

# Statins synergize with phosphodiesterase type 5 inhibitors but not with selective estrogen receptor modulators to prevent myofibroblast transformation in an in vitro model of Peyronie's disease

Marcus M. Ilg, PhD<sup>1,\*</sup>, David J. Ralph, MS, FRCS (Urol)<sup>1,2</sup>, Selim Cellek, MD, PhD, FBPhS<sup>1</sup>

<sup>1</sup>Medical Technology Research Centre, School of Allied Health, Faculty of Health, Education, Medicine and Social Care, Anglia Ruskin University, Chelmsford CM1 1SQ, United Kingdom

<sup>2</sup>Urology Department, University College Hospital, London W1G 8PH, United Kingdom

\*Corresponding author: Medical Technology Research Centre, School of Allied Health, Faculty of Health, Education, Medicine and Social Care, Anglia Ruskin University, Chelmsford, United Kingdom. E-mail: [marcus.ilg@aru.ac.uk](mailto:marcus.ilg@aru.ac.uk)

## Abstract

**Background:** Peyronie's disease (PD) is a fibrotic disorder characterized by plaque formation in the tunica albuginea (TA) of the penis, and we have previously shown that inhibition of transformation of TA-derived fibroblasts to myofibroblasts using a combination phosphodiesterase type 5 (PDE5) inhibitors and selective estrogen receptor modulators (SERMs) is effective in slowing the progression of early PD.

**Aim:** The study sought to investigate whether combinations of statins with PDE5 inhibitors or SERMs would affect myofibroblast transformation in vitro.

**Methods:** Primary fibroblasts were isolated from TA of patients with PD and stimulated with transforming growth factor  $\beta$ 1 in the absence and presence of a range of concentrations of statins, PDE5 inhibitors, SERMs, and their combinations for 72 hours before quantifying  $\alpha$ -smooth muscle actin using in-cell enzyme-linked immunosorbent assay.

**Outcomes:** The prevention of transforming growth factor  $\beta$ 1-induced transformation of TA-derived fibroblasts to myofibroblasts was measured in vitro.

**Results:** Statins (simvastatin, lovastatin) inhibited myofibroblast transformation in a concentration-dependent manner with half maximal inhibitory concentration values of  $0.77 \pm 0.07 \mu\text{M}$  and  $0.8 \pm 0.13 \mu\text{M}$ , respectively. Simvastatin inhibited myofibroblast transformation in a synergistic fashion when combined with vardenafil (a PDE5 inhibitor; log alpha >0). Combination of tamoxifen (a SERM) and simvastatin did not show synergy (log alpha <0). When 3 drugs (simvastatin, vardenafil, and tamoxifen) were combined, the effect was not synergistic, but rather was additive.

**Clinical implications:** A combination of a statin with a PDE5 inhibitor might be useful in the clinic to slow the progression of the disease in patients with early PD; however, caution should be taken with such a combination because of the reported myopathy as a side effect.

**Strengths and limitations:** The use of primary human cells from patients with PD is a strength of this study. The mechanisms by which these drug classes exert synergy when used in combination was not investigated.

**Conclusion:** This is the first demonstration of an antifibrotic synergy between statins and PDE5 inhibitors.

**Keywords:** Peyronie's disease; fibrosis; myofibroblast; drug discovery; pharmacology.

## Introduction

Peyronie's disease (PD) affects 3% to 9% of men and is characterized by a fibrotic plaque in the penile tunica albuginea (TA), that can lead to pain, curvature, and erectile dysfunction.<sup>1</sup>

PD presents in 2 phases: an acute phase (usually within the first 6 months after the first symptoms, pain during erection and appearance and gradual worsening of the curvature) and a chronic phase (after ~12 months, stable plaque and curvature).<sup>1,2</sup> Current treatment is limited to surgery, as nonsurgical medical treatment has demonstrated limited efficacy,<sup>3</sup> while the only other nonsurgical therapy intralesional injection of collagenase has been withdrawn from the European market

recently.<sup>4</sup> Collagenase and surgery are usually offered to men with PD in chronic phase after the inflammation subsided and the plaque is stabilized. Traction devices are also indicated for men with curvature or to correct lost penile length from surgery or chronic PD.<sup>5</sup> There are no effective medications that can be offered to patients who are in the acute phase.

The pathophysiological mechanism suggested for PD is repetitive trauma to the TA,<sup>6,7</sup> resulting from fibrinogen extravasation, which in turn leads to inflammation and increased expression of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1).<sup>8</sup> Overexpression of TGF- $\beta$ 1 is considered a key factor in PD<sup>9</sup> and fibrosis in general.<sup>10,11</sup>

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A key player in PD and other fibrotic diseases is the myofibroblast which is a contractile, extracellular matrix producing,  $\alpha$  smooth muscle actin ( $\alpha$ -SMA)-expressing cell type that is differentiated from tissue resident fibroblasts following activation by TGF- $\beta$ 1.<sup>12–14</sup> Targeting myofibroblasts has been described as a viable treatment option for fibrosis<sup>15</sup>; however, treatments to achieve this have been scarce. Particularly for PD, inhibition of transformation of resident fibroblasts to myofibroblasts might be a way to slow or halt the progression of the disease in the acute phase.

