Artesunate-induced antagonism of fibrotic gene expression and attenuation of myofibroblast-associated phenotypes

MAJOR QUALIFYING PROJECT REPORT
SUBMITTED TO THE FACULTY OF
WORCESTER POLYTECHNIC INSTITUTE
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF BACHELOR OF SCIENCE

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April 26, 2018
Acknowledgements

I would like to acknowledge the following individuals and organizations that ensured the success of this project:

- David Dolivo for his mentorship throughout my four years of my undergraduate and his unwavering support and guidance throughout my MQP.
- Dr. Tanja Dominko for her support and guidance as my advisor.
- David Dolivo again for his assistance in the lab and for the development of the qRT-PCR primers used in this MQP.
- Dr. Pamela Weathers for the generously allowing me to use her artemunate stocks and for her expertise on Artemisia annua.
- My boyfriend and friends for listening my endless musing on my research and for their overwhelming support.
- My parents for their endless support throughout college.
# Table of Contents

**Acknowledgements** .................................................................................................................. 2

**List of Figures** .............................................................................................................................. 4

**List of Tables** ................................................................................................................................ 5

**Abstract** ......................................................................................................................................... 6

1. **Introduction** ............................................................................................................................... 7

   1.1 Wound and scar formation ........................................................................................................ 7

   1.2 Fibrosis as a systemic disease ................................................................................................. 7

   1.3 Attenuation of fibroblast activation ......................................................................................... 8

   1.4 Therapeutic potential of *Artemisia annua* ............................................................................ 9

   1.5 Proposed mechanism of Artesunate ....................................................................................... 10

2. **Materials and methods** ............................................................................................................. 11

   2.1 Antibodies ............................................................................................................................... 11

   2.2 Cell culture ............................................................................................................................. 11

   2.3 SDS-PAGE and western blotting ............................................................................................ 11

   2.4 Fluorescence microscopy and flow cytometry ........................................................................ 12

   2.5 RNA isolation and qRT-PCR .................................................................................................. 13

   2.6 Proliferation assay .................................................................................................................. 15

3. **Results** ....................................................................................................................................... 15

   3.1 Artesunate reduces α-SMA expression .................................................................................... 15

   3.2 Artesunate decreases ECM deposition .................................................................................... 16

   3.3 Artesunate downregulates transcription of profibrotic genes .................................................. 18

   3.4 Artesunate is associated with a decrease in proliferation ......................................................... 19

   3.5 Artesunate induces apoptosis in human dermal fibroblasts .................................................... 20

4. **Discussion** .................................................................................................................................. 22

**References** ..................................................................................................................................... 24
List of Figures

Figure 1: Recruitment of fibroblasts to wound site from multiple sources ........................................... 8
Figure 2: Fibroblast-to-myofibroblast transition and myofibroblast characteristics .................................. 9
Figure 3: Annexin V/PI flow cytometry ...................................................................................................... 13
Figure 4: Artesunate-induced reduction of α-SMA expression ................................................................. 16
Figure 5: Artesunate-induced decrease in ECM proteins .......................................................................... 17
Figure 6: Transcriptional analysis of fibrosis-associated genes ................................................................. 19
Figure 7: Analysis of proliferation in artesunate-treated human dermal fibroblasts ................................. 20
Figure 8: Live cell staining for apoptotic markers .................................................................................. 21
Figure 9: Flow cytometry quantification of annexin V/PI in human dermal fibroblasts .......................... 22
List of Tables

Table 1: Primer sequences for qRT-PCR........................................................................................................... 14
Abstract

Artemisinin has been shown to have numerous medicinal effects, most known being its antimalarial effects. Artesunate, artemether and other chemical analogs of artemisinin have also demonstrated cytostatic and cytotoxic effects in bacterial and cancer cells. Recently, artemisinin-derivative compounds have also been demonstrated to attenuate fibrotic pathologies in various preclinical animal models, including those of hepatic and pulmonary fibrosis, but the mechanisms by which this inhibition of fibrosis occurs is not well-understood. Given the ability of artesunate to antagonize various fibrotic pathologies, we decided to investigate the effects of artesunate on a process common to all fibrotic pathologies: the emergence of the myofibroblast. Artesunate treatment dose-dependently attenuated expression of α-SMA, a defining molecular marker of the myofibroblast, in human dermal fibroblasts and inhibited activation of fibroblasts mediated by the profibrotic cytokine TGF-β1, which is largely responsible for the emergence of the myofibroblast in vivo. Immunofluorescent analysis for collagen I and collagen III demonstrated a marked reduction of both basal and TGF-β1-induced expression of extracellular matrix-associated proteins. Live cell staining for Annexin V/Propidium iodide and proliferation assays suggested an apoptotic mechanism is occurring in artesunate-treated cells. qRT-PCR analysis demonstrated artesunate-mediated downregulation of profibrotic genes including canonical myofibroblast markers, ECM genes, and several TGF-β receptors and ligands, and upregulated expression of cell cycle inhibitors and matrix-metalloproteinases. Taken together, these data suggest that the antifibrotic mechanisms of artesunate in vivo may be mediated by its antagonistic effects towards myofibroblast phenotypes or its induction of myofibroblast apoptosis.
1. Introduction

