked to poor prognosis and overall survival of CRC patients^[37]. Therefore, the reduction of tumor necrosis in mice treated with 250 mg/kg BZD9L1 further underlines the therapeutic potential of this benzimidazole analogue. On the other hand, Ki67 protein expression was significantly reduced in all treatment groups compared to the vehicle control group, indicating the possibility of BZD9L1 to reduce tumor growth to be *via* the impediment of tumor proliferation. In this study, the amount of vessels left post-treatment were determined *via* gene expression analyses of mCD31 and mCD34, due to the high necrotic nature of the tumors which limited the accurate interpretation of immunohistochemical staining (Figure 7B). BZD9L1 at a high dose significantly reduced mCD31 gene expression compared to the vehicle control group, while both low and high doses of the compound decreased mCD34 gene levels.

This may be due to the expression profile of CD31 and CD34, which are not limited strictly to just EC but also a subset of leukocytes and/or hematopoietic progenitor cells that express the CD31 and CD34 antigens, respectively. In addition, gene expression may not positively correlate to protein expression levels due to the turnover rate and possible post-translational modification. Despite this, anti-CD31 and CD34 antibodies are often employed as a diagnostic for vascular malignancies and are highly sensitive indicators of EC differentiation^[38,39].

Pathophysiological angiogenesis is mediated by the VEGF which correlates with increased microvessels density and metastatic spread in CRC^[40]. Clinical studies showed that resistance developed against angiogenesis inhibitor in single-agent anti-angiogenic therapy. Research has demonstrated the blockage of the VEGF pathway to normalise the tumour-associated vessels. As tumour vasculature matures, angiogenesis inhibitor temporarily reduces tumour hypoxia by improving targeted therapy's efficacy to ensure delivery of oxygen or cytotoxic or cytostatic drugs to the tumour sites^[41]. However, the normalisation of tumour vascular usually occurs only transiently at the initial time of anti-angiogenic therapy. Hypoxia increases with prolonged VEGF inhibition due to vessel pruning, which consecutively induces systemic secretion of other pro-angiogenic cytokines^[42].

The failure of anti-angiogenic monotherapy may be attributed to the induction of VEGF-independent compensatory mechanisms. The activation of other angiogenic signaling pathways may also induce the expression of other pro-angiogenic cytokines instead of VEGF^[43]. Therefore, the development of new inhibitors that target other molecules that are involved in the angiogenesis signaling pathway may be essential. In our study, BZD9L1 did not significantly affect VEGF expression. Hence the compound may be a potential candidate to be employed as an adjunct to VEGF inhibitors or chemotherapy in CRC, as previously reported^[7]. Besides, anti-angiogenic therapy combined with chemotherapy or immune checkpoint inhibitors has demonstrated promising therapeutic effects with enhanced clinical benefits for cancer patients^[44]. As such, small molecule inhibitors with anti-angiogenic and anti-cancer capabilities, such

as that portrayed by BZD9L1, could be a promising new strategy for cancer therapy by targeting the two distinct features of CRC.

CONCLUSION

BZD9L1 hindered the processes of angiogenesis in EC through the down-regulation of cell adhesion molecules, SIRT1 and SIRT2 genes. Similarly, the angiogenesis array displayed a depletion of angiogenesis cytokines angiogenin, bFGF, PDGF-BB and PIGF in BZD9L1-treated ECs. Furthermore, BZD9L1 arrested the cells at the G1 phase and induced apoptosis in EC. These results suggest that BZD9L1 displayed cytotoxic and cytostatic properties to negatively regulate the formation of new blood vessels. The sprouting potential and the sprout regression were also noted in mouse choroid tissues post-treatment. Moreover, the indirect co-culture assay demonstrated that BZD9L1 could reduce CRC spheroid invasion compared to the negative control. This result potentially suggests that suppression of angiogenesis may obstruct cancer cell progression, as further confirmed *in vivo*. In mice, BZD9L1 had the ability to retard HCT116 colorectal xenograft tumor growth. In relation to this, the tumor necrosis and Ki67 protein expression percentage in the tumor sections were reduced in the BZD9L1-treated groups compared to vehicle control. CD31 and CD34 protein expressions were not evaluated due to the limitation posed by necrosis in the tumor sections. However, gene analyses revealed a decline in these well-reported EC markers in BZD9L1-treated mice.

