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Determination of Anti-Fibrotic Effects of Possible Scar-Collagen Antagonists on TGF-β_1 Treated Dermal Fibroblasts

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Abstract

When cells are wounded, functionality is decreased due to collagen being produced at high levels. The accumulation of collagen is called scarring or fibrosis. To simulate collagen levels during scarring primary dermal fibroblasts were treated with TGF-β_1 before the addition of test compounds. Transforming growth factor beta I (TGF-β_1), a cytokine, is a known inducer of scar collagen production. Anti-fibrotic effects of test compounds were screened and evaluated *in vitro* by measuring amounts of collagen produced at the protein level. To measure secreted collagen as part of the extra-cellular matrix monoclonal antibodies against collagen I and III were used. Collagen I is the most abundant type in the human body. II is present along with type I in connective tissue, blood vessels, and skin. Anti-fibrotic effects were measured in a fluorescent microplate assay for secreted collagen I and III and confirmed by confocal microscopy for collagen I. MTT assays confirmed anti-collagen effects from the test compounds were not due to decreased cell viability. From the results of these tests epigallocatechin gallate (EGCG) and nintedanib show promising decreases in collagen production in comparison to the control, illustrating the usefulness of the assay for screening scar antagonists.

Keywords: collagen, fibrosis, scarring

1. Introduction

During wound healing damaged cells and tissues can either be replaced through regeneration or covered by a scar. Fetal development tissue regeneration is observed whereas adult organs form scar tissue in response to damage. Regeneration is the best way to repair damage, but regrowth requires too much time and the fastest and most efficient repair is a scar. Scarring, also called fibrosis, is a major unresolved problem because though it reduces infection, scar tissue can only cover a damaged area and not restore functionality to an organ. The key signaling molecule used to trigger scarring is the cytokine, transforming growth factor beta I (TGF-β_1), which stimulates the production of collagen (especially type I collagen) by mesenchymal cells (Branton, 1999, & Pietra et al., 2015). An assay was developed by treating normal human dermal fibroblasts with TGF-β_1 to induce high levels of collagen expression. This yielded an *in vitro* scar that was used to screen compounds that can alter levels of collagen. A collagen antagonist able to reduce collagen protein levels to near zero would be the ideal anti-scarring compound.

Several compounds have been identified as fibrosis antagonists; six were used in the assay. All the compounds caused responses from the tissues tested in the assay. The relative efficacy of these compounds were measured in our screening assay, and two, Epigallocatechin-3-gallate and nintedanib, were most effective. Epigallocatechin-3-gallate, commonly abbreviated as EGCG, is a polyphenol from green tea. Hashem et al. (2008) showed reduction of type I collagen and procollagen synthesis at 100µM through ELISA of normal human dermal fibroblasts. Nakamuta et al. (2005) reported that EGCG (50 µM) suppressed type I collagen production in rat hepatic stellate cells (HSCs). Syed et al. (2013) had evidence that EGCG reduced collagen I and III production of human keloid tumors. Nintedanib(BIBF-1000) is a small molecule inhibitor preventing protein synthesis. It targets basic fibroblast growth

factor. As seen in Chaudhary et al. (2007) by an *ex vivo* investigation on the gene level of rats with lung fibrosis, nintedanib can have therapeutic effects to reduce scarring. Dexamethasone (DEX) is an anti-inflammatory steroid, Syed et al. (2013) showed decreased collagen I and III at mRNA levels but not protein level when human keloids were treated with DEX. Resveratrol, like EGCG, is a polyphenol. It is derived from plants which produce it in response to stress and injury (Higdon et al., 2015). Hashem et al. (2008) showed a reduction of collagen synthesis of cells treated with 100μM resveratrol. Lactic acid is produced by the body during stress when oxygen levels are low, Hashem et al. (2008) also saw reduction of collagen synthesis by fibroblasts when treated with 100μM lactic acid. Hydrocortisone is an anti-inflammatory steroid hormone. Its immunosuppressive nature made it a logical candidate for having possible anti-fibrotic effects.

2. Methodology

2.1 Cell Culture:

All experiments were done using cultured human derived primary dermal fibroblasts from a normal skin biopsy acquired from a local hospital.