1.1 Wound and scar formation

Wounds in the human body have two pathways for recovery: they can either regenerate completely, which results in restoration of functional tissue, or they can undergo fibrosis, which results in the formation of a scar (Stocum, 2012). Fibrosis is generally caused by improper deposition of collagen by myofibroblasts and can occur both internally or externally. This deposition of collagen and other extracellular matrix proteins results in reduced function or loss of function in that particular section of tissue due to the reduced number of functional tissue-specific cells (Stocum, 2012). For instance, in the skin, hair growth and ability to perspire at the location of a dermis-penetrating scar is significantly inhibited due to the loss of epidermal secondary structures like hair follicles and sweat glands, which can pose potential dangers for the victims of large burns.

1.2 Fibrosis as a ubiquitous pathology

Approximately 45% of deaths in the developed world can be attributed to fibrosis in some way, due to the wide variety of organs and tissues that can be affected by the loss of function that is a hallmark of fibrotic tissue (Wynn, 2007). Fibrosis is a disease that has the ability to impact many tissues in the body and can have devastating effects to the organism based on the location and severity of the fibrosis. Common models for research of this disease include pulmonary, cardiac, hepatic, renal and dermal fibrosis (Distler et al., 2007; Ito et al., 1998; Khalil et al., 2017; Li, Liu, Wang, Wang, & Chen, 2014; Roulot, Sevcsik, Coste, Strosberg, & Marullo, 1999). In this study, human dermal fibroblasts were selected as a preclinical model, as the skin is an accessible model in terms of physical accessibility for future preclinical studies and for topical treatments that bypass liver metabolism.
Figure 1: Recruitment of fibroblasts to wound site from multiple sources
Fibroblasts can be recruited to a wound site through migration of fibroblasts from other sources, including from differentiation of mesenchymal progenitors, or from epithelial-to-mesenchymal transition [adopted from (Dolivo, Larson, & Dominko, 2017)].

1.3 Attenuation of fibroblast activation

Fibrosis is the result of improper wound healing in any organ, in which fibroblasts and other myofibroblast progenitor cells undergo activation into myofibroblasts, due to the presence of activating molecules such as transforming growth factor-β (TGF-β) and mechanical tension (Darby, Laverdet, Bonté, & Desmoulière, 2014). Fibroblasts are recruited from various sources after an injury (Figure 1), and are activated via TGF-β, to become a myofibroblasts (Wynn, 2007). Myofibroblasts are responsible for the development of a scar, through their increased contractile activity and improper deposition of extracellular matrix proteins (Darby et al., 2014).
Fibroblast to myofibroblast transition is a key part of the profibrotic process (Figure 2).

Therefore this transition is a common proposed target for many antifibrotic therapies. To this day, however, few of these therapies exist and scarcely few have been approved.

![Figure 2: Fibroblast-to-myofibroblast transition and myofibroblast characteristics](image)

**Figure 2: Fibroblast-to-myofibroblast transition and myofibroblast characteristics**
Fibroblast-to-myofibroblast transition induced by TGF-β, resulting in increased presence of α-SMA-positive stress fibers, deposition of ECM, increased contractile activity and an enlarged cell body.

1.4 Therapeutic potential of Artemisia annua

From the eastern Asian medicinal plant *Artemisia annua*, artemisinin and a multitude of chemical derivatives of have been developed that have been shown to be effective against a wide variety of conditions, including malaria, cancer and inflammation (Das, 2015; Shi, Li, Yang, & Hou, 2015). Recently one of its derivatives, artesunate, has been shown in a variety of preclinical studies to act as an antifibrotic, reducing the presence of profibrotic molecules, including both
fibrosis-associated signaling and extracellular matrix molecules (Wang, Xuan, Yao, Huang, & Jin, 2015).