In order to test whether such approach might be efficacious, we previously developed a phenotypic screening assay in which human primary fibroblasts obtained from the TA of patients with PD were transformed to myofibroblasts in response to TGF- $\beta$ 1. Using this assay, we screened 1954 Food and Drug Administration-approved drugs in order to find those drugs that would inhibit the transformation of fibroblasts to myofibroblasts. This screening campaign identified phosphodiesterase type 5 (PDE5) inhibitors (sildenafil, vardenafil, and tadalafil) and selective estrogen receptor modulators (SERMs) (tamoxifen and raloxifene) as possible candidates. When a PDE5 inhibitor and a SERM was tested simultaneously, a synergy was observed, meaning that the inhibition elicited by the combination of 2 drugs was greater than the arithmetic sum of the inhibitions elicited by individual drugs.<sup>16</sup> The PDE5 inhibitor + SERM combination was also effective in preventing plaque formation and development of erectile dysfunction in a rat model of PD.<sup>16</sup> Furthermore, we have recently demonstrated in a pilot clinical study that the combination of tamoxifen with a PDE5 inhibitor was able to slow the progression of the disease in men presented with acute PD.<sup>17</sup> These results suggest that combination of PDE5 inhibitors and SERMs might be effective in preventing the transformation of fibroblasts to myofibroblasts in a synergistic fashion in vitro and in vivo, and this effect was translatable to the clinic.

A previous study demonstrated that simvastatin (a HMG-CoA [hydroxy-methyl-glutaryl-coenzyme A] reductase inhibitor or statin) inhibited TGF- $\beta$ 1-induced transformation of TA-derived fibroblasts to myofibroblasts.<sup>18</sup> Statins have previously been suggested to have antifibrotic effects in lungs, kidneys, and intestine.<sup>19–21</sup>

We have therefore investigated the effect of the combinations of statins with PDE5 inhibitors or SERMs on TGF- $\beta$ 1-induced transformation of TA-derived fibroblasts to myofibroblasts.

## Methods

### Sample acquisition

Ethical approval was obtained by independent research ethics committees (NHS Research Ethics Committee East of England [12-EE-0170] and North of Scotland [15-NS-0051]). Patients that were included were between 18 and 75 years of age and able to understand the patient information sheet. TA tissue samples were acquired from patients undergoing corrective surgery for PD at University College London Hospital. The tissues would otherwise have been surgically discarded. Patients underwent Nesbit procedures, so non-plaque TA was obtained (TA opposite of the plaque). The 3 patients whose samples were used in this study were 33 years of age (with right inguinal hernia and hypertension), 58 years of age

(no comorbidities), and 67 years of age (diabetes and lichen sclerosis).

### Isolation of fibroblasts

Fibroblasts were isolated from the TA tissue samples as previously described.<sup>16,22,23</sup> Briefly, tissue samples were dissected into small pieces, ensuring that residual corpus cavernosum was removed, and submerged in cell culture medium (Dulbecco's Modified Eagle Medium [GIBCO], 10% fetal calf serum [Thermo Fisher Scientific], and 1% penicillin and streptomycin [GIBCO]) in 6-well plates. Tissue fragments were placed in an incubator at 37 °C and 5% CO<sub>2</sub> until cell outgrowth could be observed, after which the tissue was removed and the cells washed with phosphate buffered saline (PBS) and supplemented with fresh cell culture medium. Cells were incubated until 70% confluency, after which the cultures were expanded. Cells were characterized and fibroblast identity was confirmed as previously described.<sup>16,22</sup> Passages 2 to 6 were used in this study.

### Immunocytochemistry

A total of 50 000 cells per well were seeded onto sterile cover slips in a 6-well plate (NUNC; Thermo Fisher Scientific) and incubated overnight at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere. The next day cells were treated with 10 ng/mL TGF- $\beta$ 1 or control conditions and incubated at 37 °C and 5% CO<sub>2</sub> for 72 hours in a humidified atmosphere. The cover slips were washed with cold PBS, fixed using ice-cold methanol, and then washed with cold PBS. Unspecific binding of the antibodies was blocked using 10% donkey serum (Millipore) in PBS for 1 hour. The cover slips were then incubated with the primary antibody (desmin 1:500 [Abcam]; vimentin 1:1000 [Abcam];  $\alpha$ -SMA 1:1000 [Sigma-Aldrich]) for 2 hours. After 3 subsequent washes with PBS, the cover slips were incubated with FITC conjugated secondary antibody (1:250 [EMD-Millipore]) for 2 hours in a dark humidified chamber. The secondary antibody was removed, and the cover slips washed 3 times using PBS. After that, the cover slips were mounted on a glass slide using VECTASHIELD mounting medium with DAPI (Vector Laboratories) and kept in the fridge at 4 °C. Images were captured using an Olympus IX71 fluorescent microscope and a Leica DFC3000 G camera and LASX software (Leica). Fluorescent images were taken in at least 3 random fields of view of the cover slip.