2.2 Compounds:

Nintedanib (Apex Bio Teach cat no. A8252) dissolved in dH_2O to 1, 3, 5, 10, 25, 50 µM
Epigallocatechin-3-gallate (Sigma cat no. 93894) dissolved in DMSO to 5, 10, 25, 50, 100, 250 µM
Dexamethasone (Sigma cat no. D4902) dissolved in DMSO 5, 10, 25, 50, 100, 250 µM
Resveratrol (Sigma cat no. R5010) dissolved in dH_2O to 5, 10, 25, 50, 100, 250 µM
Lactic Acid dissolved in dH_2O 5, 10, 25, 50, 100, 250 µM
Hydrocortisone (Sigma cat no. H088-1G) dissolved in dH_2O to 5, 10, 25, 50, 100, 250 µM

2.3 Immunohistochemistry And Microscopy:

TGF-β_1 assay for increased collagen-I protein - Dermal fibroblasts were treated with TGF-β_1 at 50ng/mL and incubated at 37°C 5% CO_2 for one week. They were then fixed with 50% acetone/50% methanol and 0.2% triton x-100. A monoclonal antibody 1:200 with normal antibody diluent for Col-1(Thermo prod. #MAI-83073) was added and incubated at 4°C for two days. A secondary antibody 1:100 GαM 488 was added and incubated at 4°C overnight. 4',6-diamido-2-phenylindole (DAPI) mountant to stain cell nuclei was added. Microscopy was performed using a Leica inverted fluorescent microscope.

High through-put microplate assay- cells were seeded in a 96-well Costar 3300 plate containing Corning cell bind surface. All cells were treated with 50ng/mL TGF-β_1 for three days and then the six individual compounds: (1) 1, 3, 5, 10, 25, and 50 µM nintedanib (Apex Bio Teach cat no. A8252) dissolved in dH_2O, (2) 5, 10, 25, 50, 100, and 250 µM epigallocatechin-3-gallate (Sigma cat no. 93894) dissolved in DMSO, (3) 5, 10, 25, 50, 100, and 250 µM dexamethasone (Sigma cat no. D4902) dissolved in DMSO, (4) 5, 10, 25, 50, 100, and 250 µM resveratrol (Sigma cat no. R5010) dissolved in dH_2O, (5) 5, 10, 25, 50, 100, and 250 µM lactic Acid dissolved in dH_2O, (6) 5, 10, 25, 50, 100, and 250 µM hydrocortisone (Sigma cat no. H088-1G) dissolved in dH_2O, were added in triplicate at each concentration. 1:200 monoclonal antibody with normal antibody diluent for Col-1 and collagen III were added for two days. A secondary antibody 1:100 GαM 594 was added and incubated at 4°C overnight. Collagen protein levels were determined using a BMG Fluostar Omega fluorescent microplate reader.

2.4 MTT assay:

Cells were seeded into a 96-well Costar 3300 plate containing a Corning cell bind surface. 50µM and 100µM epigallocatechin-3-gallate (EGCG), and 25µM and 50µM nintedanib were added to five wells each and then allowed to incubate at 37°C for 8 days. 5mg/mL MTT was added to each well and incubated at 37° for 3hrs. The MTT was aspirated and MTT solvent (acidified isopropanol) and DMSO were added for 15min at room temperature. The MTT was solubilized and then placed in a plate reader and read at 595nm.

3. Data

To approximate scar-tissue formation, human dermal fibroblasts were plated in dishes and collagen levels were monitored through immunohistochemistry and background levels of col-I were reduced (Tacdol, 2013). The immunohistochemistry of collagen-I protein levels and DAPI staining were captured by inverted fluorescent microscopy. The staining of nuclei was used as a visual control to demonstrate consistent cellular confluency and that differences in collagen-I expression were due to TGF-β_1. Figure 1A and 1C demonstrate uniformity of cell density. The increase in col-I production attributable to TGF-β_1 (Figure 1D compared to 1B) is the basis for the assay of an *in vitro* scar, because in organisms collagen levels are responsive to TGF-β_1 concentration during would healing (Branton, 1999). The stated conditions were utilized throughout this investigation.

