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Nitric oxide synthase inhibitors 1400W and L-NIO inhibit angiogenesis pathway of colorectal cancer

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ABSTRACT

Background: It has been widely accepted that angiogenesis plays fundamental roles in colorectal cancer development, and therapeutic targeting of this pathway has achieved promising outcome. Recent reports have highlighted the involvement of nitric oxide synthases (NOS) in the development of angiogenesis in cancer; however, the mechanism and therapeutic value of NOS inhibitors in colon cancer are largely unknown.
Objective: In this study, we investigated the effects and mechanism of the NOS inhibitors 1400W and L-NIO on the angiogenesis pathway in colorectal cancer cells.
Methods: Two colorectal cancer cell lines, HT 29 and HCT 116, were used for *in vitro* study. The expression of iNOS and eNOS in cells was knocked down *via* shRNA transfection. MTS assays and wound healing assays were performed to assess cell proliferation and migration after shRNA transfection or treatment with 1400W, L-NIO, and 5-fluorouracil. Human angiogenesis PCR arrays and proteome profiler human angiogenesis arrays were used to detect changes in key genes/proteins involved in modulating angiogenesis after 1400W and L-NIO treatment.
Results: Knockdown of iNOS and eNOS significantly inhibited colorectal cancer cell growth. Treatment with NOS inhibitors inhibited colorectal cancer cell growth and migration, and was associated with suppression of the expression of key genes/proteins involved in the angiogenesis pathway. In addition, the combined use of NOS inhibitors with 5-fluorouracil showed enhanced inhibition of cell proliferation and migration.
Conclusion: NOS inhibitors could suppress colorectal cancer cell growth and migration, likely *via* suppressing the angiogenesis pathway.

1. Introduction

Colorectal cancer (CRC) is a malignant tumor arising from the inner wall of the large intestine. It is the third most common cancer and second leading cause of cancer death in US. Since the introduction of anti-angiogenic agents, there has been significant interest in understanding the molecular basis of angiogenesis in CRC and the discovery of potent agents with the capability of inhibiting the angiogenesis pathway [1–9].

Nitric oxide (NO) is an important cellular signaling molecule. It helps modulate vascular tone, insulin secretion, airway tone, peristalsis, and neural development [10–17]. In addition to its important role in maintaining physiological conditions, NO is also shown to be involved in angiogenesis [18–25]. During angiogenesis, hypoxia or the production of various angiogenic factors could induce the release of NO from endothelial cells. The released NO then modulates various processes during angiogenesis, including endothelial cell proliferation, migration, differentiation, and interaction with the extracellular matrix [18–25]. The NO is produced from L-arginine mediated by a family of nitric oxide synthase enzymes, *e.g.*, eNOS (endothelial NOS), nNOS (neuronal NOS), and an inducible isoform, iNOS. The NOS pathway has received great research attention as a promising target for anti-angiogenic therapeutic strategies, and various NOS inhibitors have been developed and tested in order to suppress angiogenesis in cancer treatment [26–29].

1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase [30]. Research has shown that 1400W diminishes the growth of established human colon cancer xenografts in

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nude mice and inhibits the growth of iNOS-expressing murine mammary adenocarcinomas [31]. L-NIO is another NOS inhibitor that inhibits eNOS and nNOS. *in vivo* [32]. The use of a combination of L-NIO and a tyrosine kinase inhibitor, E7080, was shown to result in anti-proliferative, anti-angiogenic and apoptotic effects in colorectal cancer cells [33]. However, the effects and mechanism of 1400W and L-NIO on the angiogenesis pathway in CRC have not been studied yet.

In this study, we have demonstrated that 1400W and L-NIO suppress CRC cell proliferation and migration, which is associated with systematic inhibition of angiogenesis-related gene transcription and protein expression. Using 1400W or L-NIO combined with 5-fluorouracil (5-FU) greatly enhanced the anti-proliferative effect on CRC cell proliferation and cell migration. Our research outcomes elaborate on the effects and mechanism of 1400W and L-NIO on the angiogenesis pathway in CRC, and may guide us to develop a NOS inhibitor-based colon cancer treatment strategy.

2. Methods and materials

2.1. Cell culture, transfection and reagents

Two CRC cell lines, HT 29 and HCT 116 cells, were obtained from ATCC. Cells were maintained in Minimum Essential Medium (MEM) (Cellgro) supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. For shRNA transfection, HT 29 and HCT 116 cells were seeded (1×10^6/well) in 6-well plates a day before transfection, and treated with iNOS (Santa Cruz) or eNOS (Santa Cruz) shRNA plasmids for 48 h with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The iNOS or eNOS knockdown cell population were selected with puromycin treatment for 3 days. 1400W and L-NIO were purchased from Cayman Chemical.