### In-cell enzyme-linked immunosorbent assay

Myofibroblast transformation was quantified by measuring  $\alpha$ -SMA using the in-cell enzyme-linked immunosorbent assay technique in a 96-well plate format as previously described.<sup>16,22,23</sup> Fibroblasts were seeded into wells of a 96-well optical flat-bottom black microplates (NUNC) at a density of 5000 cells/well and left to attach overnight in an incubator at 37 °C and 5% CO<sub>2</sub>. Media were replaced with blank media or media containing 10 ng/mL TGF- $\beta$ 1 in the presence or absence of various concentrations of statins (simvastatin, lovastatin [Sigma-Aldrich]) and/or a SERM (tamoxifen [Sigma-Aldrich]) or PDE5 inhibitor (vardenafil [Sigma-Aldrich]). After 72 hours' incubation at 37 °C and 5% CO<sub>2</sub>, cells were fixed using 4% paraformaldehyde for 20 minutes at room temperature, after which cells were washed using PBS containing 0.1% Triton X-100. This was followed by blocking the cells in PBS containing 0.1% Triton X-100 and 10% donkey serum (Sigma-Aldrich) for

90 minutes at room temperature. A mouse monoclonal anti- $\alpha$ -SMA antibody (Sigma-Aldrich) was added at a 1:3000 dilution, and cells were incubated for 2 hours at room temperature, after which they were washed 3 times using PBS containing 0.1% Tween 20. A secondary antibody and nuclear stain (donkey anti-mouse at 1:500 that emits at 800 nm [IRDye 800CW; LI-COR]; nuclear counterstain at 1:1000 that emits at 700 nm [DRAQ5; Biostatus]) were added and cells were incubated for 1 hour in the dark. Cells were washed thrice using PBS containing 0.1% Tween 20, and plates were scanned using infrared imaging system (Odyssey CLx imager; LI-COR) at both 700 nm and 800 nm wavelengths.

The assay measures  $\alpha$ -SMA protein expression (800-nm channel) and divides this number with nuclear stain (700-nm channel), which gives the  $\alpha$ -SMA signal per cell. The zero (negative control) is obtained from cells without TGF- $\beta$ 1 stimulation, and 100% (positive control) is obtained from cells that are stimulated with TGF- $\beta$ 1 (without any drugs or inhibitors). When the inhibition value is above 100%, this means that the compound prevents  $\alpha$ -SMA increase completely and the  $\alpha$ -SMA staining is below the negative control. Such inhibition values above 100% would be acceptable within the limitations of assay because of the variation in signal obtained from negative control samples.

### Statistical analysis

Data analysis was performed using Microsoft Excel 2013 (Microsoft Corporation) or GraphPad Prism 7 software (GraphPad Software). Statistical significance was calculated using Student's *t* test for unpaired means (2-sided). The predicted additive effect of the compounds was compared with the observed effect. A *P* value <.05 was considered statistically significant. This analysis was performed for pairwise comparisons (Tables 1, 2, and 3). For multiple analyses (Table 4), one-way analysis of variance was performed and in which *P* < .05 and Dunnett's multiple comparisons test was performed.

Experiments were performed in at least 3 independent times using samples from at least 3 patients in triplicate wells (*N*=3). Cells derived from the same 3 patients were used in all experiments. Results from all experiments were pooled, and the mean values and standard deviations were used for statistical analysis.

The synergistic potency between 2 drugs were calculated using the freely available software (<https://musyc.app.vanderbilt.edu/>) that is based on a framework for calculating drug synergy.<sup>24</sup> The software calculates log alpha value, which is a measure of synergistic potency in which values >0 are considered to be synergistically potent.

## Results

### Characterization of cells isolated from TA

As previously shown, we isolated fibroblasts from TA fragments using the explant technique.<sup>16,22,23,25</sup> The phenotype of cells was confirmed with immunocytochemistry using antibodies against vimentin, desmin, and  $\alpha$ -SMA. The cells were vimentin-positive, desmin-negative, and  $\alpha$ -SMA-negative before TGF- $\beta$ 1 stimulation and vimentin-positive, desmin-negative, and  $\alpha$ -SMA-positive after TGF- $\beta$ 1 stimulation (Supplementary Figure 1). These results confirm

that the cells we isolated are fibroblasts and they transform into myofibroblasts after TGF- $\beta$ 1 stimulation.

### Statins, PDE5 inhibitors, and SERMs show concentration-dependent decrease in myofibroblast transformation

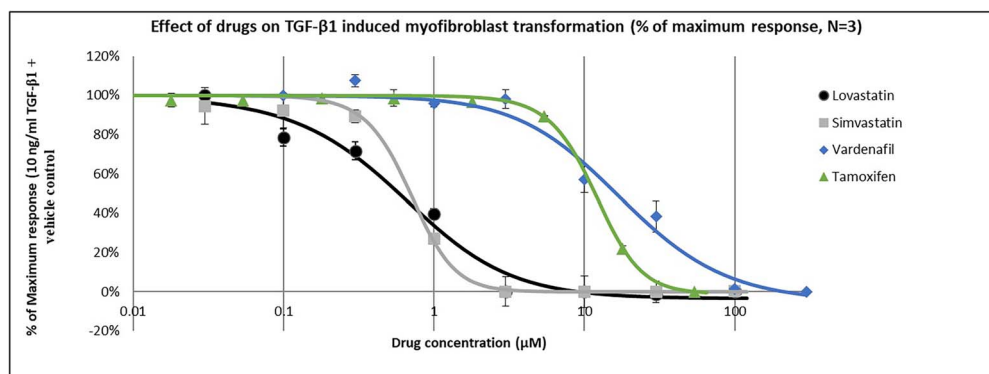
We have previously shown that statins,<sup>25</sup> PDE5 inhibitors, and SERMs<sup>16,23</sup> can prevent TGF- $\beta$ 1-induced myofibroblast transformation in TA-derived cells in *in vitro* assays. To investigate the concentration dependent effect of statins, full concentration response curves were constructed for lovastatin and simvastatin, along with a PDE5 inhibitor (vardenafil) and SERM (tamoxifen). Figure 1 depicts the concentration-dependent prevention of TGF- $\beta$ 1-induced  $\alpha$ -SMA expression increase in TA-derived fibroblasts *in vitro*. All 4 drugs prevented TGF- $\beta$ 1-induced  $\alpha$ -SMA expression increase in TA-derived fibroblasts in concentration-dependent manners. Both statins yielded inverse sigmoid curves with upper and lower plateau with half maximal inhibitory concentration (IC<sub>50</sub>) values of  $0.80 \pm 0.13$   $\mu$ M (lovastatin) and  $0.77 \pm 0.07$   $\mu$ M (simvastatin). The PDE5 inhibitor (vardenafil; IC<sub>50</sub> =  $15.0 \pm 1.10$   $\mu$ M) and SERM (tamoxifen; IC<sub>50</sub> =  $13.0 \pm 0.80$   $\mu$ M) also elicited inverse sigmoid curves with the upper and lower plateaus. Statins appeared more potent than vardenafil or tamoxifen when IC<sub>50</sub> values were compared. Because simvastatin showed lower variability than lovastatin on our initial experiments, it was used in further combination studies.

