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Antiangiogenic Activity of 4-Chloro Phenyl Carbothioamide Indole Derivative Using the In Vivo Chorioallantois Membrane Assay

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Abstract

Background New blood vessels are formed by two basic processes; namely vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of initial vascular networks. Once the vascular networks are formed, new blood vessels are formed by sprouting or splitting from pre-existing vessels in an angiogenesis process.

Objective To investigate the anti-angiogenic activity of 2-(5-bromo - 1H- indole -2- carbonyl) -N- (4-chlorophenyl) hydrazine -1- carbothioamide indole derivative.

Methods The antiangiogenic activity of the test Indoles derivative was evaluated using the in-vivo chorioallantois membrane assay

Results The tested compound significantly (P <0.05) distorted and suppressed the growth of blood vessels in the chorioallantois membrane assay

Conclusion This study showed that (4-chloro phenyl carbothioamide indole derivative) significantly inhibits the angiogenesis process and could be a potentially promising angiogenesis inhibitor lead compound.

Keywords Anti-angiogenesis, vasculogenesis, chorioallantois membrane assay, 4-chloro phenyl carbothioamide indole derivative

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List of abbreviations: 2-NHC = 2(5-bromo-1H-indole-2-carbonyl)-N-(4-chlorophenyl) hydrazine-1-carbothioamide, BMP4 = Bone morphogenetic protein 4, CAM = Chorioallantois membrane, DMSO = Dimethyl sulfoxide, ECs = Endothelial cells, EGFR = Epidermal growth factor receptor, FGF2 = Fibroblast growth factor 2, HIFs, Hypoxia inducible factors, HIV = Human immunodeficiency virus, mTOR = Mammalian target of Rapamycin, SAR = Structure-activity relationship, TKIs = Tyrosine kinase inhibitors, VEGF-A = Vascular endothelial growth factor-A

Introduction

Endothelial cells (ECs) originate from mesodermal precursor cells termed angioblasts [(1)]. Fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 4 (BMP4) initiate the specification of the mesoderm and differentiation toward ECs [(2)]. Angioblasts, which form the outer layer of blood islands, differentiate into ECs, aggregate, and generate a primitive vascular network through vasculogenesis [(3,4)].

Angiogenesis is the process that involves the formation of new blood vessels from pre-existing ones. The primary step is thought to be initiated by the activation of ECs of pre-existing vessels in response to angiogenic stimuli. This process is typically initiated within hypoxic tissues where additional new blood vessels are

required to maintain oxygenation and nutritional supply [5]. When the tissue is hypoxic, cellular oxygen sensing mechanisms are activated, which induce gene expression of various pro-angiogenic proteins. The primarily activated factors are hypoxia-inducible factors (HIFs), which up-regulate multiple pro-angiogenic genes directly or indirectly [6]. Among the up-regulated genes, vascular endothelial growth factor–A (VEGF-A) is the major one and is also responsible for the proliferation and migration of cells during this process [7].

ECs, which are the principal building blocks of vasculature in the process of angiogenesis, must undergo four chief steps, which include breaking through of the basal lamina that wraps surviving blood vessels, migration toward a source signal, proliferation, and formation of tubes [8]. The angiogenic inhibitors can be divided into 3 categories [9]: monoclonal antibodies, small molecule tyrosine kinase inhibitors (TKIs), and inhibitors of mammalian targets of rapamycin (mTOR).

Indole is a naturally present heterocyclic compound with a benzene ring attached to a pyrrole ring with electrophilic substitution exposure. An indole derivative (5-bromoindole-2-carboxylic acid) was used as the starting chemical compound for the synthesis of other new indole derivatives. Among the synthesized derivatives; carbothioamides were selected for the study. Carbothioamides derivatives were synthesized previously in an earlier study [10]. Due to the wide distribution of indole derivatives in nature, it has acceptability among the organic and medicinal industries. Numerous indole moiety drug molecules are under investigation to control disease conditions such as bacterial, malaria, fungal, viral, tubercular, and HIV infections [11].

2(5-bromo-1H-indole-2-carbonyl)-N-(4-chlorophenyl) hydrazine-1-carbothioamide (2-NHC), a novel synthesized carbothioamide indole derivative as a white powder, was selected for the study based on the findings of Hassan et al. [10], the selected agent has good pharmacokinetic profiles, such as high absorption levels with no cytochrome P450 inhibitory activity and no in silico hepatotoxicity. The agent was found to be an epidermal growth factor receptor (EGFR), tyrosine kinase inhibitor (TKI), with an EC50 (Half maximum effective concentration) comparable to that of erlotinib, a standard TKI. The structure-activity relationship (SAR) studies of the new compound revealed that the presence of an aryl or heteroaryl fragment attached to the hydrophilic linker is critical for anti-cancer activity as shown in figure (1).