2.2. NOS assay

HT 29 and HCT 116 cells and the cells with stable knockdown of iNOS or eNOS were analyzed for NO production with a NOS assay using an Ultra-sensitive assay for nitric oxide synthase kit (Oxford Biomedical Research). Cell lysates were extracted with cell lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 5% glycerol, 100 mM NaCl) supplemented with protease inhibitor cocktail (Roche Applied Science) and were subjected to the NOS assay. 100 μL of standards or samples were loaded onto the 96 well microplate in triplicate. After adding the color reagents, the absorbance values were read at 540 nm in a microtiter plate reader (SpectraMax M5; Molecular devices).

2.3. Cell viability assay

The MTS cell proliferation assay (Promega) was performed according to the manufacturer's instructions. Briefly, cells were seeded at 8000 cells (in 100 μL medium) per well into 96-well plates, incubated overnight and exposed to treatments for the indicated time periods. Then 20 μL of CellTiter® 96 Aqueous One Solution Reagent was added into each well. After 4 h incubation at 37 °C, the quantity of formazan product was measured by recording the absorbance at 490 nm with a 96-well plate reader (SpectraMax M5; Molecular Devices). Cell viability was calculated as a percentage of the control group (normalized to 100%).

2.4. Wound healing assay

A wound healing assay was used to assess cell migration of both the HT 29 and HCT 116 cancer cell lines upon treatment with 1400W, L-NIO, 5-FU, and their combinations. HT29 and HCT 116 cells were seeded at 1×10^6 cells per well (6-well plate). After the cells reached 90% confluence, the cells were wounded by scratching with a sterile pipette tip and washed with phosphate buffered saline (PBS) subsequently to eliminate the impaired cells. The medium was changed to medium with 1% fetal bovine serum. The cells were subjected to different treatments and observed for 24 h. The wound area was measured using Image-J software (NIH, Bethesda, MD, USA). The wound area percentage was calculated as the wound area at 24 h vs. the wound area at 0 h in each group.

2.5. Human angiogenesis PCR array

HT 29 cells were treated with 1400W or a control for 24 h. RNA was then extracted from the cells and converted into complementary DNA using a cDNA synthesis kit (Invitrogen). The cDNAs were then subjected to a Human Angiogenesis RT^2 Profiler™ PCR Array (Qiagen). This array profiles the expression of 84 key genes involved in modulating the biological processes of angiogenesis, including growth factors and their receptors, chemokines and cytokines, matrix and adhesion molecules, and proteases and their inhibitors, as well as transcription factors.

2.6. Proteome profiler human angiogenesis array

HT 29 cells were treated with NOS inhibitors or a control for 24 h. Proteins were then extracted from cells using cell lysis buffer. Protein concentrations were analyzed by a BCA assay using a Pierce™ BCA Protein Assay Kit (Thermo Fisher). The proteins were then subjected to a proteome profiler angiogenesis array (R&D Systems) according to the manufacturer's instructions. Briefly, samples were mixed with a cocktail of biotinylated detection antibodies and then incubated with the array membrane, which was spotted in duplicate with capture antibodies to specific target proteins. Captured proteins were visualized using chemiluminescent detection reagents. The image quantification was performed by Image-J software (NIH, Bethesda, MD, USA).

2.7. Statistics

SPSS 23.0 statistical software was used for statistical analysis. The measurement data were expressed as mean ± standard deviation. Comparison between groups used analysis of variance, while two pairs of comparison used Student's *t*-test. When the variance was not uniform, comparisons were performed using a rank sum test. $P < 0.05$ for the difference was considered statistically significant.

3. Results

3.1. shRNA knockdown of iNOS or eNOS suppresses CRC cell proliferation

HT 29 and HCT 116 cells were transfected with iNOS shRNA, which led to significantly suppressed NO production in both iNOS-*KD* HT 29 and iNOS-*KD* HCT 116 cells compared to their corresponding controls (Fig. 1A). An MTS assay showed that iNOS-*KD* suppressed cell proliferation by ~30% in iNOS-*KD* HT 29 cells and ~50% in iNOS-*KD* HCT 116 cells compared to their corresponding controls (Fig. 1B).

Similarly, HT 29 and HCT 116 cells were also transfected with eNOS shRNA to create stable eNOS-*KD* cell lines. We observed significantly decreased NO production in both eNOS-*KD* HT 29 and HCT 116 cells (Fig. 1C), associated with suppressed cell proliferation by about 30% in eNOS-*KD* HT 29 cells and more than 50% in eNOS-*KD* HCT 116 cells (Fig. 1D).

3.2. NOS inhibitors (1400W and L-NIO) suppresses CRC cell proliferation and migration

After confirming that inhibition of NO production by NOS-*KD* could suppress colon cancer cell growth, we then tested whether NOS inhibitors (1400W and L-NIO) could also result in a similar anti-cancer