### Combining statins with PDE5 inhibitors leads to synergy

To investigate whether PDE5 inhibitors would synergize with statins, a full concentration response curve of vardenafil was constructed in the presence of 0.3  $\mu$ M of simvastatin (concentration that generates 10% of maximum response (IC<sub>10</sub>)). As can be seen in Figure 2A, addition of simvastatin led to a downward and leftward shift of the concentration response curve, while lowering the IC<sub>50</sub> value of vardenafil from  $15 \pm 1.1$   $\mu$ M to  $8.0 \pm 1.6$   $\mu$ M (*P* = .034). Cell viability was not affected by addition of simvastatin. Table 1 lists the predicted and observed effects along the entire concentration response curve and demonstrates that the observed effect was greater than the predicted effect at all concentrations below 100  $\mu$ M. A log alpha value, which calculates synergistic potency between 2 drugs,<sup>24</sup> of 1.87 (95% confidence interval, 1.38–2.68) was calculated, which confirms synergistic interaction.

### Combining statins with SERMs does not lead to synergy

To assess whether a similar synergistic effect could be achieved in combination with SERMs, a full concentration response curve of tamoxifen was created in the presence of 0.3  $\mu$ M simvastatin (IC<sub>10</sub>). Figure 2B shows the effect of tamoxifen in co-incubation with 0.3  $\mu$ M simvastatin on TGF- $\beta$ 1-induced myofibroblast transformation. The curve shows a downward and shift, while IC<sub>50</sub> values were not affected ( $13.0 \pm 0.80$   $\mu$ M tamoxifen only vs  $11 \pm 1.3$   $\mu$ M tamoxifen in the presence of 0.3  $\mu$ M simvastatin) (*P* = .0858). Cell viability was unaffected by the addition of simvastatin. The predicted and observed values for the entire concentration response curve are listed in Table 2. Although a synergistic effect for the combination could be observed only at 2 concentrations 0.018  $\mu$ M or



**Figure 1.** Statins, phosphodiesterase type 5 (PDE5) inhibitors, and selective estrogen receptor modulators (SERMS) can prevent transforming growth factor  $\beta 1$  (TGF- $\beta 1$ )–induced myofibroblast transformation in tunica albuginea (TA)–derived cells. TA-derived cells were treated with 10 ng/mL TGF- $\beta 1$  in the presence or absence of a range of concentrations of lovastatin (0.03–100  $\mu$ M; black), simvastatin (0.03–100  $\mu$ M; gray), vardenafil (0.1–300  $\mu$ M; blue), or tamoxifen (0.018–54  $\mu$ M; green). After 72 hours' incubation, cells were stained for  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) and expression was normalized to nuclear staining. Data points are plotted as average  $\pm$  SD of  $\alpha$ -SMA/nuclear staining ratio. Data were obtained via odyssey plate reader. N = 3. For clarity purposes, cell viability data are not shown. None of the drugs affected the cell viability at any concentration.

**Table 1.** Statistical comparison using Student's *t* test between predicted and observed effects when combining vardenafil with 0.3  $\mu$ M simvastatin.

Concentration of vardenafil ( $\mu$ M)	Inhibition by vardenafil only (%)	SD (%)	Predicted additive effect (%)	Predicted SD (%)	Observed effect (%)	Observed SD (%)	<i>t</i> test predicted vs observed
300	100	0	111	3	96	3	.0036*
100	99	1	110	4	101	3	.0356*
30	62	8	73	11	92	3	.0447*
10	43	7	54	10	79	5	.0179*
3	2	5	13	8	53	3	.0013*
1	4	2	15	5	40	13	.0359*
0.3	–8	3	3	6	36	9	.0062*
0.1	0	0	11	3	33	7	.0075*
0.03	0	5	11	8	33	7	.0231*

Predictive effect is the arithmetic sum of percentage inhibition by vardenafil and 11% inhibition by 0.3  $\mu$ M simvastatin. \**P* < .05.

0.18  $\mu$ M, the overall interaction between simvastatin and tamoxifen was deemed not to be synergistic because the log alpha value  $-0.01$  (95% confidence interval, 0.12–0.13) was below 0.