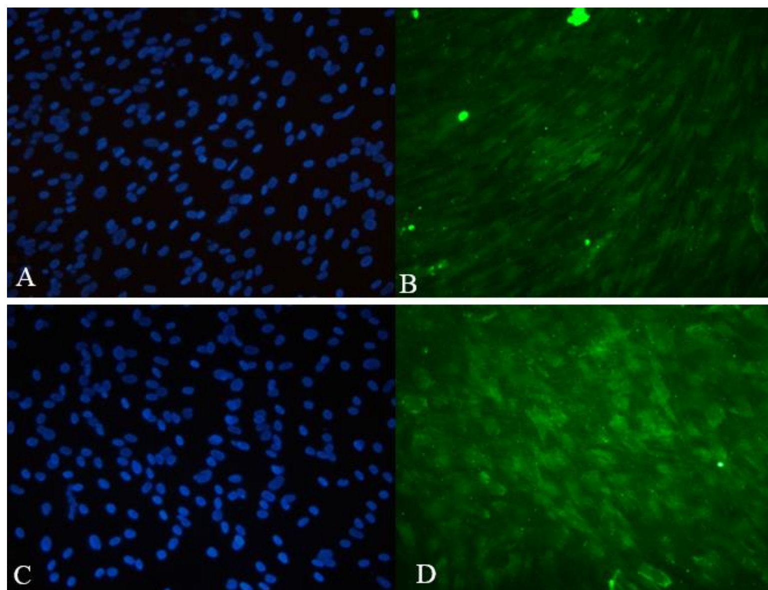

Figure 1. TGF-β_1 treatment increases collagen-I protein expression. A and C are DAPI stained showing nuclei (blue). B and D show immunohistochemistry on dermal fibroblasts treated with primary monoclonal antibody and secondary antibody GαM 488. The amount of green is proportional to the amount of collagen-I protein. B is not treated with TGF-β_1, D is.

Compounds with anti-fibrotic effects were identified through a literature review. The compounds were resveratrol, lactic acid, hydrocortisone, epigallocatechin-3-gallate, dexamethasone, and nintedanib. The conditions used to create high collagen-I production were adapted to a 96-well high through-put microplate assay, with TGF-β_1 induced collagen present in all wells. Resveratrol, lactic acid, hydrocortisone, EGCG, DEX were screened at six different concentrations (5, 10, 25, 50, 100, 250 µM) and nintedanib was tested at 1, 3, 5, 10, 25, 50 µM (Chaudhary, 2007; Hashem, 2008; Syed, 2013). Nintedanib was tested at different concentrations than the others because through the literature review those lower concentrations were most effective for that compound. Each compound was added to the 96-well microplate in triplicate. Water was the control (Figure 2 green line). The amount of collagen protein (types I and II) in each well was assessed through IHC by a goat anti-mouse secondary antibody detected at 594nM in a plate reader.

EGCG showed a stimulation in collagen at 25 µM but increases at all other tested concentrations Figure 2), Hashem et al. (2008) also observed a decrease at 100 µM . Nintedanib decreased collagen levels in comparison to the control at all concentrations tested (Figure 2). Hydrocortisone showed very little alteration to collagen levels in comparison to the water control except at 5µM it showed a slight stimulation and at 10µM a decrease(Figure 2). Resveratrol decreased collagen levels at 100 and 250 µM, and slightly at 10µM with 250 µM having the lowest average value of all compounds tested at that concentration (Figure 2). Hashem et al. (2008) saw a decrease in COL1A2 mRNA expression in dermal fibroblasts treated with 100µM of resveratrol. DEX reduced collagen levels at 10, 25, 100, and

250 µM (Figure 2), Syed (2013) showed reduction of collagen-I and -III at mRNA levels by 50µg/mL on human keloids. Lactic acid decreased collagen levels at concentrations, 5, 25, and 50 µM but stimulated them at 100 and 250 µM (Figure 2 orange line).