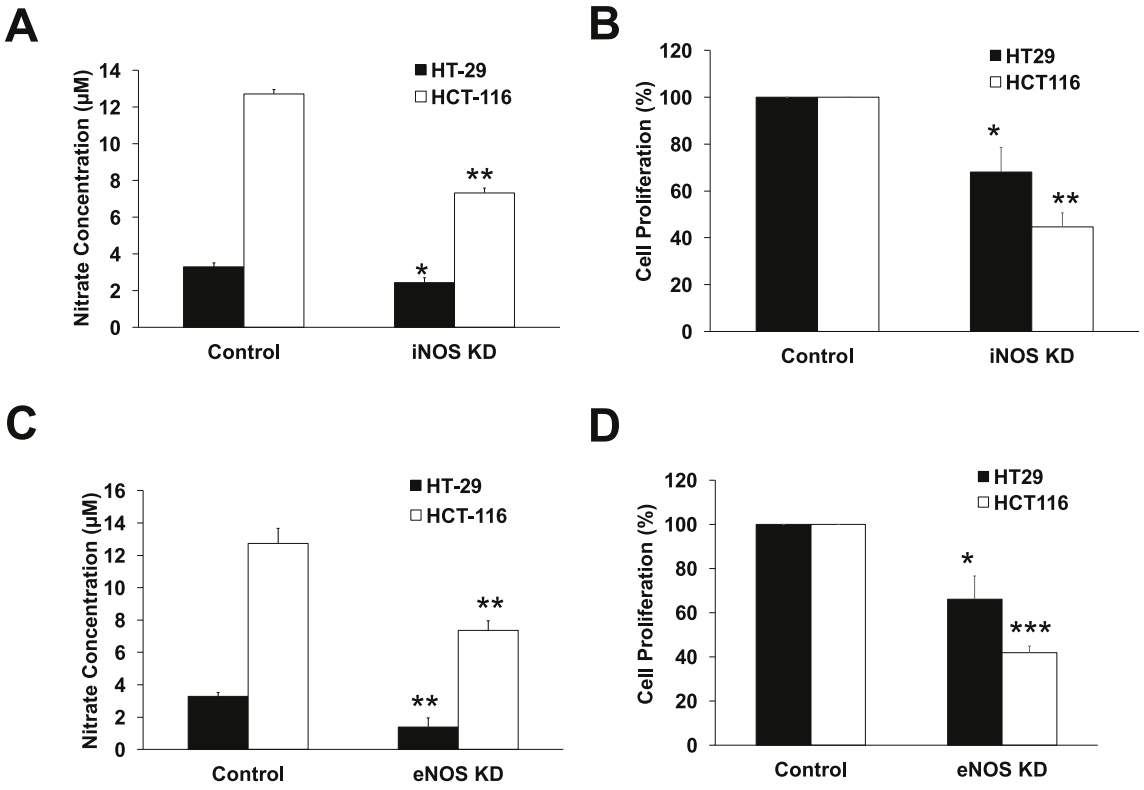

Fig. 1. Knockdown of iNOS or eNOS suppresses CRC cell proliferation. A: HT 29 and HCT 116 cells were treated with iNOS shRNA plasmids for 48 h, and the iNOS knockdown cell populations were selected with puromycin treatment for 3 days. The cells with stable knockdown of iNOS were then analyzed for NO production by NOS assay. B: MTS assay of iNOS knockdown HT 29 and HCT 116 cells. C: HT 29 and HCT 116 cells were treated with eNOS shRNA plasmids for 48 h, and the eNOS knockdown cell populations were selected with puromycin treatment for 3 days. The cells with stable knockdown of eNOS were then analyzed for NO production by NOS assay. D: MTS assay of eNOS knockdown HT 29 and HCT 116 cells. *: significant difference with $p < 0.05$, **: significant difference with $p < 0.01$, ***: significant difference with $p < 0.001$.

effect. A dosage-dependent inhibition of cell proliferation was observed when HT 29 and HCT 116 cells were treated with 1400W for 48 h at 1–4 mM (Fig. 2A). When the cells were treated with L-NIO, an eNOS inhibitor, a slight inhibition of HCT 116 cell growth were observed at 48 h, but no inhibition in HT 29 cell proliferation was detected (Fig. 2B).

A wound healing assay showed that 1400W treatment (0.1–1 mM) in HCT 116 cells resulted in larger remaining wound areas at 24 h compared to control cells (Fig. 2C and D). Similarly, we also observed larger remaining wound areas in L-NIO treated HCT 116 cells at 24 h compared to control cells (Fig. 2E).

3.3. 1400W and L-NIO inhibit key genes involved in modulating angiogenesis

A PCR array analysis was performed to test whether 1400W and L-NIO could affect angiogenesis in colon cancer cells. Data showed that 1400W systematically inhibits angiogenesis-related gene transcription in HT 29 cells. For example, among the 86 genes tested in the angiogenesis pathway, 1400W inhibits the gene transcription of a number of angiogenesis related growth factors and receptors, with the most affected genes being HGF and KDR (Fig. 3A). 1400W also inhibits many adhesion molecules, with COL4A3, THBS2, and ccl2 being the most affected genes (Fig. 3B). In addition, 1400W also inhibits many matrix proteins and transcription factors (Fig. 3C and D).

Similarly, L-NIO also inhibits gene transcription of various angiogenesis-related growth factors and receptors, adhesion molecules, matrix proteins and transcription factors, with the most affected genes being HGF, ccl11, ccl2, TIMP3, MMP9 and SPHK1. (Fig. 4A–D).