1.5 Proposed mechanisms of Artesunate

Artesunate, along with artemisinin’s other chemical derivatives, artemether, arteether and dihydroartemisinin, contains an endoperoxide bridge in its structure, which is key to its efficacy and functionality in malaria patients and cancer models (Das, 2015). In these pathologies, the compound undergoes a Fenton reaction, in which ferrous iron breaks the endoperoxide bridge, and produces and causes aggregation of reactive oxygen species, which results in death of afflicted cells (Shi et al., 2015). It has been theorized that artemisone induces apoptosis in myofibroblasts, as apoptosis-associated molecules such as Fas, FasL and Caspase-3 have been shown to be upregulated in artemisone-treated fibroblasts in vitro (Chang-ming, Hong-xiu, & Xiao-fei, 2011). Our lab theorizes that this same mechanism contributes to the efficacy of artemisone in the reduction of fibrotic gene expression and protein levels, as many previous studies have shown an increase in apoptosis and a reduction of cellular growth during artemisone treatment of preclinical animal models and fibroblasts (Chang-ming et al., 2011; Chen et al., 2016). However, the effects of artemisone on profibrotic gene expression, particularly in human dermal fibroblasts, are not well-studied. Thus, this major qualifying project was designed to better understand the mechanisms through which artemisone reduces myofibroblast phenotypes through investigation of proliferation, apoptosis, and expression of profibrotic and myofibroblast-associated genes.
2. Materials and Methods

2.1 Antibodies

The primary antibodies used were the following: sc-32251 α-SMA, sc-8654-R Histone H3, sc-8783 Collagen I, sc-59826 ED-A Fn, sc-271249 Collagen III (all from Santa Cruz Biotechnology). The secondary antibodies used were Alexafluor488-conjugated or Alexafluor568-conjugated (Invitrogen) for immunofluorescence and HRP-conjugated for Western blotting (Bio-Rad). The sc-4252 AK fluorescent annexin V (Santa Cruz) was also used.

2.2 Cell Culture

CRL-2097 and CRL-2352 human dermal fibroblasts were obtained from ATCC, and CT-1005 human dermal fibroblasts were obtained from the University of Massachusetts Medical School tissue distribution program in Worcester, MA. The cultures were all maintained with 1:1 DMEM:Ham’s F12 (Corning) supplemented with 4mM L-glutamine (Mediatech) and 10% Fetal Clone III (Hyclone) on Nunclon Delta tissue culture plastic (ThermoFisher). The cultures were incubated at 37°C, 19% O₂, 5% CO₂ until treatment with artesunate or human recombinant TGF-β1 (Peprotech). Artesunate was obtained at 100 mM concentration from Dr. Pamela Weathers. Cultures were treated with supplemented media one day after plating to allow for cells to adhere to the plate. Treated cultures were then moved to incubate at 37°C, 5% CO₂ 5% O₂ and were harvested after four days unless otherwise noted.

2.3 SDS-PAGE and Western Blotting

Samples were prepared by washing in DPBS and snap-freezing cell pellets in liquid nitrogen and storing at -80°C for use at time of experiment. Pellet were resuspended in protein lysis buffer and sonicated. A Bradford assay was performed to determine the protein concentration of each
sample. 5X lamelli dye was added to each sample and the samples were boiled at 100 °C for five minutes. Equal amounts of protein for each sample were separated on an SDS-PAGE gel and transferred onto a PVDF membrane (Millipore) in a semi-dry apparatus (GE Healthcare). The membrane was blocked in 5% fat-free dry milk in TBS-T buffer for one hour and incubated overnight in a pre-determined dilution of primary antibody in 1% fat-free dry milk in TBS-T. After washing, the membrane was incubated in a 1:5000 HRP-conjugated secondary antibody in the 1% fat-free dry milk/TBS-T solution for two hours. The membrane was washed three times with TBS-T, then visualized using a ChemiDoc XRS system (Bio-Rad) and developed with SuperSignal West Dura Extended Duration Substrate (ThermoFisher).

