

1 Title: In vitro effect of a non-immunosuppressive FKBP ligand, FK1706, on SARS-CoV-2  
2 replication in combination with antivirals

3 Running Title: FK1706-remdesivir in vitro synergy against SARS-CoV-2

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33 **Abstract**

34 FKBP, a naturally occurring ubiquitous intracellular protein, has been proposed as a potential  
35 target for coronavirus replication. A non-immunosuppressive FKBP ligand, FK1706, was studied  
36 in vitro in a Vero cell model to assess potential activity alone and in combination with antivirals  
37 against SARS-CoV-2 replication. When combined with remdesivir, synergistic activity was seen  
38 (summary synergy score  $24.7 \pm 9.56$ ). FK1706 warrants in vivo testing as a potential new  
39 combination therapeutic for the treatment of COVID-19 infections.

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56 FKBP is one of the naturally occurring ubiquitous intracellular proteins called immunophilins that  
57 has enzymatic activity as a peptidyl prolyl cis-trans isomerase and is also essential to the  
58 pharmacologic activity of immunosuppressants. The binding of tacrolimus, everolimus, and  
59 sirolimus, to FKBP is necessary but not sufficient to produce immunosuppression (1,2).

60 Replication of human coronaviruses is dependent on active immunophilin binding and inhibition  
61 of cyclophilins, an intracellular immunophilin, by cyclosporine blocks the replication of CoVs of  
62 all genera tested, including SARS-CoV, human CoV-229E and -NL-63, feline CoV, as well as  
63 avian infectious bronchitis virus (3-6). More recently, the immunophilin FKBP has been  
64 described as one of the potential targets for SARS-CoV-2 (7,8).

65 Two ligands to FKBP that are not immunosuppressive, FK1706 (9,10) and ElteN378 (11,12)  
66 were studied. These compounds are structurally distinct; both bind to the core structure for  
67 FKBP but do not have intact calcineurin or mTOR binding domains that produce  
68 immunosuppression. Because these drugs target host cells and may work by a unique  
69 mechanism to inhibit coronavirus replication, the additive or synergistic effect with known virus-  
70 targeting antivirals with mechanisms of RNA polymerase inhibition (e.g., remdesivir), viral error  
71 catastrophe or viral lethal mutagenesis (e.g., molnupiravir), or protease inhibition (e.g.,  
72 M128533) were evaluated.

73 Vero E6 cells were infected with the live SARS-CoV-2 virus (USA-WA1/2020; World Reference  
74 Center for Emerging Viruses and Arboviruses (WRCEVA)) at low MOI (multiplicity of infection)  
75 and multiple rounds of viral replication occurred over the course of the assay. Percent CPE in  
76 compound-treated virus-infected cells were normalized to infected untreated cells as 0% and  
77 uninfected cells as 100% CPE protection. Based on these data, a concentration-response curve  
78 was created. Toxicity was assessed and compared in untreated, uninfected cells compared to  
79 treated cells.

80 In vitro testing was conducted at two independent laboratories in sequence. The details of the  
81 protocol followed by each laboratory are included in the appendix materials.

82 FK1706 (Shanghai SIMR Biotechnology Co. LQY20200910), ElteN378 (Glixx Laboratories Inc.  
83 GLXC -20448), remdesivir, molnupiravir, and M128533 were solubilized in DMSO and were  
84 diluted in culture test media to prepare compound concentrations.

85 Synergy was calculated using SynergyFinder 2.0 software (13). A summary synergy score  
86 greater than 10 was considered synergistic.

87 The initial results of FK1706 alone and in combination with remdesivir, molnupiravir, and  
88 M128533 are summarized in Table 1.

89 When combined, FK1706 (11-90  $\mu\text{M}$ ) and remdesivir (3  $\mu\text{M}$ ) were effective in inhibiting SARS  
90 CoV-2 viral CPE (93-100%, see Appendix Fig A1). FK1706 (2.85-90  $\mu\text{M}$ ) and molnupiravir (0.3  
91  $\mu\text{M}$ ) inhibited SARS CoV-2 CPE (up to 70% reduction in viral CPE at 90  $\mu\text{M}$  FK1706 with 0.3  
92  $\mu\text{M}$  molnupiravir; see Appendix Fig A2). FK1706 (11-90  $\mu\text{M}$ ) and M128533 (1  $\mu\text{g}/\text{mL}$ ) reduced  
93 SARS CoV-2 CPE (64-100%, see Appendix Fig A3).