3.4. 1400W and L-NIO suppress human angiogenesis-related proteins

A proteome profiler angiogenesis array was used to analyze the effects of 1400W and L-NIO on the key molecules in the angiogenesis pathway at the protein level. The results showed that 1400W significantly down-regulated the protein expression levels of various important molecules in the angiogenesis pathway, including CXCL16, GDNF, GM-CSF, HB-EGF, Serpin B5 and uPA (Fig. 5A–B). In comparison, while L-NIO inhibited some of the same molecules, *e.g.,* Serpin B5 and uPA expression, it also down-regulated some other molecules such as amphiregulin and endothelin-1 (Fig. 5C).

3.5. NOS inhibitors improve 5-FU's efficacy in CRC cells

Colon cancer cells were treated with NOS inhibitors, 5-FU, or their combination for 24 h, and the cell proliferation was analyzed by the MTS assay. For HT 29 cells, the combination of 1400W and 5-FU resulted in enhanced effects (~40% growth inhibition) compared to 1400W alone (~15% inhibition) and 5-FU alone (~20%, Fig. 6A). For HCT 116 cells, the combination of 1400W and 5-FU resulted in ~60% growth inhibition compared to 1400W alone (~20% inhibition) and 5-FU alone (~40%, Fig. 6A). Similarly, the combination of L-NIO and 5-FU also resulted in improved anti-cancer effects in both HT 29 and HCT 116 cells compared to 1400W alone and 5-FU alone (Fig. 6B).

Consistent with the cell proliferation data, the wound healing assay demonstrated that a combination of NOS inhibitors and 5-FU led to improved anti-migration effects compared to NOS inhibitor treatment alone or 5-FU alone.