2.4 Florescence microscopy and flow cytometry

Cells were plated in VisiPlate 24-well plates (Perkin Elmer) and grown in the presence or absence of artesunate and TGF-β (Peprotech). The cells were washed with DPBS and fixed with ice-cold methanol at room temperature for 20 minutes with agitation then permeabilized with 1.5 M HCl for 30 minutes at RT with agitation. The cells were then blocked with 5% BSA in TBST for 30 minutes with agitation and treated with 250 µL primary antibody, diluted in 1xPBS-Tween overnight at 4C with agitation (dilution specific to primary antibody). Cells were then treated with 250 µL of 1:500 dilution of Alexa Fluro-conjugated secondary antibody in PBS-T for 30 minutes in the dark with agitation. Cells were then washed three times for five minutes with PBS-T then treated with 500ng/mL Hoechst 33342 in PBS-T (ThermoFisher) for 15 minutes. Cells were washed an additional two times for five minutes with PBS then imaged on Axiovert (Zeiss) with identical exposure times and setting between control and treatment. Alternatively, cells were stained for FITC-annexin V/PI according to manufacturer’s protocols
and analyzed on an Axiovert 200M or an Accuri C6 flow cytometer (BD Biosciences) (Figure 2).

**Figure 3: Annexin V/Propidium iodide flow cytometry**
Figure shows a healthy fibroblast, compared to a fibroblast with increasing levels of fluorescent Annexin V-conjugated to FITC bound to the phosphatidylserine localized on the surface, characteristic of apoptotic cells. The propidium iodide can enter the nucleus of dead cells, fluorescing red and increasing along the x-axis.

2.5 RNA isolation and qRT-PCR

Samples were prepared by washing pellets in DPBS and snap-freezing cell pellets in liquid nitrogen and stored at -80°C until time of the experiment. RNA was isolated using the E.Z.N.A. RNA Isolation Kit (Omega Bio-Tek) and the concentration of the isolated RNA was measured.
using the Nanodrop 2000 (Thermo Scientific). The RNA was then converted to cDNA in 20 uL reactions using SuperScript VILO Master Mix (ThermoFisher). After conversion to cDNA, the samples were processed in 25 uL PCR reactions with PowerUP SYBR Master Mix Reagent and primers from Table 1. The PCR reactions were performed using the following procedure: 50C for 2 minutes, 95C for two minutes, then 40 cycles of denaturing at 95C for 15 seconds and anneal/extend at 60C for 1 minute.

Table 1: Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein encoded</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
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<tr>
<td>ACTA2</td>
<td>α-SMA</td>
<td>ACTGCTTGGTGTGATGCAAA</td>
<td>CACCACCCCCTGATGTC</td>
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</tr>
<tr>
<td>CNN1</td>
<td>Calponin</td>
<td>AGTGAAGAAGCTGGGCC</td>
<td>GAGGCCGCATGAAATGTC</td>
<td>113</td>
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<tr>
<td>TALGN</td>
<td>SM22α</td>
<td>CACAAGGGTGTGTAAGGTTG</td>
<td>GGCTCATGCTAGGAAGGAC</td>
<td>132</td>
</tr>
<tr>
<td>CCN2</td>
<td>CTGF</td>
<td>GTGCTGTCATTACAACCTGTC</td>
<td>TCTCACTCTGATCAGC</td>
<td>98</td>
</tr>
<tr>
<td>ED-A Fn</td>
<td>Fibronectin (ED-A)</td>
<td>CAGTGGAGATGTTAGTTAGTC</td>
<td>GTGACCTGAGTAACCTCAGG</td>
<td>119</td>
</tr>
<tr>
<td>Col1A1</td>
<td>Collagen 1 (α1)</td>
<td>GTCAGGCTGGTGATGGGG</td>
<td>GCCTGTTCACCTGTCGC</td>
<td>182</td>
</tr>
<tr>
<td>Col1A2</td>
<td>Collagen 1 (α2)</td>
<td>CTGGAGGGCTGGCTGACTGCT</td>
<td>AGCACCAGAGACCTGAGG</td>
<td>62</td>
</tr>
<tr>
<td>Col3A1</td>
<td>Collagen III (α1)</td>
<td>GGACACAGAGCTCAGTGGG</td>
<td>GATAAAGTATAGATAGGTT</td>
<td>190</td>
</tr>
<tr>
<td>MMP1</td>
<td>Matrix metalloproteinase 1</td>
<td>GCATATCGATGCTGCTTTC</td>
<td>GATAACCTGATAGGTT</td>
<td>110</td>
</tr>
<tr>
<td>MMP3</td>
<td>Matrix metalloproteinase 3</td>
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</tr>
<tr>
<td>MYOCDC</td>
<td>Myocardin</td>
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<td>GGGTCTTACCTGAGTCC</td>
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<tr>
<td>TGFB1</td>
<td>TGF-81</td>
<td>CATTGCTGAAATCTGCTG</td>
<td>TGACACTCACCATTGTTTTTC</td>
<td>110</td>
</tr>
<tr>
<td>TGFB2</td>
<td>TGF-82</td>
<td>GAGCCAGAAGACTGACTACG</td>
<td>TGTAACAAGCTGAGCACAGA</td>
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<tr>
<td>TGFBR1</td>
<td>TGF-8R1</td>
<td>GCAACTTACGCCATGCAATGAG</td>
<td>AGAACCTCAGCAGCATGTG</td>
<td>104</td>
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<tr>
<td>TGFBR2</td>
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<td>TGGACCTACTGCTGCTG</td>
<td>CTGAGCCATGATCTGCA</td>
<td>72</td>
</tr>
<tr>
<td>TGFBR3</td>
<td>Betaglycan</td>
<td>CGGTCTGTCCTGAGACATG</td>
<td>GAGCCAGCAACCTGAGCA</td>
<td>79</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>p21</td>
<td>CACTGCTTGGTACCATGAGG</td>
<td>TCTTCATGAGAGATGAGC</td>
<td>148</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>p16</td>
<td>GAGACCTACGCCCTAAGC</td>
<td>AGTGTGACTCAAGAAGGACC</td>
<td>139</td>
</tr>
</tbody>
</table>
2.6 Proliferation assay