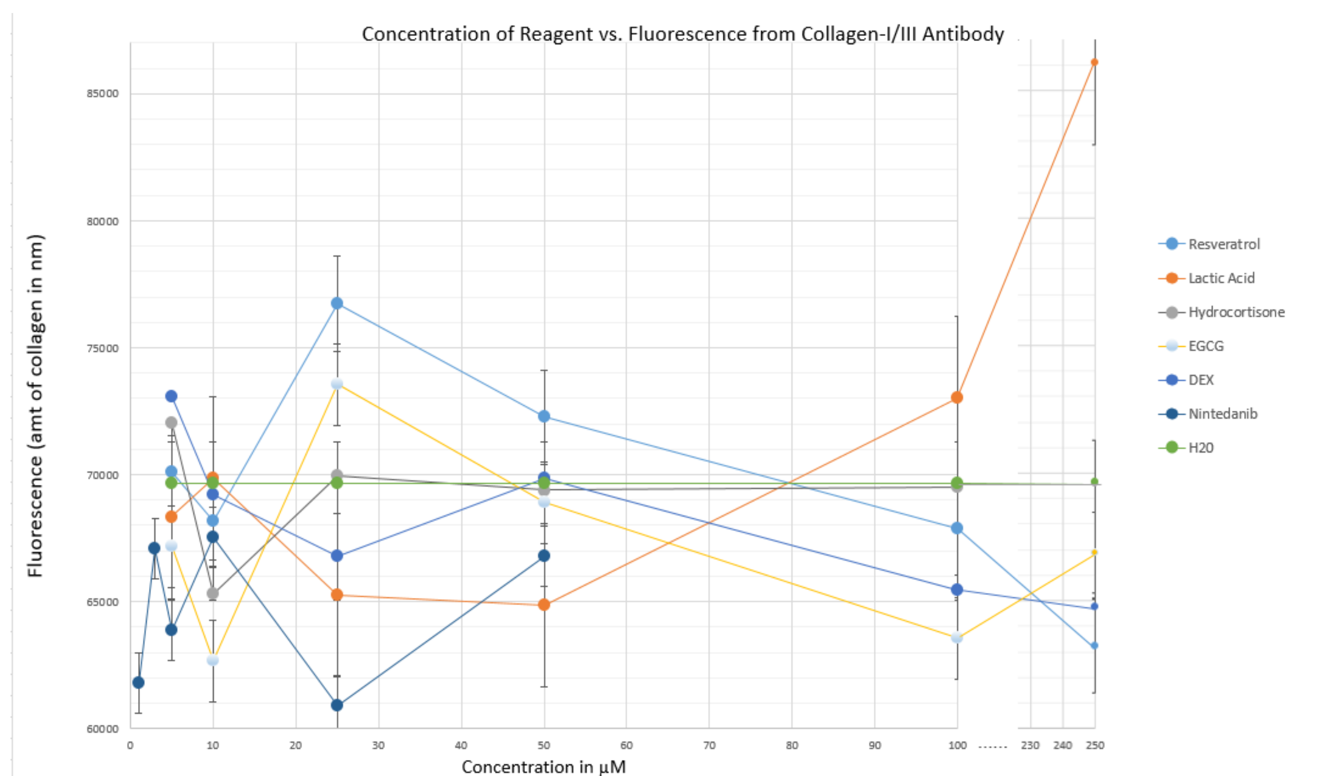

Figure 2. Plot of concentration of reagent vs. fluorescence from col-I. Shown are mean values for triplicates of each compound at each concentration in micromoles. H_2O (in green) is a negative control. Error bars represent standard error between values.

EGCG and nintedanib were determined to be particularly effective at decreasing collagen. To confirm this effect by microscopy 50µM, and 100µM EGCG, and 25 µM and 50 µM nintedanib were added to a chamber slide with TGF-β_1 treatment to all wells. To act as a control of collagen-I protein produced without antagonists, one well had no compounds added (Figure 3B), showing high collagen levels without any compound added. EGCG inhibited collagen-I protein synthesis at both 50 and 100µM in comparison to collagen-I protein with only TFG-β_1 treatment (Figure 3. D&F compared to B). 100µM EGCG (F) was inhibited more than 50µM (D), confirming concentration dependent inhibition seen in figure 2. Nintedanib also inhibited collagen-I protein at both concentrations tested (Figure 3. H&J compared to B). Nintedanib 25µM induced a response similarly to 50µM, and less than both concentrations of EGCG. The wells were DAPI stained to show similar numbers of cells in each well, excluding the possibility that intensity of staining was due to differences in cell number (Figure 3. Panels A, C, E, G, &I).