94 Although FK1706 alone did not exhibit inhibitory activity against SARS-CoV-2, when combined  
95 with suboptimal concentrations (less than the  $\text{EC}_{50}$ ) of all three antivirals, increased inhibition  
96 was observed. Additive effects of ElteN378 with either remdesivir or M128533 were also  
97 demonstrated (see Appendix Table A1).

98 In follow-up confirmatory combination studies, FK1706 at multiple concentrations was tested in  
99 combination with multiple concentrations of remdesivir. When combined, remdesivir and  
100 FK1706 exhibited synergistic activity inhibiting SARS-CoV-2 and shifting the  $\text{EC}_{50}$  value of both  
101 compounds when in combination with the other (Figures 1A,B). The summary synergy scores  
102 were  $24.7 \pm 9.56$  by the ZIP (Supplementary Fig A4,A5),  $24.8 \pm 9.56$  by the Bliss and  $24.9 \pm 9.56$   
103 by the HSA models. Scores  $>10$  in all 3 models indicate synergy.

104 Molnupiravir alone, nor in combination with FK1706, did not demonstrate activity in the follow-up  
105 confirmatory study. There was no evidence of cytotoxicity with FK1706 or remdesivir alone or in  
106 combination (Appendix Figs A6,A7).

107 The synergistic effects of FK1706 in combination remdesivir were demonstrated in a live SARS-  
108 CoV-2 virus assay measuring the ability of compounds to inhibit viral-induced CPE in Vero E6  
109 host cells in vitro. The CPE reduction assay is a popular and widely used assay format to  
110 screen for antiviral agents because of its ease of use in quantitative high-throughput screening.  
111 The CPE reduction assay indirectly monitors the ability of compounds to inhibit viral replication  
112 and infection through various mechanisms, including direct inhibition of viral entry or enzymatic  
113 processes as well as acting on host pathways that modulate viral replication. This assay was  
114 previously used to screen 8,810 approved and investigational drugs from the National Center for  
115 Advancing Translational Sciences (NCATS) small molecule collections (14). A cytotoxicity  
116 counter-screen was conducted in parallel in host cells without addition of virus and  
117 demonstrated no substantial cytotoxicity of any of the test agents alone or in combination.

118 Since two chemically distinct FKBP ligands, FK1706 and ElteN378, both demonstrated activity,  
119 it is likely that FKBP is the key target. This target is in the host cells and complements the virus-  
120 targeted antivirals. The combination activity of these FKBP ligands was not limited to a single  
121 virus-targeted mechanism as the three antivirals have distinctly different mechanisms.  
122 Remdesivir (Veklury), currently the only FDA-approved antiviral for COVID-19 infections, is  
123 administered intravenously to patients (15). Molnupiravir has received Emergency Use  
124 Authorization as oral therapy for outpatient COVID-19 infections (16). Although both of these  
125 antivirals have demonstrated clinical efficacy, there is a need for higher response rates and  
126 FK1706 may have utility in both settings. Additionally, these combinations should be active  
127 against variants with mutations in spike protein.

128 Both live virus assays use Vero E6 as host cells. Vero E6 cells have been shown to have high  
129 drug efflux transporter P-glycoprotein (P-gp) activity, which can reduce cellular concentrations of  
130 test articles, and remdesivir is a known P-gp substrate (17). Therefore, synergy observed in  
131 Vero E6 cells could be due to P-gp inhibition, which enhances the exposure of remdesivir *in*  
132 *vitro*, and warrants repeating in other cell-based models.

133 FK1706 has completed all nonclinical safety pharmacology, ADME, and GLP toxicity studies to  
134 support clinical development. Phase 1 healthy volunteer and Phase 2 studies in patients with  
135 neuropathy have been completed (9). This clinical experience would expedite the introduction of  
136 FK1706 into clinical studies of patients infected with SARS-CoV-2.