Sample were prepared seeding CRL-2097 human dermal fibroblasts at a density of 2000 cells/well in a tissue culture-treated 96 well plate and treated for four days prior to the assay. For the resazurin assay, media was aspirated and 100 µL of fresh media was added to each well. Twenty microliters of 6x (0.15 mg/mL) resazurin solution was added to each well. Cells were incubated for two hours at 37°C and the fluorescence was read at excitation/emission of 544nm/590nm on a Victor 1420 multilabel counter (Perkin Elmer). Proliferation rates were estimated by averaging replicates and calculating relative fluorescence of treated condition to the control.

3. Results

3.1 Artesunate is effective in reducing α-SMA in human dermal fibroblasts and can antagonize TGF-β-induced fibroblast activation

As smooth muscle alpha actin (α-SMA) is one of the key functional markers that defines myofibroblast phenotypic identity (Figure 1), this was the chosen target to determine whether artesunate was able to antagonize profibrotic phenotypes in human dermal fibroblasts.

Artesunate did indeed dose-dependently decrease protein expression of α-SMA, when compared to α-SMA expression of the negative control and TGF-β positive control (Figure 4a, 4b). The 50 µM concentration of artesunate was chosen for use moving forward. To confirm that this effect
was not idiosyncratic and did not affect only the CRL-2097 human dermal fibroblast line, two other lines of human dermal fibroblasts, CRL-2352 and CT-1005, were also examined and showed qualitatively similar decreases α-SMA protein expression when treated with artesunate (Figure 4b). Each cell line was co-treated with artesunate and TGF-β, and artesunate successfully antagonized TGF-β-mediated α-SMA expression as well. Immunofluorescent analysis of CRL-2097 fibroblasts for α-SMA showed a decrease in α-SMA protein expression in the artesunate-treated fibroblasts. Together, these data suggest that artesunate has the ability to antagonize fibroblast activation by TGF-β in multiple fibroblast lines, due to its ability to antagonize α-SMA expression.

**Figure 4: Artesunate-induced reduction of α-SMA expression**
(a) CRL-2097 human dermal fibroblasts were cultured under control conditions or in the presence of indicated concentration of artesunate and analyzed at day 4. Protein lysates were examined via Western blotting for α-SMA and with Histone H3 as a loading control. (b) CRL-2097, CRL-2352 and CT-1005 human dermal fibroblasts were cultured under control conditions or in the presence of 50 μM artesunate and/or 10 ng/mL TGF-β1, and analyzed at day 4. Protein lysates were examined via Western blotting for α-SMA and with Histone H3 as a loading control.