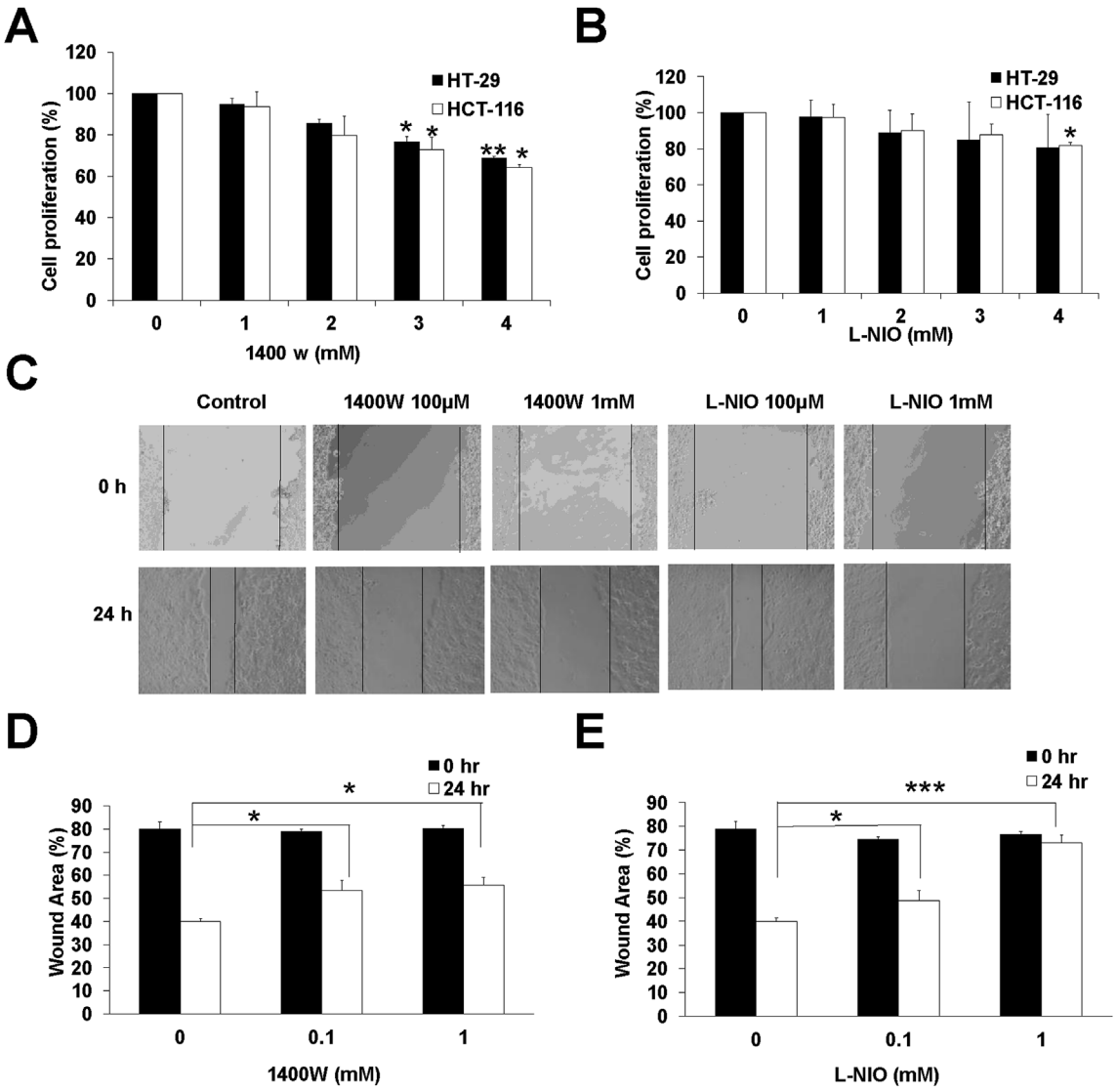

Fig. 2. 1400W and L-NIO suppress CRC cell proliferation and migration. A: CRC cells were treated with 1400W for 48 h at the indicated concentrations, followed by MTS assay. B: CRC cells were treated with L-NIO for 48 h at the indicated concentrations, followed by MTS assay. C: Wound healing assay of HCT 116 cells treated with 1400W or L-NIO at the indicated concentrations. Images of the scratch were taken at time 0 and 24 h later. D&E: Quantification of the wound healing assay for 1400W and L-NIO treated cells, respectively. *: significant difference with $p < 0.05$, **: significant difference with $p < 0.01$, ***: significant difference with $p < 0.001$.

4. Discussion and conclusion

In this study, we demonstrated that the NOS inhibitors 1400W and L-NIO suppress CRC cell proliferation and migration, which is associated with systematic inhibition of angiogenesis-related gene transcription and protein expression. The use of a combination of 1400W or L-NIO with 5-FU showed greatly enhanced anti-proliferative effects on CRC cell proliferation and cell migration.

We first confirmed that knockdown of iNOS or eNOS significantly impaired CRC cell proliferation and decreased the production of NO (Fig. 1). Then we tested and demonstrated that 1400W and L-NIO, two NOS inhibitors, also suppress growth and migration in CRC cells (Fig. 2). However, while 1400W led to a significant growth inhibition effect in both HT 29 and HCT 116 cells, L-NIO did not affect HT 29 cell growth and only showed a moderate inhibitory activity in HCT 116 cells. This observation may be caused by the difference in NOS inhibitory specificity/efficacy between 1400W and L-NIO [30,32].

We then conducted PCR arrays and proteome profiler angiogenesis arrays to test whether 1400W and L-NIO could affect the key molecules in the angiogenesis pathway at the gene and protein levels (Figs. 3–5). The results showed that both 1400W and L-NIO systemically inhibit angiogenesis-related gene transcription and protein levels, and the most affected genes include HGF, KDR, COL4A3, THBS2, ccl11, ccl2, TIMP3, MMP9 and SPHK1. Interestingly, while both compounds could inhibit some same target molecules, a different inhibitory specificity/efficacy was also observed from 1400W *vs.* L-NIO. In addition, from the PCR array test, despite systemic inhibition in angiogenesis-related gene transcription, we also observed that 1400W and L-NIO treatment resulted in a slight increase in certain genes (Figs. 3–4). Therefore, further investigation will be necessary to test whether and how these increases would contribute to angiogenesis.

Drug resistance is one of has been the major obstacles for cancer chemotherapy [34]. For example, insensitivity to 5-FU, a front-line chemo-drug for colon cancer treatment, has been reported in many colon cancer research studies [35–37]. Therefore, there is an urgent need to develop a combination treatment in order to improve 5-FU's efficacy. Here we have made the first attempt to test and demonstrate that the combination of 1400W (or L-NIO) and 5-FU resulted in

