Communicative & Integrative Biology 5:2, 122–126; March/April 2012; © 2012 Landes Bioscience

Comparative analysis of calcineurin signaling between *Candida dubliniensis* and *Candida albicans*

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Keywords: ER stress, pH homeostasis, virulence, hyphal growth, serum survival, Crz1, Crz2

Candida dubliniensis, an emerging fungal pathogen, is the closest known species to the established pathogenic species *Candida albicans*. Despite the fact that these two species share > 80% genome sequence identity, they exhibit distinct properties such as less hyphal growth, reduced pathogenicity and increased sensitivity to sodium stress and elevated temperatures in *C. dubliniensis* compared with *C. albicans*. It is, however, largely unknown whether signaling pathways are conserved in the two *Candida* species. Calcineurin signaling is known to be required for hyphal growth in *Cryptococcus neoformans* and *Aspergillus fumigatus* but remains elusive in *C. albicans*. Our recent study showed that calcineurin plays a clearly demonstrable role in controlling hyphal growth, drug tolerance and virulence in *C. dubliniensis*. Here, we extend our studies and show that calcineurin is conserved in controlling endoplasmic reticulum stress but distinct in governing pH homeostasis. Furthermore, we demonstrate that azole or echinocandin drugs in combination with the calcineurin inhibitor FK506 exhibit a synergistic effect against *C. dubliniensis* wild-type and echinocandin-resistant strains. The involvement of calcineurin in a variety of fungal virulence attributes and as a target for fungicidal synergism with azoles and echinocandins highlights the potential of combination therapy with calcineurin inhibitors for treating Candida infections.

Calcineurin is a eukaryotic Ca^{2+}-calmodulin-dependent serine/threonine specific protein phosphatase that is required for fungal virulence in a variety of human fungal pathogens including *C. albicans*,[1-3] *C. neoformans*,[4,5] and *A. fumigatus*.[6] We recently investigated the role of calcineurin in *Candida dubliniensis*, a sibling species of *C. albicans* that often infects the oral cavities of immunocompromised patients, such as those with HIV/AIDS.[7,8] In *C. dubliniensis,* we found that calcineurin is required for cell wall integrity, hyphal growth, serum survival and virulence.[9] Calcineurin played a greater role in serum survival of *C. albicans* compared with *C. dubliniensis*[9] (**Fig. 1**). Similar to results in *C. albicans,* the calcineurin downstream target Crz1 was not required for serum survival in *C. dubliniensis*. Furthermore, calcineurin and *crz1*/*crz1* mutants had reduced tolerance to azole and echinocandin antifungal drugs in both species (**Fig. 1**).

While *C. albicans* is still the major cause of Candida infections, *C. dubliniensis* now causes 2 to 7% of all clinical cases of candidemia.[10,11] *C. dubliniensis* isolates are susceptible to azole antifungal drugs but can rapidly develop resistance during clinical therapy. Thus, it is important to explore novel therapies and drug combinations for treatment. Studies in *C. albicans* have shown that calcineurin is required for azole and/or echinocandin tolerance.[12] Here, we demonstrate that FK506 exhibits synergism with fluconazole, posaconazole, and caspofungin based on in vitro minimum inhibitory concentration (MIC) and checkerboard assays against a wild-type and an echinocandin-resistant strain, suggesting the potential for drug combination therapy.

We are also interested in further characterizing the roles of calcineurin, especially with respect to endoplasmic reticulum (ER) stress and pH homeostasis. The ability to switch to hyphal growth in response to ER stress and to maintain pH homeostasis are both critical for virulence of *C. albicans*.[13] Previously, we found that calcineurin and Crz1 are required for hyphal growth in *C. dubliniensis* on nutrient limiting media.[9] However, the roles of calcineurin and Crz1 in *C. albicans* hyphal growth remain unclear with conflicting data reported on possible roles of calcineurin and Crz1 in *C. albicans* morphogenesis (**Fig. 1**).[2,3,12,14] Here, we characterize two stress responses associated with hyphal growth in *C. dubliniensis* and *C. albicans*.

Calcineurin is Essential for ER Stress Response in *C. dubliniensis* and *C. albicans*

ER stress due to an accumulation of unfolded or misfolded proteins activates the unfolded protein response (UPR) signaling pathway in eukaryotic cells[15,16] and has been shown to be involved in *C. albicans* morphogenesis.[17] UPR signaling activates a variety of downstream transcripts, including the bZIP transcription factor Hac1 that impacts *C. albicans* hyphal growth in response to ER stress.[17] Tunicamycin (TM) and dithiothreitol (DTT) induce the

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Submitted: 10/09/11; Revised: 11/22/11; Accepted: 11/22/11
http://dx.doi.org/10.4161/cib.18833