Overall, this project has provided insights into the potential of BZD9L1 to reduce EC growth and progression *in vitro* and hinder CRC tumor growth *in vivo*. It is noteworthy that this study should be recapitulated using at least one other primary ECs or ECs derived from CRC to confirm the findings. Moreover, BZD9L1 may regulate other angiogenesis players and cancer pathways to impact cancer progression, as suggested in a recent study where 58 other BZD9L1-regulated targets were identified^[45]. *In vivo* study in an orthotopic model where the tumor cells will be engrafted into the organ, which matches the cancer cell type may also provide a more realistic model to study the

mechanism of action of BZD9L1 and its impact on the tumor microenvironment. Additionally, real-time vessel perfusion imaging may be performed to more accurately assess the efficacy of BZD9L1 treatment^[46]. This study provides valuable insights into BZD9L1 as a potential anti-angiogenic agent in CRC.

Figure Legends

Figure 1 Cell viability and adhesion analyses of Ea.HY926 endothelial cells treated with different concentrations of BZD9L1. A: Cell viability was determined using the MTT assay. The inhibitory concentration of BZD9L1 in Ea HY926 is $5.20 \mu\text{M} \pm 0.38 \mu\text{M}$ ($n = 3$) at 72 h; B: Real-time xCELLigence impedance analysis of the area under the curve of BZD9L1-treated Ea.HY926 cells in suspension over 5 h. BZD9L1 at $10 \mu\text{M}$ reduced the ability of the endothelial cells to adhere to the microtiter plates (E-Plates®); C: Real-time xCELLigence impedance analysis of normalized cell index of BZD9L1-treated Ea.HY926 cells over 72 h. BZD9L1 at $2.5 \mu\text{M}$ and $5.0 \mu\text{M}$ had no significant impact on cell proliferation at 24 h and 48 h. Statistical analysis (^a $P < 0.05$; ^b $P < 0.01$), one-way ANOVA with Bonferroni posthoc test, $n = 3$ independent experiments using GraphPad Prism 9.4.0. Error bars represent SEM. VC: Vehicle control.

Figure 2 Quantitative polymerase chain reaction analysis of Ea.HY926 cells treated with BZD9L1 for 4 h. A: BZD9L1 reduced the gene expression levels of sirtuin 1 (SIRT1) and SIRT 2; B: Intercellular adhesion molecule 1 (ICAM-1), vascular endothelial cadherin (VE-cadherin) and integrin-alpha V (ITGA5) cell adhesion markers including ICAM-1, VE-cadherin and ITGA5, compared to the negative control (NC). Statistical analysis (^c $P < 0.001$), one-way ANOVA with Bonferroni posthoc test, $n = 3$ independent experiments using GraphPad Prism 9.4.0. Error bars represent SEM. SIRT: Sirtuin; ICAM-1: Intercellular adhesion molecule 1; NC: Negative control; VE-cadherin: Vascular endothelial cadherin; ITGA5: Integrin-alpha V.

Figure 3 Representative cytograms and endothelial cells sprouting spheroids depict the negative impact of BZD9L1 on endothelial cell functions. A: BZD9L1 at 2.5 μ M and 5.0 μ M induced apoptosis; B: Cell cycle arrest at G1 phase; C: But reduced Ea.Hy 926 spheroid sprouting compared to the negative control at 72 h. Sunitinib anti-angiogenic agent was used as the positive control. Statistical analysis ($^cP < 0.001$), one-way ANOVA with Bonferroni posthoc test, $n = 3$ independent experiments using GraphPad Prism 9.4.0. Error bars represent SEM. NC: Negative control.

Figure 4 Analyses of mouse choroidal endothelial sprouts in sprouting and regression models. A: BZD9L1 impeded mouse choroidal endothelial sprouting 96 h post-treatment; B: Regressed choroid sprouting 24 h post-treatment compared to the negative control. A receptor like type 1 anti-angiogenic agent was used as the positive control regression and sprouting assays. Statistical analysis ($^aP < 0.05$; $^cP < 0.001$), one-way ANOVA with Bonferroni posthoc test, $n = 2$ independent experiments using GraphPad Prism 9.4.0. Error bars represent SEM. ALK1: A receptor like type 1; NC: Negative control.

Figure 5 Refreshed conditioned media from Ea.Hy 926 endothelial cells 48 h post-treatment with Sunitinib positive control, BZD9L1 at 2.5 μ M, 5.0 μ M, or negative control for 48 h prior, were subjected to Quantibody Human Angiogenesis array and indirect co-culture with HCT116 colorectal cancer spheroids. A: BZD9L1 at 5 μ M

down-regulated angiogenin, fibroblast growth factor, platelet-derived growth factor and placental growth factor cytokine concentrations in Ea.Hy 926 conditioned media; B: The endothelial conditioned media from BZD9L1 or sunitinib positive control-treated groups impeded HCT116 tumor invasion at 72 h. Statistical analysis (^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$), one-way ANOVA with Bonferroni posthoc test, $n = 2$ independent experiments for protein array analysis and $n = 3$ independent replicates for co-culture analysis using GraphPad Prism 9.4.0. Error bars represent SEM. NC: Negative control. bFGF: Fibroblast growth factor; PDGF-BB: Platelet-derived growth factor; PIGF: Placental growth factor; NC: Negative control.