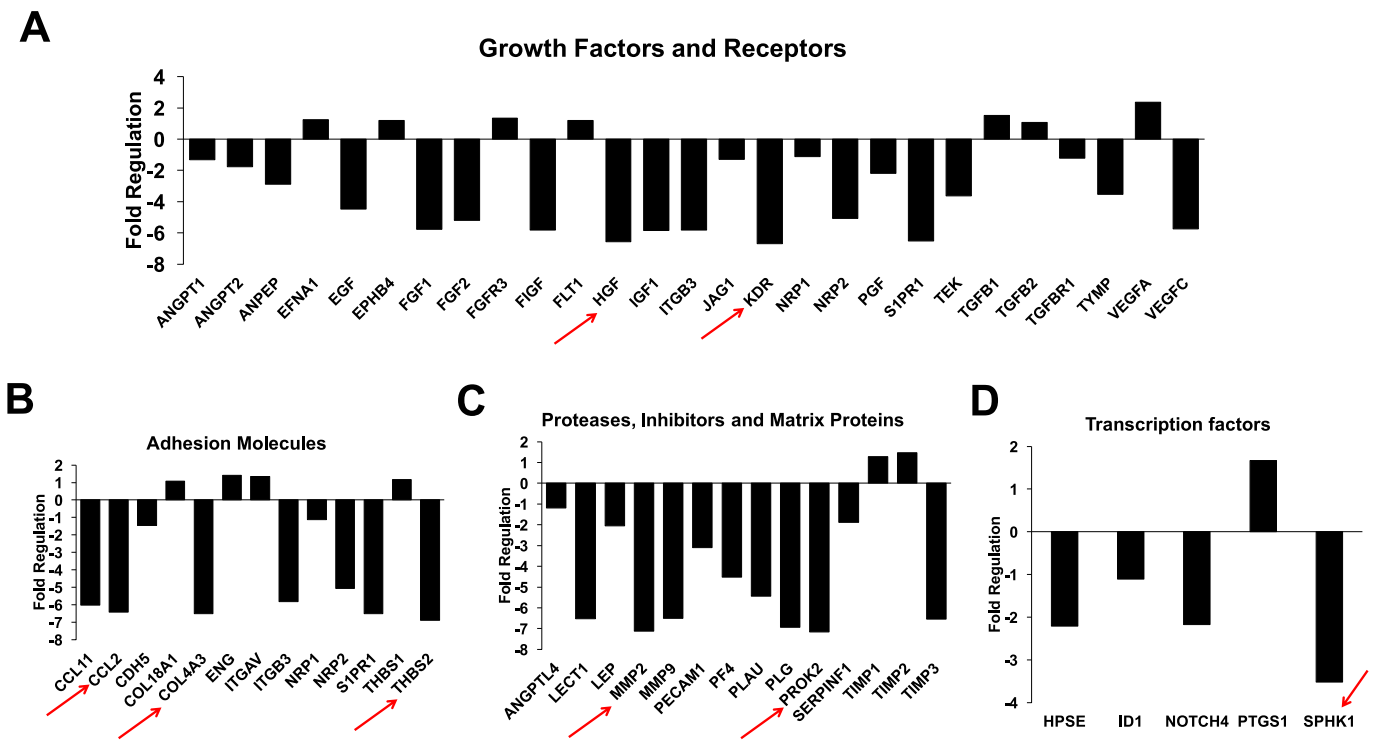

Fig. 3. 1400W inhibits key genes involved in modulating angiogenesis. HT 29 cells were treated with 1400W or control for 24 h. RNA was then extracted from cells and converted into complementary DNA using a cDNA synthesis kit. The cDNAs were then subjected to angiogenesis PCR array. Among the 86 genes involved in the angiogenesis pathway, 1400w inhibits A: the angiogenesis-related growth factors and receptors for gene transcription, with the most affected genes HGF and KDR. B: adhesion molecules, with COL4A3, THBS2, and ccl2 as the most affected genes. C: matrix proteins, with the PROK2 and MMP2 downregulated the most. D: transcription factors, with the SPHK1 gene transcription down-regulated the most.

enhanced anti-proliferation and anti-migration effects in CRC cells compared to 5-FU alone (Fig. 6). Our research outcome may guide us to develop a novel combination treatment strategy for colon cancer.

In conclusion, for the first time, we have investigated the effects and mechanism of 1400W and L-NIO on the angiogenesis pathway in CRC, and demonstrated that NOS inhibitors can be a promising anti-angiogenesis treatment strategy for cancer therapy. In our future study, we will investigate the mechanism related to the regulation of