### PDE5 inhibitors and SERMs synergize

As previously demonstrated, PDE5 inhibitors and SERMs synergize in in vitro models of PD.<sup>16,23</sup> This was replicated in this study as shown in Figure 2C. When concentration response curve to vardenafil was constructed in the presence of 1  $\mu$ M of tamoxifen (concentration that generates 5–10% of maximum response ( $IC_{5-10}$ )), a downward and leftward shift of the curve was observed and the  $IC_{50}$  value was reduced from  $15.0 \pm 1.10$   $\mu$ M to  $8.0 \pm 1.5$   $\mu$ M (*P* = .029). Table 3 shows that the observed inhibition was greater than predicted inhibition at all concentrations of vardenafil below 100  $\mu$ M when they were combined with 1  $\mu$ M tamoxifen. The synergistic interaction was confirmed with a log alpha value of 8 (95% confidence interval, 0–7.99).

### Statin-PDE5 inhibitor-SERM combination mostly not synergistic

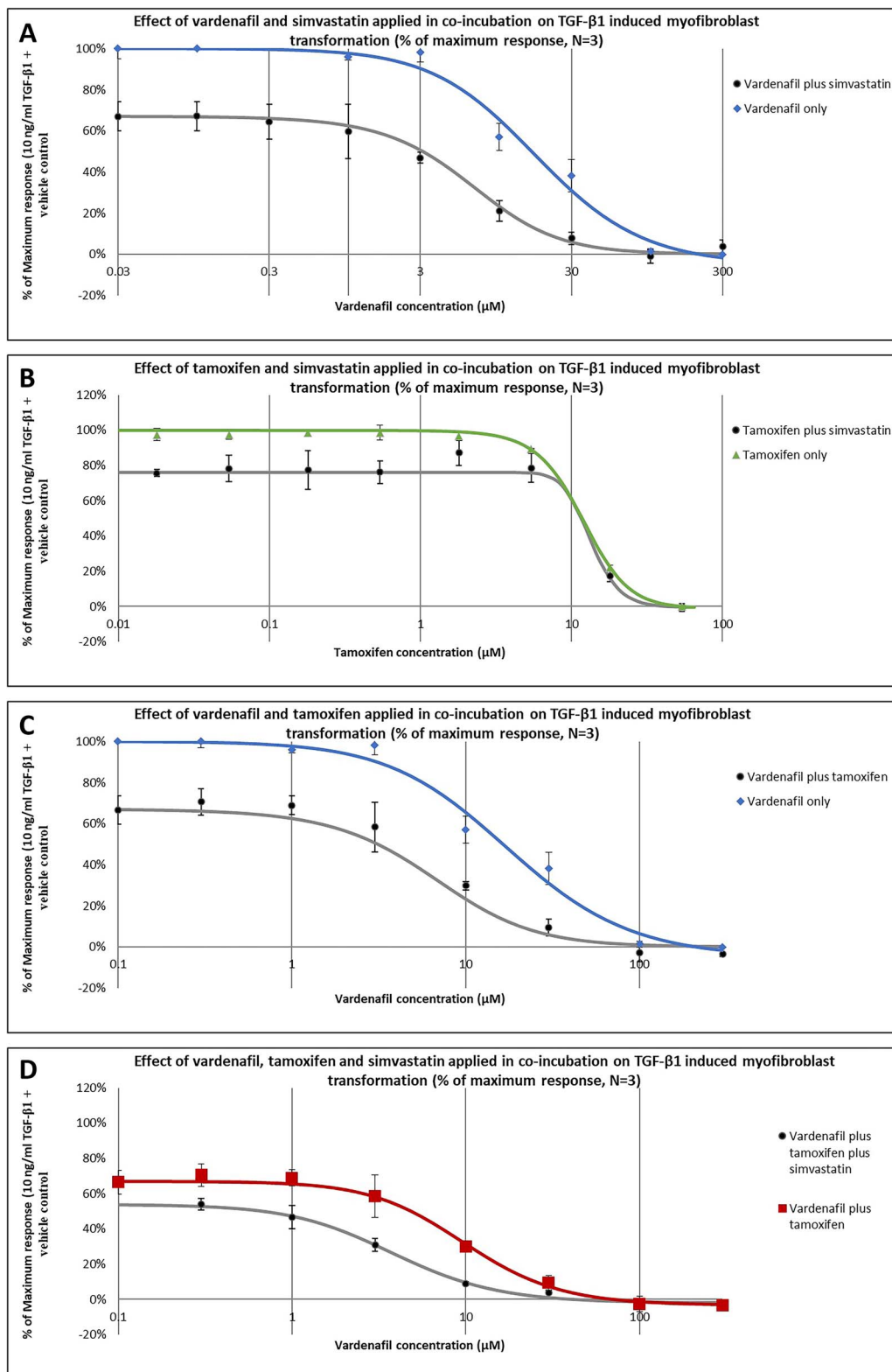
To further investigate the effects of the interplay between the 3 drug classes, a triple combination of statin, PDE5 inhibitor, and SERM was investigated. The data on the triple combination are summarized in Figure 2D. A concentration response curve to vardenafil (0.1–300  $\mu$ M) was constructed in

the presence of 1  $\mu$ M tamoxifen and 0.3  $\mu$ M simvastatin. This curve was shifted downward and leftward compared with the control double combination curve (PDE5 inhibitor plus tamoxifen). The  $IC_{50}$  value was reduced from  $8.0 \pm 1.5$   $\mu$ M to  $3 \pm 1.6$   $\mu$ M (*P* = .0168). The statistical analysis is summarized in Table 4. When the predicted effect of the triple combination was compared with the observed effect of the triple combination, the only significant difference was observed at 10  $\mu$ M vardenafil with 1  $\mu$ M tamoxifen and 0.3  $\mu$ M simvastatin (*P* = .0274). When comparing the observed double combination (vardeafil and tamoxifen) with the observed triple combination (vardeafil, tamoxifen, simvastatin) the triple combination showed significantly higher inhibition at ranges between 0.3 to 10  $\mu$ M of vardenafil, suggesting an additive effect in these ranges. The log alpha value could not be calculated in these triple drug experiments because this algorithm can calculate potency of synergy between 2 drugs only.