Tumor growth analyses of HCT116 tumor xenograft in nude mice. Treatments were administered when the tumors reached 100 mm³. A: Relative tumor volume; B: Tumor weight and; C: Percentage body weight change in mice treated with vehicle control (0.5% carboxymethylcellulose), BZD9L1 (50 mg/kg per 3 d, BZD9L1 (250 mg/kg per 3 d) and sunitinib (40 mg/kg per 3 d) as the positive control; D: Tumor necrosis percentage in sections stained with haematoxylin and eosin. N indicates necrotic tissues; E: Ki67 proliferation protein expression in treated and vehicle control groups. Statistical analysis (^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$). The non-linear fit was used to track the tumor size and body weight change over time, one-way ANOVA with Bonferroni posthoc test for tumor weight, $n = 6$ animals per group) using GraphPad Prism 9.4.0. Error bars represent SEM. VC: Vehicle control.

Figure 7 Quantitative polymerase chain reaction analyses of murine SIRT1, mSIRT2 and endothelial cell markers in HCT116 tumor xenograft in nude mice. A: BZD9L1 did not significantly affect murine SIRT1 (mSIRT1) gene expression in the colorectal cancer xenograft tumors but down-regulated mSIRT2 at low (50 mg/kg) and high (250 mg/kg) doses and reduced human SIRT1 (hSIRT1) and hSIRT2 gene expression at 250

mg/kg, compared to vehicle control; B: BZD9L1 at the high dose significantly reduced cluster of differentiation 31 (CD31) gene expression. Both low and high doses of BZD9L1 decreased mCD34 gene expression compared to vehicle control. ³ Statistical analysis ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$), one-way ANOVA with Bonferroni posthoc test, $n = 6$ animals per group) using GraphPad Prism 9.4.0. Error bars represent SEM. VC: Vehicle control.

Table 1 List of primers

Genes	Primer sequences	
	Forward sequences (5' to 3')	Reverse sequences (3' to 5')
mPpia	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
m18SrRNA	GGACCAGAGCGAAAGCATTTGCC	TCAATCTCGGGTGGCTGAACGC
18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
mCD31	CCAAAGCCAGTAGCATCATGGTC	GGATGGTGAAGTTGGCTACAGG
mCD34	CTTCCCCAACTGGCATACTGC	TCCAGAGCATTTGATTTCTCCC
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTGTGCTGTA
ICAM	CGACTGGACGAGAGGGATTG	TTATGACTGCGGCTGCTACC
VE-cadherin	CCCTTCTTCACCCAGACCAA	CCGGTCAAACCTGCCCATACT
ITGA5	CGGGCCCCCTGCACCAACAAG	CAGCTGTGGCCACCTGACGC
mSIRT 1	CGGCTACCGAGGTCCATATAC	CAGCTCAGGTGGAGGAATTGT
mSIRT 2	GAGCCGGACCGATTGAGAC	AGACGCTCCTTTTGGGAACC
SIRT 1	TCTAACTGGAGCTGGGGTGT	TGGGAAGTCTACAGCAAGGC
SIRT 2	GCCCTTTACCAACATGGCTG	TTCGTACAACACCCAGAGCG

ITGA5: Integrin-alpha V; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SIRT: Sirtuin; ICAM: Intercellular adhesion molecule; VE-cadherin: Vascular endothelial cadherin; CD31: Cluster of differentiation 31.

26%

SIMILARITY INDEX

PRIMARY SOURCES

- 1 Wei Kang Chen, Chern Ein Oon, Gurjeet Kaur, Richard C.A. Sainson, Ji-Liang Li. "Downregulation of Manic fringe impedes angiogenesis and cell migration of renal carcinoma", Microvascular Research, 2022 268 words — 3%
Crossref
- 2 www.ncbi.nlm.nih.gov 180 words — 2%
Internet
- 3 journals.sagepub.com 145 words — 2%
Internet
- 4 www.frontiersin.org 140 words — 2%
Internet
- 5 Ashwaq Hamid Salem Yehya, Ayappa V Subramaniam, Muhammad Asif, Gurjeet Kaur, AMS Abdul Majid, Chern Ein Oon. " Anti-tumour activity and toxicological studies of combination treatment of and gemcitabine on pancreatic xenograft model ", World Journal of Gastroenterology, 2022 110 words — 1%
Crossref
- 6 Wei Kang Cheng, Gurjeet Kaur, Elin Sjöberg, Magnus Frodin, Lars Egevad, Ulrika Harmenberg, Ji-Liang Li, Chern Ein Oon. "Nuclear and stromal expression of Manic fringe in renal cell carcinoma", Experimental and Molecular Pathology, 2021 100 words — 1%