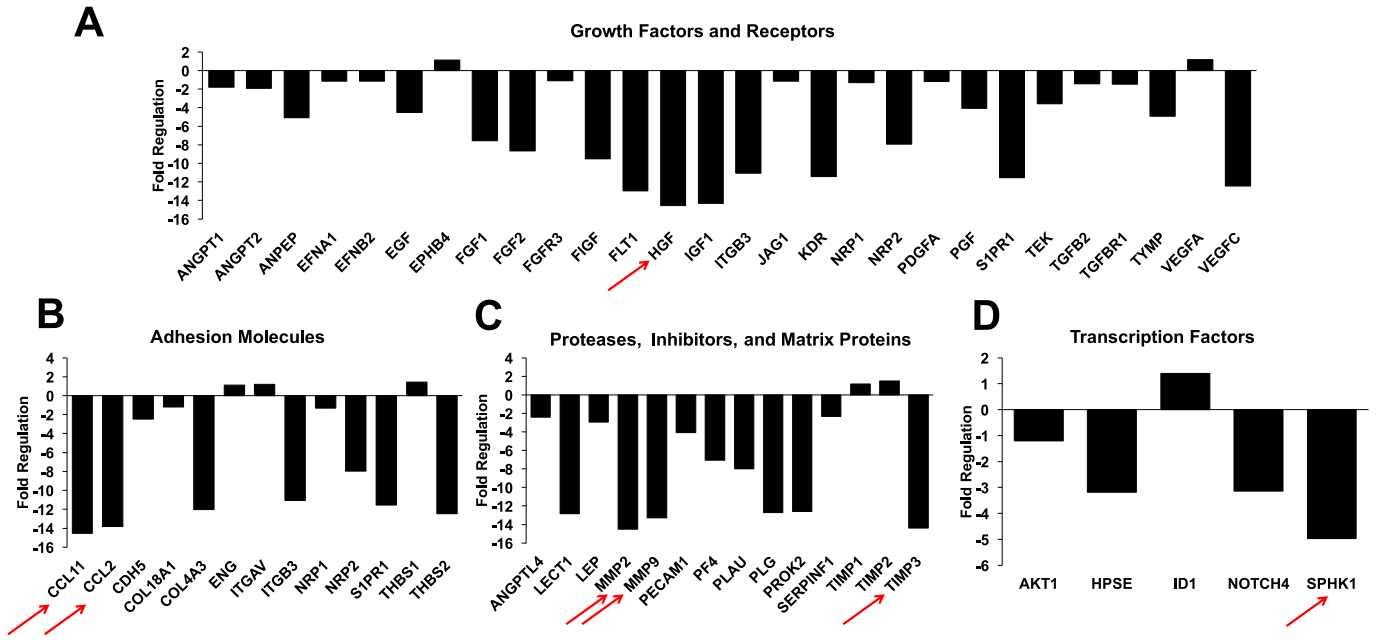

Fig. 4. L-NIO inhibits key genes involved in modulating angiogenesis. HT 29 cells were treated with L-NIO or control for 24 h. RNA was then extracted from cells and converted into complementary DNA using a cDNA synthesis kit. The cDNAs were then subjected to angiogenesis PCR array. Among the 86 genes involved in the angiogenesis pathway, L-NIO inhibits A: the angiogenesis-related growth factors and receptors gene transcription, with the most affected gene HGF. B: adhesion molecules, with ccl11 and ccl2 as the most affected genes. C: matrix proteins, with the TIMP3 and MMP9 downregulated the most. D: transcription factors, with the SPHK1 gene transcription down-regulated the most.

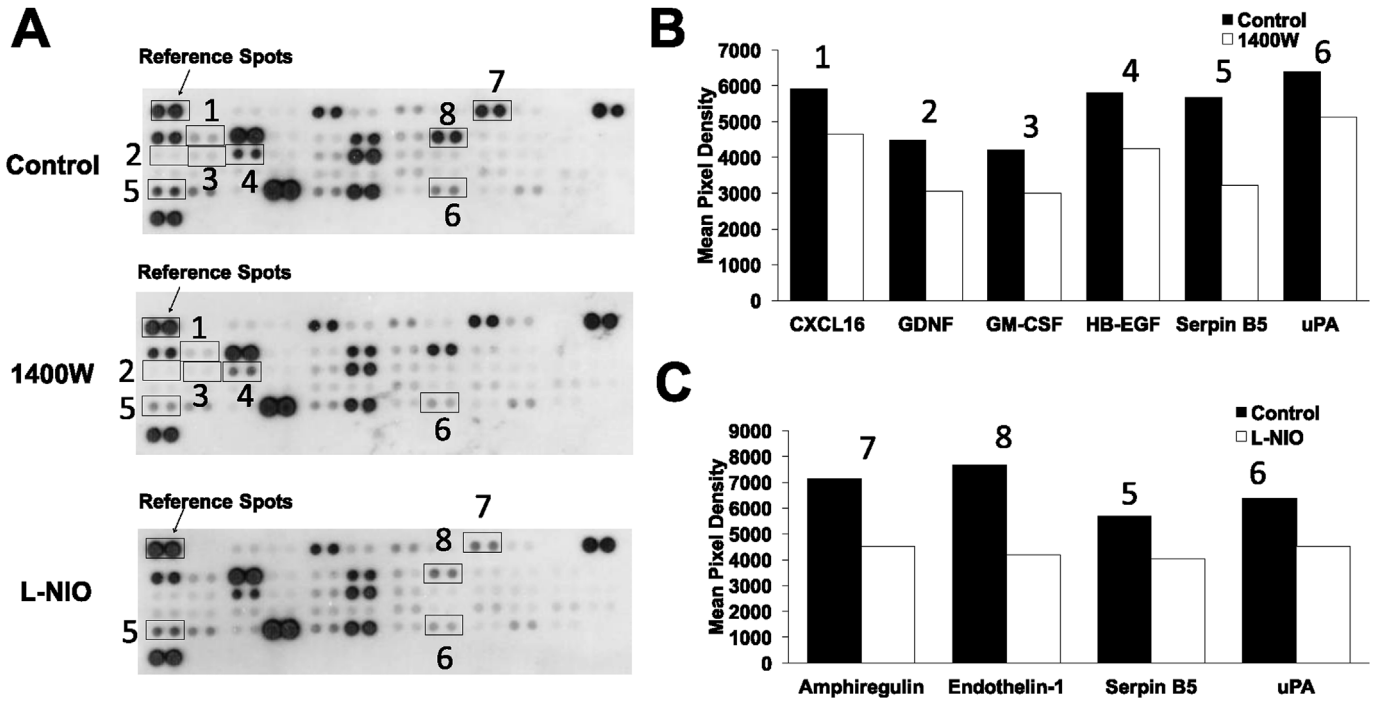

Fig. 5. 1400W and L-NIO suppress human angiogenesis-related proteins. HT 29 cells were treated with NOS inhibitors or control for 24 h. Proteins were then extracted from cells using cell lysis buffer. Protein concentrations were analyzed by BCA assay. The proteins were then subjected to proteome profiler angiogenesis array. A: the comparison between the membrane of the control and the NOS inhibitor-treated ones. B & C: The images from Fig. 5A were analyzed by ImageJ. The pixel intensity of each spot was analyzed and normalized to reference spots. The most affected proteins are shown here.