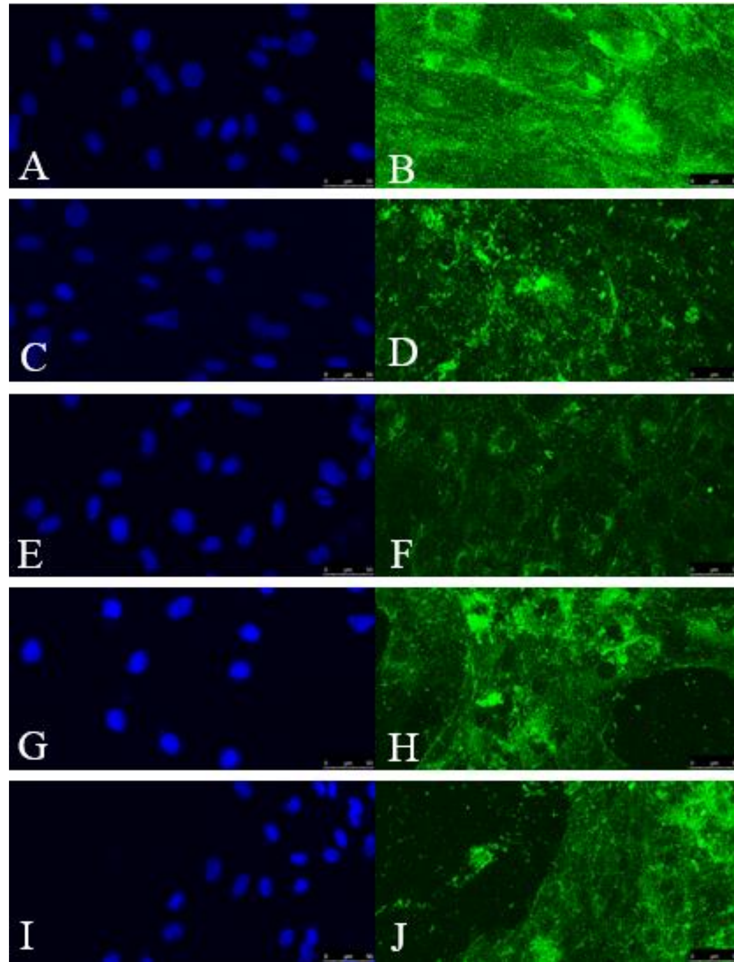

Figure 3. Primary dermal fibroblasts were plated in a chamber slide and treated with TGF-β_1. A, C, E, G, and I show nuclei through DAPI staining. B, D, F, H, and J show collagen expression through IHC of a primary monoclonal antibody for col-I, and secondary antibody GαM 488. B is the basal expression of col-I when treated with TGF-β_1 without test compounds. D is expression 50µM EGCG, F is 100 µM EGCG, H is 25 µM nintedanib, and J is 50 µM nintedanib.

To determine if the decrease in collagen production of cells treated with EGCG and nintedanib was due to antagonistic effects on collagen protein synthesis and not decreased cell viability an MTT assay was performed. MTT assays are colorimetric cell viability tests in which live cells uptake and metabolize MTT converting it to a purple compound. An optical plate reader can quantify the amount of purple based on the optical density at 595nm. Higher absorbance reflects higher cell viability. EGCG and nintedanib were tested in ten replicates of 50 and 100µM for EGCG and 25 and 50µM for nintedanib. The averages of the results from the MTT assay are shown in Figure 4. Cell viability was minimally effected at the concentrations tested.