-
- 7 Ashwaq H.S. Yehya, Muhammad Asif, Amin M.S. Abdul Majid, Chern E. Oon. "Complementary effects of Orthosiphon stamineus standardized ethanolic extract and rosmarinic acid in combination with gemcitabine on pancreatic cancer", Biomedical Journal, 2020
Crossref 86 words — 1%
-
- 8 downloads.hindawi.com
Internet 59 words — 1%
-
- 9 www.mdpi.com
Internet 56 words — 1%
-
- 10 Yi Jer Tan, Yeuan Ting Lee, Keng Yoon Yeong, Sven H Petersen, Koji Kono, Soo Choon Tan, Chern Ein Oon. "Anticancer activities of a benzimidazole compound through sirtuin inhibition in colorectal cancer", Future Medicinal Chemistry, 2018
Crossref 43 words — 1%
-
- 11 www.dovepress.com
Internet 43 words — 1%
-
- 12 Ikhlas A. El karim, Gerard J. Linden, Chris R. Irwin, Fionnuala T. Lundy. "Neuropeptides Regulate Expression of Angiogenic Growth Factors in Human Dental Pulp Fibroblasts", Journal of Endodontics, 2009
Crossref 42 words — 1%
-
- 13 T. L. Guo, J. A. McCay, R. D. Brown, D. L. Musgrove, L. Butterworth, A. E. Munson, D. R. Germolec, K. L. White. "GLYCIDOL MODULATION OF THE IMMUNE RESPONSES IN FEMALE B6C3F1 MICE", Drug and Chemical Toxicology, 2000
Crossref 42 words — 1%
-

14	Internet	37 words — < 1 %
15	www.science.gov Internet	32 words — < 1 %
16	docksci.com Internet	26 words — < 1 %
17	refubium.fu-berlin.de Internet	24 words — < 1 %
18	stemcellres.biomedcentral.com Internet	24 words — < 1 %
19	www.freepatentsonline.com Internet	24 words — < 1 %
20	epub.uni-bayreuth.de Internet	23 words — < 1 %
21	Yeuang Ting Lee, Yi Jer Tan, Pei Yi Mok, Gurjeet Kaur, Sasidharan Sreenivasan, Marco Falasca, Chern Ein Oon. "Sex-divergent expression of cytochrome P450 and SIRTUIN 1–7 proteins in toxicity evaluation of a benzimidazole-derived epigenetic modulator in mice", Toxicology and Applied Pharmacology, 2022 Crossref	21 words — < 1 %
22	Supamate Tarasuntisuk, Tanapat Palaga, Hakuto Kageyama, Rungaroon Waditee-Sirisattha. "Mycosporine-2-glycine exerts anti-inflammatory and antioxidant effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages", Archives of Biochemistry and Biophysics, 2018 Crossref	20 words — < 1 %

23	biologydirect.biomedcentral.com Internet	20 words — < 1%
24	patents.justia.com Internet	20 words — < 1%
25	westminsterresearch.westminster.ac.uk Internet	19 words — < 1%
26	www.thermofisher.com Internet	19 words — < 1%
27	Federico Perche, Vladimir P. Torchilin. "Cancer cell spheroids as a model to evaluate chemotherapy protocols", Cancer Biology & Therapy, 2014 Crossref	18 words — < 1%
28	www.spandidos-publications.com Internet	18 words — < 1%
29	Jung, Y.O.. "Toll-like receptor 2 and 4 combination engagement upregulate IL-15 synergistically in human rheumatoid synovial fibroblasts", Immunology Letters, 20070315 Crossref	17 words — < 1%
30	d-nb.info Internet	17 words — < 1%
31	mspace.lib.umanitoba.ca Internet	17 words — < 1%
32	www.caymanchem.com Internet	17 words — < 1%
33	www.researchsquare.com Internet	17 words — < 1%