### 3.2 Artesunate qualitatively reduces expression of extracellular matrix proteins

Another hallmark of myofibroblasts and a key indicator of fibrosis is the accumulation of extracellular matrix proteins, such as collagen I, collagen III and ED-A fibronectin (Figure 1). Treatment of human dermal fibroblasts with artesunate results in a substantial decrease that was
observed in both collagen I and collagen III protein expression between the untreated fibroblasts, TGF-β-treated positive control and the artesunate-treated cells. In the co-treatments of artemesunate and TGF-β, both collagen I and collagen III expression is attenuated, when compared to control fibroblasts (Figure 5b, 5c), which suggests that artemesunate can antagonize TGF-β-mediated increases in collagen expression. There was no change observed in ED-A Fn expression (Figure 5d). Taken together, it can be determined that artemesunate decreases transition of fibroblasts to myofibroblasts, as it attenuates collagen I and collagen III extracellular matrix deposition.

Figure 5: Artesunate-induced decrease in extracellular matrix-associated proteins
CRL-2097 human dermal fibroblasts were cultured in control conditions or in the presence of 50 μM artemesunate, 10 ng/mL TGF-β 1 or both, and analyzed at day 4. Fibroblasts were fix and stained for (a) α-SMA, (b) Collagen I, (c) Collagen III or (d) ED-A Fibronectin. DNA was stained with Hoechst. Scale bar = 100μm.
3.3 Artesunate downregulates transcription of fibrosis-associated genes

To confirm that the reduction of fibrosis-associated proteins resulting from downregulation of gene expression at the transcriptional level, qRT-PCR was performed. It was determined that many of the genes that encode fibrosis-associated proteins were downregulated (Figure 6). This includes several major hallmark proteins of myofibroblasts, including ACTA2, TAGLN, MYOCD and CNN1, which encode α-SMA, SM22α, pro-myogenic transcription factor myocardin and calponin, respectively. Extracellular matrix genes, such as a COL1A1, COL1A2 and COL3A1 were found to be downregulated, along with the significant upregulation of several genes associated with the degradation of fibrillar collagens, MMP1 and MMP3 (Figure 6). A significant downregulation was also detected in the gene encoding major profibrotic inflammatory cytokine IL-6, profibrotic CCN2, which encodes CTGF, and in ITGA11, an integrin that has been shown to be necessary for fibroblast activation (Rodriguez et al., 2009), (Bansal et al., 2017). Pro-myofibroblastic transcription factor SRF is also downregulated significantly, as it has been shown that inhibition of these protein is effective in reducing scarring in preclinical models (Yu-Wai-Man et al., 2017). Several genes encoding profibrotic TGF-β ligands and receptors are also downregulated, including TGFB2 and TGFBRI. Taken together, these data suggest that artesunate treatment antagonizes profibrotic gene expression, inhibits fibroblast activation, as well as increases expression of cell cycle inhibitors.
Figure 6: Transcriptional analysis of fibrosis-associated genes
CRL-2097 human dermal fibroblasts were cultured in control conditions or in the presence of 50μM artesunate and harvested at day 4. Gene expression levels of artesunate-treated fibroblasts were determined relative to that of control fibroblasts using qRT-PCR. GAPDH was used as an internal control. Fold change was calculated using the ∆∆Ct method (Livak & Schmittgen, 2001). Statistical significance was determined by a two-tailed t-test per gene. n=4-5. Error bars=standard deviation. *p<.05, **p<.01, ***p<.001, ****p<.0001.

3.4 Artesunate is associated with a decrease in proliferation
A previous report detailed artesunate-induced apoptosis in human embryonic lung fibroblasts (Chang-ming et al., 2011). This report, paired with observation of reduced cell density of artesunate-treated fibroblasts compared to control fibroblasts (Figure 5) and a significant upregulation in cell cycle inhibitor genes CDKN1A and CDKN2A, suggested that artesunate may
impact fibroblast proliferation. Considering these data, a resazurin metabolic assay to measure proliferation confirmed that there is a significant decreased cellular metabolism in the artesunate-treated fibroblasts (Figure 7). Taken together, these data suggest artesunate-mediated inhibition of growth, based on the upregulation of key cell cycle inhibitors and the overall decreased growth.