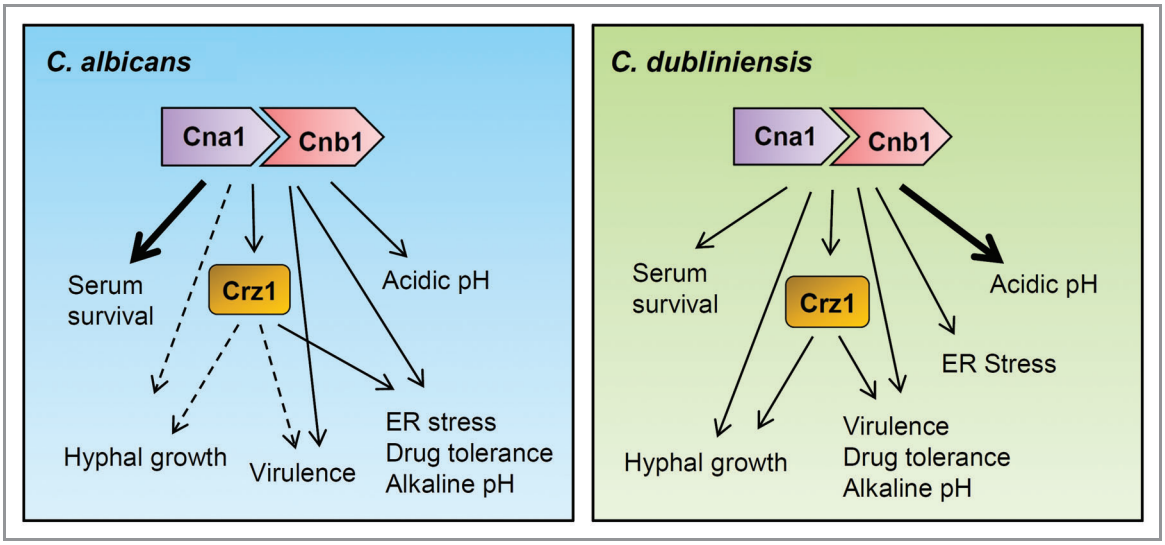

Figure 1. Comparison of the calcineurin pathway in *C. albicans* and *C. dubliniensis*. The roles of calcineurin and Crz1 in hyphal growth are unclear in *C. albicans* (dotted arrows) while their roles in hyphal growth of *C. dubliniensis* (right panel) are demonstrable. Calcineurin is required for virulence in both Candida species. ER stress responses are controlled by Crz1-dependent and Crz1-independent calcineurin signaling in *C. albicans* and *C. dubliniensis*, respectively. Drug tolerance and alkaline pH homeostasis are governed by the Crz1-dependent calcineurin pathway in both Candida species. *C. dubliniensis* calcineurin plays a larger role (bold arrow) in controlling acidic pH homeostasis compared with *C. albicans*. In *C. albicans* calcineurin plays a greater role (bold arrow) in serum survival compared with *C. dubliniensis*.

UPR by inhibiting N-linked glycosylation of secreted proteins and preventing disulfide bond formation, respectively.[16] We found that *C. albicans* and *C. dubliniensis* calcineurin mutants are hypersensitive to tunicamycin and DTT compared with the wild-type (**Fig. 2**). Furthermore, the role of Crz1 in ER stress is different in *C. albicans* compared with *C. dubliniensis* (**Fig. 2**).