### Discussion

With the aim of finding new therapeutics for PD, we developed a phenotypic screening assay that measures the transformation of TA-derived fibroblasts to myofibroblasts in response to TGF- $\beta 1$ , the key profibrotic cytokine in PD pathophysiology. Using this assay, we ran several screening campaigns including 1954 Food and Drug Administration–approved drugs that revealed PDE5 inhibitors and SERMs as hits. When these





**Figure 2.** Effect of combinations of phosphodiesterase type 5 (PDE5) inhibitors, selective estrogen receptor modulators (SERMS), and statins on transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1)-induced myofibroblast transformation in tunica albuginea (TA)-derived cells. **(A)** TA-derived cells were treated with 10 ng/mL TGF- $\beta$ 1 in the presence or absence of a range of concentrations of vardenafil (0.1–300  $\mu$ M) in the presence of 0.3  $\mu$ M simvastatin. **(B)** TA-derived cells were treated with 10 ng/mL TGF- $\beta$ 1 in the presence or absence of a range of concentrations of tamoxifen (0.018–54  $\mu$ M) in the presence of 0.3  $\mu$ M simvastatin. **(C)** TA-derived cells were treated with 10 ng/mL TGF- $\beta$ 1 in the presence or absence of a range of concentrations of vardenafil (0.1–300  $\mu$ M) in the presence of 1  $\mu$ M tamoxifen. **(D)** TA-derived cells were treated with 10 ng/mL TGF- $\beta$ 1 in the presence or absence of a range of concentrations of vardenafil (0.1–300  $\mu$ M) in the presence of 1  $\mu$ M tamoxifen. After 72 hours' incubation, cells were stained for  $\alpha$  smooth muscle actin ( $\alpha$ -SMA), and expression was normalized to nuclear staining. Data points are plotted as average  $\pm$  SD of  $\alpha$ -SMA/nuclear staining ratio or nuclear staining only (cell viability). Data were obtained via odyssey plate reader. N = 3. For clarity purposes, cell viability data are not shown. None of the drugs affected the cell viability at any concentration.

**Table 2.** Statistical comparison using Student's *t* test between predicted and observed effects when combining tamoxifen with 0.3  $\mu$ M simvastatin.

Concentration of tamoxifen ( $\mu$ M)	Inhibition by tamoxifen only (%)	SD (%)	Predicted additive effect (%)	Predicted SD (%)	Observed effect (%)	Observed SD (%)	<i>t</i> test predicted vs observed
54	100	0	111	3	101	2	ns
18	78	1	89	4	83	3	ns
5.4	11	0	22	3	21	8	ns
1.8	3	3	14	6	13	7	ns
0.54	2	4	13	7	24	7	ns
0.18	2	2	13	5	23	2	.0324*
0.054	3	3	14	6	22	7	ns
0.018	2	3	13	6	24	2	.0395*

Predictive effect is the arithmetic sum of percentage inhibition by vardenafil and 11% inhibition by 0.3  $\mu$ M simvastatin. Abbreviation: ns, not significant.

\**P* < .05.

**Table 3.** Statistical comparison using Student's *t* test between predicted and observed effects when combining vardenafil with 1  $\mu$ M tamoxifen.

Concentration of vardenafil ( $\mu$ M)	Inhibition by vardenafil only (%)	SD (%)	Predicted additive effect (%)	Predicted SD (%)	Observed effect (%)	Observed SD (%)	<i>t</i> test predicted vs observed
300	100	0	102	4	103	3	ns
100	99	1	101	5	103	3	ns
30	62	8	64	12	90	3	.0220*
10	43	7	45	11	70	5	.0231*
3	2	5	4	9	42	3	.0023*
1	4	2	6	6	31	13	.039*
0.3	-8	3	-6	7	29	9	.006*
0.1	0	0	2	4	33	7	.0026*

Predictive effect is the arithmetic sum of percentage inhibition by vardenafil and 2% inhibition by 1  $\mu$ M tamoxifen. Abbreviation: ns, not significant.

\**P* < .05.

**Table 4.** Statistical comparison using ANOVA with Dunnett's test between predicted and observed effects when combining vardenafil with 1  $\mu$ M tamoxifen and 0.3  $\mu$ M simvastatin and comparison between observed double combination (varafenafil + tamoxifen) and observed triple combination.

Concentration of statin ( $\mu$ M)	Inhibition by double combination (varafenafil plus tamoxifen) (%)	SD (%)	Predicted effect of triple combination (%)	SD (%)	Observed effect of triple combination (%)	SD (%)	ANOVA predicted triple combination vs observed triple combination	ANOVA observed double combination vs observed triple combination
300	103	1	114	4	103	1	.0027*	ns
100	103	5	114	8	103	0	ns	ns
30	90	4	101	7	96	1	ns	ns
10	70	2	81	5	91	1	.0146*	.0004*
3	42	12	53	15	69	4	ns	ns
1	31	5	42	8	53	7	ns	.0131*
0.3	29	6	40	9	46	3	ns	.0322*
0.1	33	7	44	10	34	8	ns	ns

Abbreviation: ANOVA, analysis of variance; ns, not significant. \**P* < .05.