![Graph showing proliferation](image)

**Figure 7: Analysis of proliferation in artesunate-treated human dermal fibroblasts**
CRL-2097 human dermal fibroblasts were cultured in control conditions or in the presence of 50μM artesunate and assayed at day 4. Samples were assayed in each respective well with resazurin solution. Fluorescence was read at excitation/emission of 544nm/590nm. Proliferation rates were estimated by averaging replicates and calculating relative fluorescence of each condition to the control. Error bars = standard deviation. Statistical significance was determined with student’s t-test. N = 4 biological replicates/condition, **** = p<0.0001

### 3.5 Artesunate induces apoptosis in human dermal fibroblasts

Considering the previous data and the hypothesis of an apoptotic-mediated mechanism, the next logical step was to measure fibroblast apoptosis induced by artesunate treatment. Live cell fluorescent imaging for Annexin V/PI qualitatively confirmed an increase in apoptotic death in
artesunate-treated fibroblasts compared to control fibroblasts (Figure 8). It should also be noted that in the phase view of the fluorescence images, there is a notable reduction in cell density from the artesunate-treated fibroblasts, as compared to the control fibroblasts.

![Image of fluorescence images showing live-cell staining for apoptotic markers.](image)

**Figure 8: Live-cell staining for apoptotic markers**
CRL-2097 human dermal fibroblasts were cultured in control conditions or in the presence of 50μM artesunate and assayed at day 4. Samples were live-stained with FITC-Annexin V/Propidium iodide and imaged on the Axiovert 200M (Zeiss).

Propidium iodide and annexin V-FITC fluorescence were quantified by flow cytometry, which showed that artesunate-treated cells were had significantly higher median PI and annexin V fluorescence compared to control cells (Figure 9), suggesting that the cell death induced by artesunate was mediated at least in part by apoptotic mechanisms. Apoptotic fibroblasts treated with the highly cytotoxic chemotherapeutic agent vinblastine sulfate were used as a positive control. Considering these data, it has been shown that artesunate effectively reduces proliferation of profibrotic myofibroblasts and induces apoptosis of myofibroblasts.
Figure 9: Flow cytometry quantification of annexin V/PI in human dermal fibroblasts
Samples were assayed for Annexin V/Propidium iodide flow cytometry and the median fluorescence relative to the control was calculated. Error bars = standard deviation. Statistical significance was determined with an ANOVA analysis and post-hoc Tukey’s test. n = 2 biological replicates/condition. **p<.01, ****p<.0001.

4. Discussion
The development and presence of myofibroblasts is a hallmark of fibrosis in all tissues, and represents a potential therapeutic target for fibrosis-associated diseases and prevention of scarring. These data demonstrate that artesunate targets myofibroblasts by antagonizing fibroblast activation and deposition of types I and III collagen, resulting a decrease in the expression of canonical myofibroblast marker α-SMA (Figure 4-5). Artesunate treatment also results in decreased profibrotic gene expression, associated with downregulation of myofibroblast markers, and other profibrotic genes, including genes associated with TGF-β signaling, a central regulator of fibrosis. Among other target genes, TGF-β induces expression of type 1 collagen (Ignotz & Massague, 1986) and inhibits expression of MMP1 (White, Mitchell,
& Brinckerhoff, 2000) and MMP3 in fibroblasts (Kerr, Miller, & Matrisian, 1990), whereas these data demonstrate that artesunate downregulates type 1 collagen and upregulates MMP1 and MMP3. Paired with the qRT-PCR data that suggest artesunate downregulates several TGF-β receptors and ligands, these data suggest that artesunate may inhibit TGF-β activity, which is significant, as TGF-β can be responsible for the fibroblast-to-myofibroblast transition (Meng, Nikolic-Paterson, & Lan, 2016).

These data also demonstrate that artesunate increases expression of cell cycle inhibitor genes, (Figure 6) reduces proliferation (Figure 7), and induces apoptosis (Figure 8, Figure 9) in fibroblasts. Previous literature has shown that artesunate increases p53 expression in rat primary hepatic stellate cells, known myofibroblast progenitors in liver fibrosis, resulting in the induction of growth arrest and apoptosis (Longxi, Buwu, Yuan, & Sinan, 2011). Other studies propose inhibition of the PI3K/Akt pathway as a possible means of decreasing proliferation in airway smooth muscle cells and murine lung tissue (Cheng et al., 2011; Tan et al., 2014). Taken together these data suggest that the antifibrotic effects of pharmacologic artesunate likely proceed at least in part via downregulation of profibrotic genes in myofibroblasts and myofibroblast progenitors, as well as via induction of myofibroblast apoptosis.
References