137 In conclusion, these data demonstrate that FKBP is a valid target for coronavirus infections in  
138 combination with virus-targeted antivirals such as remdesivir and molnupiravir. FK1706 warrants  
139 testing in an *in vivo* animal model of SARS-CoV-2 and if promising, rapid introduction into  
140 COVID-19 infection clinical trials.

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148 **Acknowledgements:**

149 The authors thank Diane M. Coniglio, Pharm.D., President, Opus Medical Communications for  
150 editorial assistance with the manuscript.

151

152 **Funding:**

153 This research was funded by Tutela Pharmaceuticals Inc., a 501(c)(3) not-for-profit  
154 pharmaceutical company. William E. Fitzsimmons is the Founder and Chair of Tutela  
155 Pharmaceuticals Inc. This work was supported by the Intramural Research Program of National  
156 Center for Advancing Translational Sciences, Sciences, National Institutes of Health.

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158 **Figure Captions:**

159 Figure 1A. Concentration response of FK1706 when combined with remdesivir (RDM).

160 Figure 1B. Concentration response of remdesivir when combined with FK1706.

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231 hydroxy-3-methoxycyclohexyl]-1-methylvinyl-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(2-  
232 oxopropyl)-11,28-dioxa-4-azatricyclo[22.3.1.0(4.9)]octacos-18-ene-2,3,10,16-tetrone (FK1706),  
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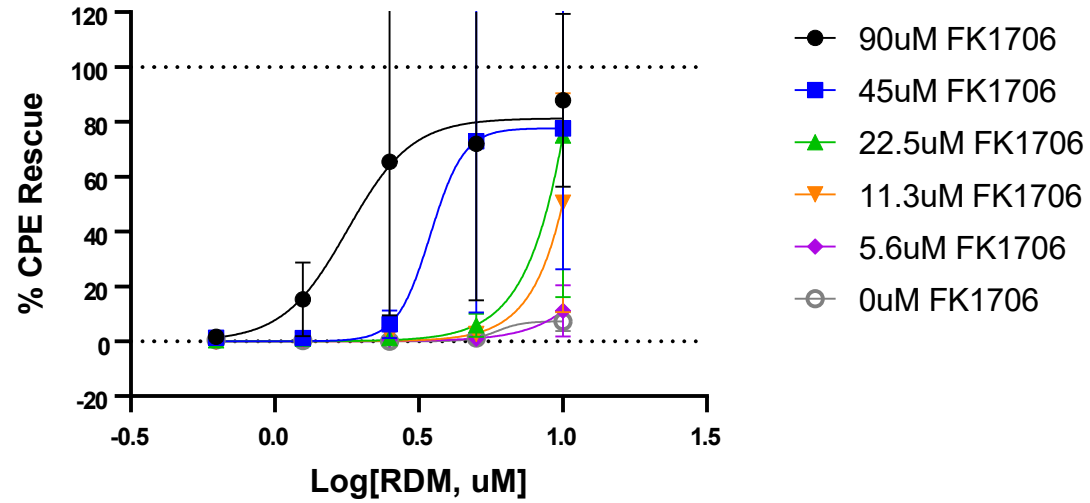
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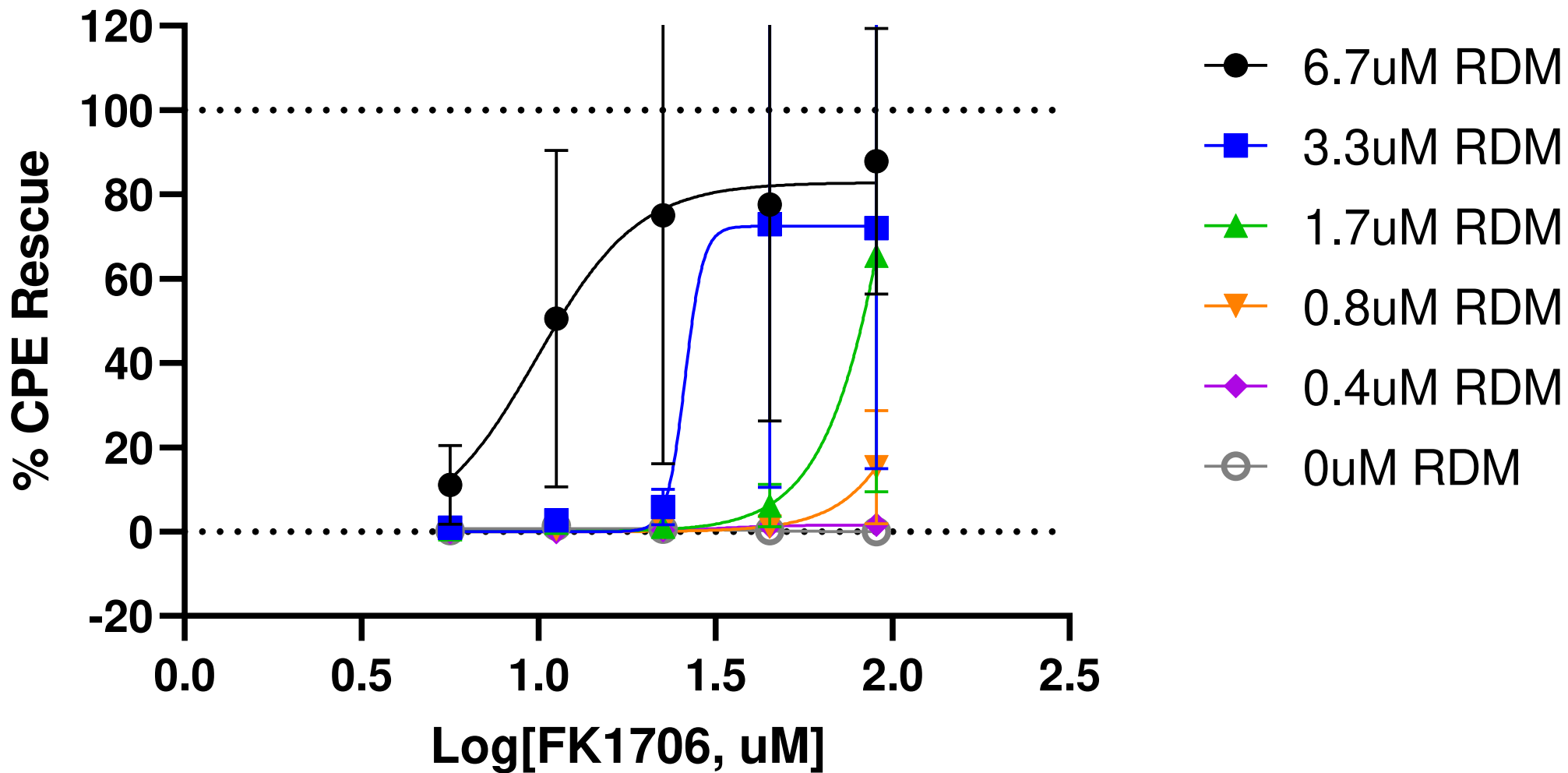
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### Remdesivir dose response (CPE)



	90uM FK1706	45uM FK1706	22.5uM FK1706	11.3uM FK1706
EC50	1.789	3.468	18.55	20.76

# FK1706 dose response (CPE)



	6.7uM RDM	3.3uM RDM	1.7uM RDM
EC50	9.926	25.96	137.9

**Table 1. Anti-SARS-CoV-2 Cytoprotection Assay Results for FK1706 and antivirals against SARS-CoV-2 (USA-WA1/2020).**

Compound or combination	EC <sub>50</sub> (μM)	TC <sub>50</sub> (μM)	TI
FK1706	>90	>90	--
FK1706 + Remdesivir (3 μM)	<11.3	>90	>7.96
Remdesivir (3 μM)	>3	>3	--
Remdesivir	3.63	>100	>27.5
FK1706 + M128533 (1 μg/mL)	<11.3	>90	>7.96
M128533 (1 μM)	>1	>1	--
M128533 (μg/mL)	1.53	86.8	56.7
FK1706 + Molnupiravir (0.3 μM)	28.7	>90	>3.14
Molnupiravir (single conc.)	>0.3	>0.3	--