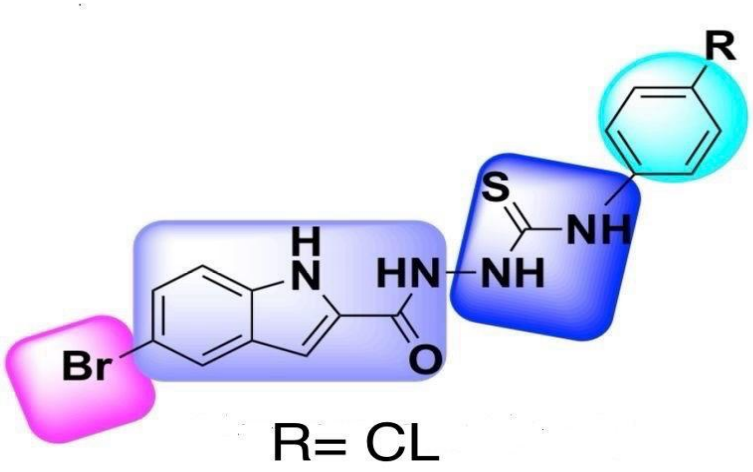

Figure 1. The structure activity relationship (SAR) of the novel indole derivative (2-NHC) exhibited several common features [10]

The objective of the study was to investigate the anti-angiogenic activity of 2-NHC indole derivative.

Methods

Study settings and ethical consideration

The investigation was carried out in The Tissue Culture Laboratory of the Department of Pharmacology at the College of Medicine, Al-Nahrain University. The project commenced in August 2023 and was completed in March 2024. All procedures were carried out after the approval of the Institute Review Bourd (IRB) of the College of Medicine, Al-Nahrain University (approval number: UNCOMIRB202405015, date: 22 August 2023).

All procedures were carried out in a strictly controlled environment and a class II laminar flow safety cabinet. Sterilization by both autoclave and ultraviolet light ensued for all equipment included in the experiments, as well as all solutions and compounds were filter sterilized using a 0.22 µM syringe filter. In addition to that, all surfaces and lab items were disinfected with 70% ethanol before commencing and when ceasing any experiments.

Chemicals and reagents

The indole derivative 2-NHC was obtained from the Department of Pharmaceutical Chemistry, College of Pharmacy, Kirkuk University, Kirkuk, Iraq. The compound´s chemical synthesis and characterization were previously mentioned by Hassan et al. [10]. Dimethyl sulfoxide (DMSO) was used to dissolve the tested chemical agent to create a stock solution with a concentration of (10 mg of the agent in 1 ml of DMSO). (Romil®, UK).

Chorioallantois membrane (CAM) in vivo assay

The modified method of Marchesan and colleagues [12] was used. Fertilized chicken eggs (n = 12) (6 eggs used as negative non treated group,and 6 eggs used as treated geoup) were obtained from a local hatchery in Baghdad (Saif Hatchery for Poultry). The eggs were cleared of dirt/debris with 70% ethanol and Povidone iodine 10% and then incubated in a horizontal position for about 72 hr at 37°C with a relative humidity of 60%. After 72 hr, 2 ml of albumin was aspirated out through a pinpoint hole punctured down at the pointed end of the egg by using a 2 ml syringe with a 21G needle and sealed with surgical adhesive tape to allow the CAM to detach from the shell, then eggs were incubated horizontally for further 24 hr. After that, a relatively small square window (3-4 cm diameter) of the shell was made by using a fine cutting drill that was removed with sharp pointed forceps, then exposed to the CAM of 6 fertilized eggs with the test sample that was soaked previously in filter paper discs, and the window was covered with sterile surgical adhesive tape, and embryos will be incubated again horizontally with the window uppermost for 72 hr at 37°C. The inhibition zone was calculated using the ruler provided in the Snipping Tool application, where the inhibition zone was determined by measuring the distance (in mm) of retracted blood vessels from the disc to the point of a visible blood vessel. The test agent was prepared as 10 mg/ml and placed on the filter paper discs and left to dry before being transferred to the CAM.

Quantification and chick CAM

The responses were categorized into three grades: + (3-6 mm), ++ (6-9 mm), and +++ (>10 mm). The size of the zone of inhibition was measured using an image analyzer (BIOCOM Visiolab- TM 2000) [12].

Statistical analysis

The CAM assay results analysis was done using unpaired Welch´s t-test to compare the two groups and considered significant at $P < 0.05$. The analysis was executed using GraphPad Prism 8.0.

Results

Chick CAM served as the in-vivo model for investigating the anti-angiogenic potentials of test agents. Fertilized chicken eggs (n = 6) were used in the experiment, where the CAM of each egg was treated with 100 µg/ml of the test agent (2-NHC) by placing an impregnated disk with the test agent (4-chloro phenyl carbothioamide indole derivative) on the edges of the CAM as shown in figure (2).

The inhibition zone was measured according to the scoring system mentioned previously. The results of the assay revealed that the agent produced an inhibition zone of 7.63±1.18 mm which is statistically highly significant (P <0.0001) regression in the blood vessels of the CAM compared to the negative control, which have zero inhibition zone as shown in table (1) and figure (2).