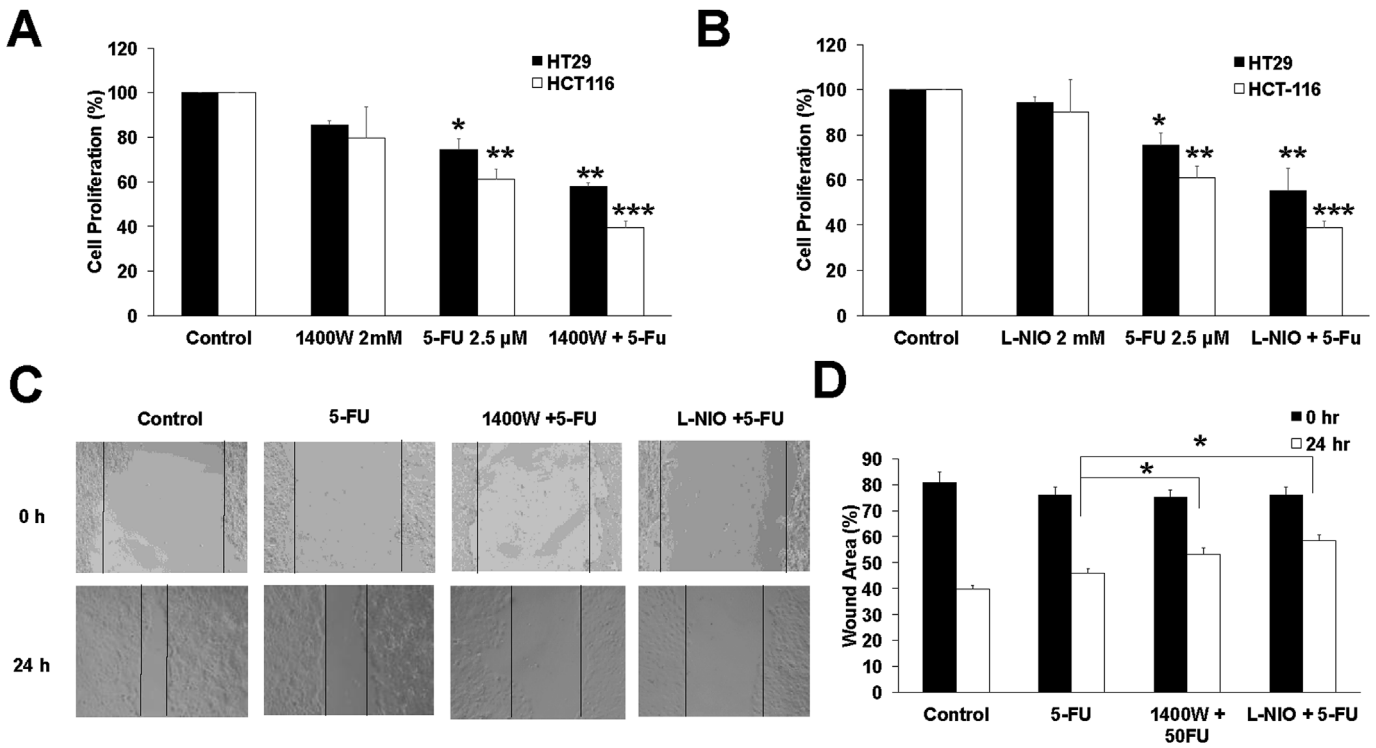

Fig. 6. NOS inhibitors improve 5-FU's efficacy in CRC cells. **A:** CRC cells were treated with 1400W and 5-FU or a combination for 24 h, and the cell proliferation was analyzed by MTS assay. **B:** CRC cells were treated with L-NIO and 5-FU or a combination for 24 h, and the cell proliferation was analyzed by MTS assay. **C:** HCT 116 cells were seeded into 24 well plates. The cell surface was scratched with a pipette tip. Images of the scratch were taken at time 0 and 24 h later. **D&E:** Quantification of the wound healing assay for 1400W- and L−NIO−treated cells respectively. *: significant difference with $p < 0.05$, **: significant difference with $p < 0.01$, ***: significant difference with $p < 0.001$.

angiogenesis-related genes and proteins by NOS inhibitors, and analyze the effects and mechanisms of these inhibitors on angiogenesis in a CRC xenograft mouse model. Our research outcome elaborates on the mechanisms and may guide us to develop a NOS inhibitor-based cancer treatment strategy.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Author contributions

Y Gao, S Zhou, S Qian, and X Huo conceived and designed the experiments. Y Gao and S Zhou performed the experiments. Y Gao, S Zhou, Y Xu, and S Sheng analyzed the data. Y Gao and X Huo contributed reagents/materials/analysis tools. Y Gao, S Zhou, Y Xu, S Qian and X Huo wrote the paper.

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