2 classes were tested simultaneously, we observed a synergy in both in vitro and in vivo animal models.<sup>16</sup> We then retrospectively analyzed the clinical effect of a combination of tamoxifen and PDE5 inhibitors in men presenting with acute PD. The initial results suggest that the combination treatment was able to slow the progression of the disease in patients with early PD.<sup>17</sup> These results suggest that phenotypic assay of myofibroblast transformation is able to detect drugs that can be translated into the clinic. In the current study, we utilized the same assay, which has been validated previously.<sup>16,22</sup>

Statins have previously been reported to have antifibrotic effects in animal models of intestinal fibrosis,<sup>26</sup> kidney,<sup>27</sup> and cardiac fibrosis<sup>28,29</sup> and in in vitro models of lung fibrosis.<sup>30</sup> In the context of PD, a previous study demonstrated

that simvastatin inhibited TGF- $\beta$ 1-induced transformation of TA-derived fibroblasts to myofibroblasts.<sup>18</sup> Although the authors of the previous study did not report the IC<sub>50</sub> value of simvastatin, from their published figures, we estimate it to be somewhere between 0.5 and 1  $\mu$ M, which is similar to our findings. Although the exact mechanism by which statins exert their antifibrotic effect is unknown, it has been suggested that statins suppress expression of connective tissue growth factor (CTGF), a potent profibrotic mediator downstream of TGF- $\beta$ 1, through inhibition of the nuclear translocation of YAP/TAZ.<sup>18</sup>

Our experiments suggest that when simvastatin was combined with vardenafil, the effect was synergistic. A synergy between these drug classes was demonstrated in improving

fine motor function in a mouse ischemic stroke model<sup>31</sup> and in reducing right ventricular hypertrophy and pulmonary vascular muscularization in a rat model of hypoxia-induced pulmonary hypertension.<sup>32</sup> Furthermore, in a clinical context, a synergy between statins and PDE5 inhibitors has been suggested for treatment of erectile dysfunction.<sup>33</sup> Although exact molecular mechanism by which the 2 drug classes exert synergy is not known, an increased bioavailability of NO, decreased ROCK expression, and increased intracellular cGMP concentrations have been suggested.<sup>32</sup> Whether such a molecular interaction would result in inhibition of myofibroblast transformation warrants further research.

SERMs such as tamoxifen have previously been investigated in clinical studies on men with PD. A nonrandomized study of 36 men demonstrated that patients with early disease responded better to tamoxifen than patients with a longer history in painful erection scores, curvature degree, and plaque scores.<sup>34</sup> In a randomized placebo-controlled study of 25 men showed no statistically significant differences between the tamoxifen and placebo groups with regard to the reduction of penile deformity, decrease penile pain, and decrease in plaque size.<sup>35</sup> Although the authors selected patients with no calcified plaques, the mean duration of PD was 20 months, which may explain the discrepancy between the results reported by these authors and a previous study<sup>34</sup> in which the patients were in the early phase of PD. Another study showed that tamoxifen decreased plaque size and slowed the progression of the disease but did not affect the penile curvature in acute PD.<sup>36</sup>

In the current study, we showed that simvastatin and tamoxifen did not have synergistic action in preventing transformation of fibroblasts to myofibroblasts. A previous study showed a synergy between tamoxifen and simvastatin in inducing apoptosis in estrogen receptor (ER)-positive breast cancer cell line.<sup>37</sup> In a different breast cancer cell line, an antagonistic interaction was observed between simvastatin and tamoxifen.<sup>38</sup> This has been attributed to differential expression of ER $\alpha$  in these cell lines, whose expression is known to be affected by statins.<sup>39</sup> Because TA-derived fibroblasts we use in our experiments do not express ER $\alpha$ , but rather express ER $\beta$ ,<sup>23</sup> we do not think that simvastatin or vardenafil alone or in combination caused cell apoptosis in our experiments.

In this study, we tested a combination of statins, SERMs, and PDE5 inhibitors. We could observe an additive effect in a certain concentration range, but no synergy was observed. Combination therapies have been described to be more successful in drug-repurposing efforts.<sup>40</sup> This has led to suggestions of using combinations to increase the success in drug repositioning, as it allows to lower high IC<sub>50</sub> values or reduce concentrations of individual drugs. It has also been suggested that polypharmacology might be exploited by computational approaches in the future to lead to more promising drug combinations to find new synergies and redirect stalled drug discovery projects to treat niche diseases.<sup>41</sup>

### Limitations and suggestions for future research

We have not investigated the mechanism of action of statins in this study. Previous studies have suggested the involvement of the RhoA-ROCK pathway in inhibition of myofibroblast differentiation and contraction.<sup>42–44</sup> Further studies are required to better understand the molecular

pathways involved in statin-induced inhibition of myofibroblast transformation and function.