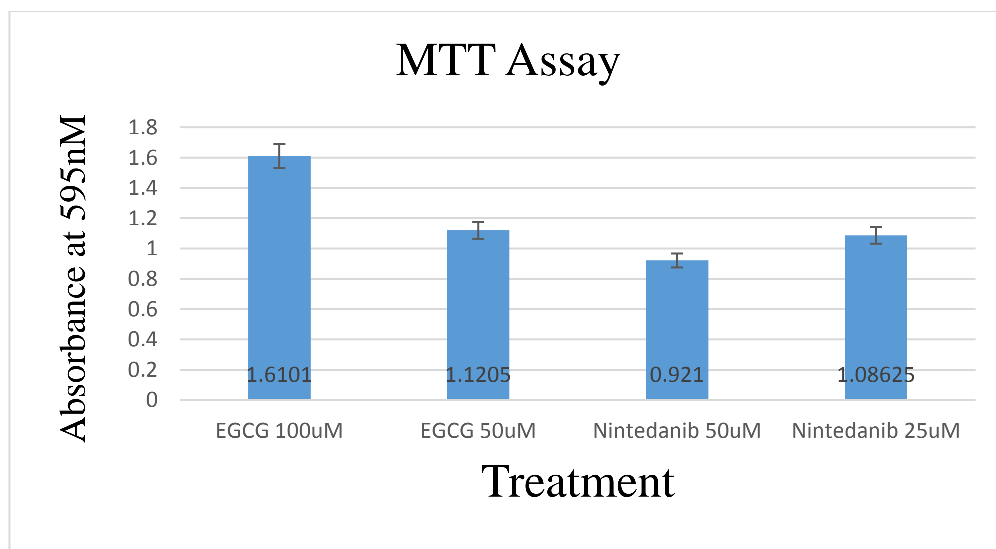

Figure 4. EGCG and nintedanib did not show significant effects upon cell viability. Each concentration of each compound was tested 10 times. Histogram values consist of ten replicates.

4. Discussion

The induced collagen conditions by TGF-β_1 is the basis for our *in vitro* scar. The cells were not otherwise under stress, yet TGF-β_1 caused them to produce collagen as they would during wound healing. Some investigators have used cells from keloid samples that are producing mass amounts of collagen (Syed, 2013). Others have looked at anti-collagen effects at the transcriptional or translational level (Chaudhary, 2007, & Hashem et al., 2008). Collagen is an extra-cellular matrix protein so its form in a scar is as a protein. The assay used here is particularly unique because the immunohistochemistry performed directly measured the amount of collagen produced. These levels in comparison to basal production by TGF-β_1 treated cells and those treated with various compounds were observed both by microscopy and quantitatively in a fluorescent plate reader.

Scarring, as well as causing unsightly blemishes on the skin, reduces functionality of any organ that has been wounded. If TGF-β_1 is not active the body could attempt to regenerate damaged cells because scarring is a result of TGF-β_1 activity that increases collagen levels. Regeneration may be favored because unlike scarring where the damage is filled with protein which cannot perform cell duties like circulation, the organ would regain full function with new cells.

All the compounds tested showed an effect on collagen levels. Resveratrol greatly decreased collagen-I levels in the fluorescent reading at 100µM and 250µM, yet it increased levels below 50µM. This agrees with Hashem et al. (2008) who saw a decrease in COL1A2 mRNA expression in dermal fibroblasts treated with 100µM of resveratrol. EGCG at 50 and 100µM were the most promising scar-collagen antagonists from our tests, with 100µM showing the greatest decrease in collagen-I protein in the confocal microscopy. This confirms effects seen by Nakamuta et al. (2005) on rat hepatic stellate cells treated with 50µM EGCG and Hashem et al. (2008) on dermal fibroblasts through ELISA for type I collagen and procollagen. Decrease in collagen-I protein levels in cells treated with nintedanib at 25 and 50µM supports Chaudhary et al.'s (2005) *ex vivo* analysis on the gene level of rats with lung fibrosis that nintedanib can have therapeutic effects to reduce scarring. EGCG and nintedanib were tested in an MTT viability assay that resulted in no major effect on viability. Further tests would benefit from all the compounds at different concentrations being evaluated trough an MTT assay to determine any effects on cell viability. If the anti-fibrotic effects of low collagen levels were because the cells were dead, and thus not producing collagen, the compounds would not be effective. Nintedanib and EGCG were also the only compounds where confocal pictures were taken to observe effect on collagen levels. Further experimentation at more concentrations between 100µM and 250µM or greater than 250µM could also enhance or change the findings. The effect of mixing certain compounds together could also be experimented. Overall, the observed effect of the collagen antagonists in a dose-dependent manner to reduce collagen protein levels supports their possible usefulness as anti-fibrotic treatments.

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