34	www.hindawi.com Internet	16 words — < 1%
35	Ashwaq H.S. Yehya, Muhammad Asif, Gurjeet Kaur, Loiy E.A. Hassan, Fouad S.R. Al-Suede, Amin M.S. Abdul Majid, Chern E. Oon. "Toxicological studies of Orthosiphon stamineus (Misai Kucing) standardized ethanol extract in combination with gemcitabine in athymic nude mice model", Journal of Advanced Research, 2018 Crossref	15 words — < 1%
36	academic.oup.com Internet	15 words — < 1%
37	aging-us.com Internet	15 words — < 1%
38	vdoc.pub Internet	15 words — < 1%
39	advbiores.net Internet	13 words — < 1%
40	Sara E Walker. "Accelerated appearance of neoplasms in female nzb/nzw mice treated with high-dose cyclophosphamide", Arthritis & Rheumatism, 12/1979 Crossref	12 words — < 1%
41	mdpi-res.com Internet	12 words — < 1%
42	www.oncotarget.com Internet	12 words — < 1%
43	open.bu.edu Internet	11 words — < 1%

44	patents.google.com Internet	11 words — < 1%
45	apps.dtic.mil Internet	10 words — < 1%
46	preview-jneuroinflammation.biomedcentral.com Internet	10 words — < 1%
47	spandidos-publications.com Internet	10 words — < 1%
48	www.liai.org Internet	10 words — < 1%
49	Segovia Mendoza Mariana. "Calcitriol o sus análogos aumentan la actividad antineoplásica de gefitinib, lapatinib y neratinib en células de cáncer de mama", TESIUNAM, 2016 Publications	9 words — < 1%
50	Zheng, Yi, Yubing Sun, Xinwei Yu, Yue Shao, Ping Zhang, Guohao Dai, and Jianping Fu. "Angiogenesis in Liquid Tumors: An In Vitro Assay for Leukemic Cell Induced Bone Marrow Angiogenesis", Advanced Healthcare Materials, 2016. Crossref	9 words — < 1%
51	discovery.dundee.ac.uk Internet	9 words — < 1%
52	link.springer.com Internet	9 words — < 1%
53	spiral.imperial.ac.uk Internet	9 words — < 1%

54	www.longdom.org Internet	9 words — < 1%
55	www.thno.org Internet	9 words — < 1%
56	Chern Ein Oon, Carina Strell, Keng Yoon Yeong, Arne Östman, Jai Prakash. "SIRT1 inhibition in pancreatic cancer models: Contrasting effects in vitro and in vivo", European Journal of Pharmacology, 2015 Crossref	8 words — < 1%
57	download.atlantis-press.com Internet	8 words — < 1%
58	ediss.uni-goettingen.de Internet	8 words — < 1%
59	insights.ovid.com Internet	8 words — < 1%
60	www.cibic.com.ar Internet	8 words — < 1%
61	www.wjgnet.com Internet	8 words — < 1%
62	Benachour, Hamanou, Thierry Bastogne, Magali Toussaint, Yosra Chemli, Aymeric Sève, Céline Frochot, François Lux, Olivier Tillement, Régis Vanderesse, and Muriel Barberi-Heyob. "Real-Time Monitoring of Photocytotoxicity in Nanoparticles-Based Photodynamic Therapy: A Model-Based Approach", PLoS ONE, 2012. Crossref	7 words — < 1%
63	Z. Adzemovic, Milena, Manuel Zeitelhofer, Ulf Eriksson, Tomas Olsson, and Ingrid Nilsson.	7 words — < 1%

"Imatinib Ameliorates Neuroinflammation in a Rat Model of Multiple Sclerosis by Enhancing Blood-Brain Barrier Integrity and by Modulating the Peripheral Immune Response", PLoS ONE, 2013.

Crossref

64 Sun-Ah Jung, Jongdae Shin, Ungsoo Samuel Kim, Joon H. Lee. "MicroRNA-193b, a critical factor in cell aging, in human lymphatic endothelial cells", Research Square Platform LLC, 2023

6 words — < 1%

Crossref Posted Content

65 Yi Jer Tan, Yeuan Ting Lee, Sven H. Petersen, Gurjeet Kaur, Koji Kono, Soo Choon Tan, Amin M. S. Abdul Majid, Chern Ein Oon. "BZD9L1 sirtuin inhibitor as a potential adjuvant for sensitization of colorectal cancer cells to 5-fluorouracil", Therapeutic Advances in Medical Oncology, 2019

6 words — < 1%

Crossref

EXCLUDE QUOTES OFF

EXCLUDE BIBLIOGRAPHY OFF

EXCLUDE SOURCES OFF

EXCLUDE MATCHES OFF