In this study and our phenotypic screening assays,<sup>25,45</sup> we have chosen TGF- $\beta$ 1 as the stimulatory cytokine, as its role in pathophysiology of PD has been well defined.<sup>46,47</sup> It should be noted, however, that other cytokines or factors than TGF- $\beta$ 1 have been reported to induce myofibroblast transformation.<sup>48</sup> CTGF (a factor downstream of TGF- $\beta$ 1) is such a factor that has been shown to be associated with the severity of fibrotic diseases<sup>49</sup> and that enhances TGF- $\beta$ 1's profibrotic properties,<sup>50</sup> and its downregulation has been shown to protect animal models from developing fibrosis.<sup>51</sup> Although overexpression of CTGF has been reported to lead to exaggerated myofibroblast transformation and function in several fibrotic conditions,<sup>52</sup> its expression has been shown to be unaltered in TA of patients with PD.<sup>53</sup> Another factor is platelet-derived growth factor (PDGF), which is secreted by endothelial cells and macrophages and released from platelets upon degranulation. Although PDGF is a well-known profibrotic factor and has been shown to be increased in mouse model of tight skins that develops spontaneous PD,<sup>54</sup> to our knowledge its expression has not been shown to be increased in TA of patients with PD. Monocyte chemoattractant protein-1 (MCP-1), a chemokine, is upregulated in fibrosis of the lung,<sup>55</sup> skin,<sup>56</sup> kidney,<sup>57</sup> and liver.<sup>58</sup> MCP-1 expression has been shown to be increased in TA tissue of patients with PD and cells isolated from those tissues and to be further elevated in response to TGF- $\beta$ 1.<sup>53,59</sup> These previous studies suggest that CTGF, PDGF, and MCP-1 may be released from different cells in an inflammatory microenvironment in PD, and they all are likely to make some contribution to drive the fibrotic processes with strong links to TGF- $\beta$ 1. Further studies are required to compare the effect of these factors on myofibroblast transformation and function in PD.

In our previous study, using the same phenotypic assay that was deployed in this study, we identified a synergistic combination of PDE5 inhibitors and SERMs that was then tested in a rat PD model in which fibrosis was induced with intratunical TGF- $\beta$ 1 injection.<sup>16</sup> The PDE5 inhibitor + SERM combination was taken to the clinical studies after positive results from the animal study were obtained. Similarly, the combination of PDE5 inhibitors and statins should be tested in animal models before it can be taken to clinical studies.

### Clinical translation

Approximately 20% of men with PD are likely to be on statins due to comorbidities such as hypercholesterolemia and hyperlipidemia.<sup>33</sup> Therefore, it is likely that some men with early PD will be on statins. The addition of PDE5 inhibitors to statins, according to our data, is likely to decrease the progression of PD in these patients. Similarly, we have observed that 5 mg tadalafil once daily in combination with 20 mg tamoxifen twice daily was able to slow the progression of early (acute) PD.<sup>17</sup>

Clinical translation of our findings very much depends on the pharmacokinetic properties of the 3 drugs. Oral 20 mg vardenafil and 40 mg simvastatin have been reported to elicit to maximum plasma concentration of ~20 ng/mL (~40 nM)<sup>60</sup> and ~2 ng/mL (~5 nM),<sup>61</sup> respectively. In our experiments, much higher concentrations of vardenafil and simvastatin were required to show an effect on myofibroblast transformation. However, a direct comparison of plasma concentrations to in vitro concentrations can be misleading because several

factors such as drug distribution, plasma protein binding, and tissue penetrance can influence tissue concentrations. Moreover, the artificial in vitro setting is far from an intact organism, and there seems to be large variation in the sensitivity of the assays used. For example, 1 to 10  $\mu$ M sildenafil<sup>62</sup> or vardenafil<sup>63</sup> has been reported to relax human corpus cavernosum (HCC) strips in vitro, which are significantly higher than the peak plasma concentrations that can be achieved in vivo in human. On the other hand, sildenafil, tadalafil, and vardenafil at nanomolar concentrations have been reported to produce significant relaxation of HCC,<sup>64</sup> and vardenafil potentiates nitric oxide donor-induced, endothelial, and neurogenic relaxation in HCC at 10 nM.<sup>65</sup> Further clinical studies are required to demonstrate clinically meaningful effect of combinations of statins with PDE5 inhibitors in treatment of early phase PD.

Statins are a well-established class of drugs in the treatment of hypercholesterolemia and have been shown to reduce the risk of cardiovascular morbidity and mortality in patients with or at risk for coronary heart disease in several clinical trials.<sup>66,67</sup> Myopathy, described as unexplained muscle pain, tenderness, or weakness, is one of the most important adverse effects associated with statins, which occurs in 0.1% to 0.2% of the patients and typically fully resolves on cessation of statin therapy.<sup>68</sup> One of the reasons for statin-associated myopathy has been suggested to be concomitant drug usage. Sildenafil<sup>69</sup> and tadalafil<sup>70</sup> have been reported to cause such a drug-drug interaction and increase the incidence of myopathy in patients taking statins. Therefore, caution should be taken when combining a statin and a PDE5 inhibitor for treatment of early-phase PD.

## Conclusion

This is the first demonstration of a synergistic inhibition of myofibroblast transformation with a statin and PDE5 inhibitor combination. Because drug repurposing is more likely to succeed when drugs are combined, combining statins with PDE5 inhibitors may be a potential pathway to novel treatments for PD. However, care must be taken, as there have been adverse events reported when combining statins and PDE5 inhibitors.

## Supplementary Material

Supplementary material is available at *The Journal of Sexual Medicine* online.

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