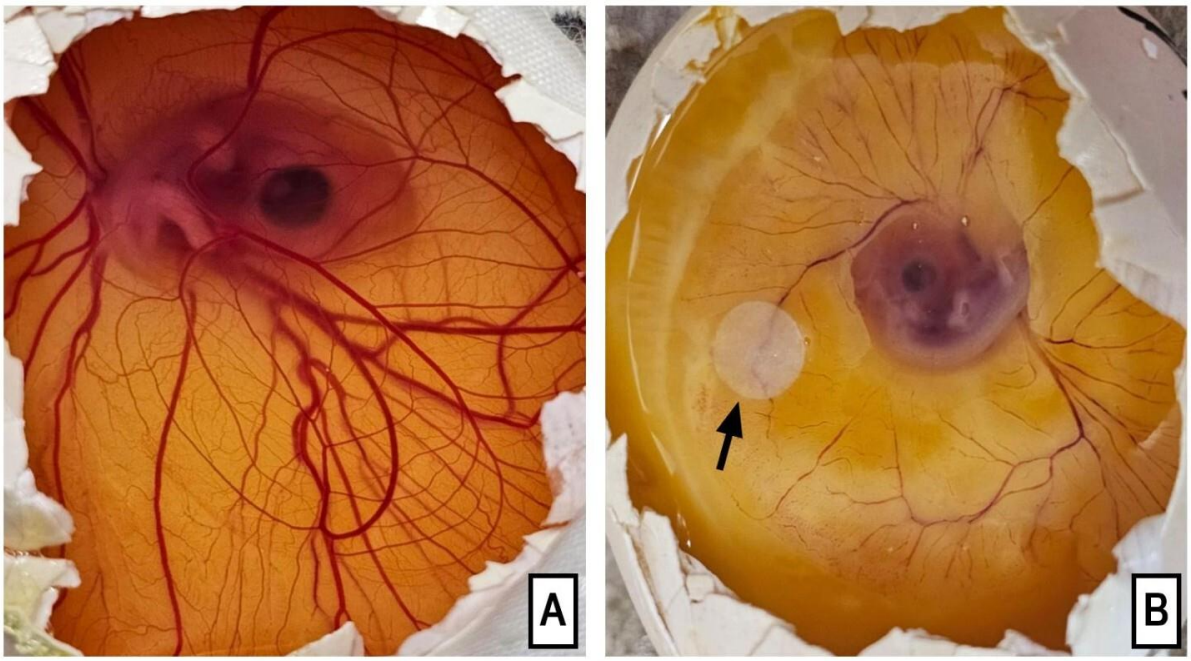

Figure 2. Images of the in vivo CAM assay showing a marked regression in the blood vessel growth surrounding the implanted disk (indicated by the black arrows) of test agents. (A) represents the negative control CAM, (B) represents CAM treated with 2-NHC

Table 1. Scoring of the inhibition zone of blood vessel growth in the in vivo CAM assay for the test agent (2-NHC)

2-NHC	
Distance of inhibition (mm)	Score
8.6	++
5.6	+
7.4	++
8.2	++
8.8	++
7.2	++
7.63±1.18	++

Results are presented as mean±SD. The agent was tested with 6 eggs (n = 6) and statistical significance was set at (P <0.0001) in comparison to the negative control eggs (n = 6), which have zero distance of inhibition

Discussion

It is important to search for and discover novel agents that may show a promising effect as antiangiogenic activity. This study examined an emerging novel compound that is 2-NHC and evaluated its anti-angiogenesis leverage. The chick embryo CAM is a highly vascularized extraembryonic membrane, that carries out several functions during embryonic development, including the exchange of respiratory gases, calcium transport from the eggshell, acid-base homeostasis in the embryo, and ion and water reabsorption from the allantois fluid. Due to its easy accessibility, and affordability and given that it constitutes an immunodeficient environment [13].

In the present study, 2-NHC exerted a pronounced effect on CAM assay, in which it produced a significant zone of inhibition in most of the samples due to a noticeable decrease in the number of blood vessels as compared with the negative control group. substance at a concentration of 10 mg/ml has stopped the radiation of a large number of blood vessels in CAM underneath the disc containing the substances. The current results were consistent with earlier research by Wanegaonkar et al. (14), where they performed the CAM assay on the 11 compounds of indolyl chalcones showed very good anti-angiogenic activities, and showed a visible inhibition of the development of capillaries. Another recent study for Indoloquinazoline alkaloids was done by zebrafish embryos with fluorescent blood vessels as an efficient model used for in vivo screening of antiangiogenic compounds and showed significant inhibitory activity and most efficacy to suppress the zebrafish vascular outgrowth in zebrafish embryos [15].

In conclusion, 2-NHC revealed significant inhibitory zones of blood vessels in vivo chick CAM assay.

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Author contribution

Ali: Conceptualization, investigation, and Manuscript preparation. Dr. Kadhim Supervision, manuscript review, and editing. Both authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare there is no interfering conflict.

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