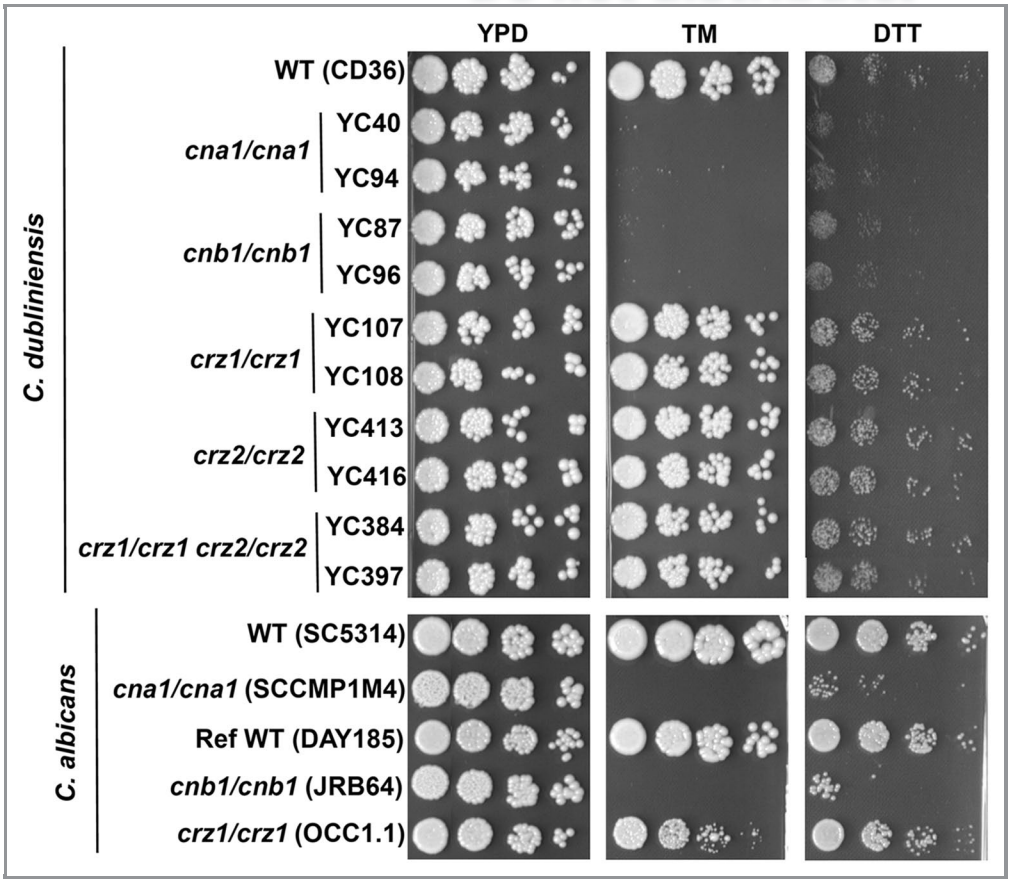

Figure 2. Calcineurin functions are conserved whereas Crz1 has different roles during ER stress in *C. albicans* and *C. dubliniensis*. *C. dubliniensis* and *C. albicans* calcineurin mutants were hypersensitive to the ER stress inducers tunicamycin (TM) and dithiothreitol (DTT). The *C. albicans crz1/crz1* mutants, but not *C. dubliniensis crz1/crz1* mutants, exhibit TM sensitivity intermediate between wild-type and calcineurin mutants. Cells were grown overnight in YPD medium at 24°C, washed twice in dH_2O, 5-fold serially diluted, and spotted onto YPD medium containing TM (1 μg/ml) or DTT (45 mM). All strains were spotted on the same media. Strains tested are described in reference.[9]

While *C. dubliniensis crz1/crz1*, *crz2/crz2*, and *crz1/crz1 crz2/crz2* double mutants grew similarly to wild-type in the presence of TM, the *C. albicans crz1/crz1* mutant exhibited attenuated growth that was intermediate between wild-type and calcineurin mutants (**Figs. 1 and 2**), indicating a divergent function of Crz1 in ER stress responses in both Candida species. Interestingly, we found that the *C. dubliniensis* wild-type strainis hypersensitive to the ER stress inducer DTT compared with the *C. albicans* wild-type strain (**Fig. 2**), indicating that the tolerance to protein misfolding has been reduced in *C. dubliniensis* compared with *C. albicans*.

Calcineurin Plays a Greater Role in Acidic pH Homeostasis in *C. dubliniensis* Compared with *C. albicans*

Another crucial factor for hyphal growth and virulence of *C. albicans* is the ability to respond to environmental pH changes. Both calcineurin and Crz1 have been demonstrated to play a role in *C. albicans* tolerance to changes in environmental pH through the Rim101/pacC pH-sensing pathway.[18,19] Previoius studies have shown that Rim101 acts in parallel with calcineurin and Crz1 to allow for adaption to alkaline pH in *C. albicans*.[19] Furthermore, Crz1, Crz2, and calcineurin are needed for adaptation to acidic pH in *C. albicans*. The role of calcineurin in pH homeostasis of *C. dubliniensis* was unknown. *C. dubliniensis* forms true hyphae less efficiently than *C. albicans* in response to pH shifts in vitro.[20,21] Here, we show that *C. dubliniensis cna1/cna1* and *cnb1/cnb1* mutants are hypersensitive to acidic and alkaline pHs compared with wild-type (**Fig. 3**), whereas *C. albicans* calcineurin mutants did not show attenuated growth at acidic pH. This result differs from previous studies where calcineurin was shown to be required for growth at acidic pH[19] and may be attributable to the use of different pH buffering systems or experimental procedures. Taken together, we conclude that in *C. dubliniensis* calcineurin plays a greater role in acidic pH homeostasis compared with its role in *C. albicans* (**Figs. 1 and 3**). Interestingly, while *C. dubliniensis* and *C. albicans crz1/crz1* mutants grew similarly to wild-type at acidic pH, they exhibited attenuated growth that was intermediate between wild-type and calcineurin mutants at alkaline pH. However, *C. dubliniensis crz2/crz2* mutants did not have growth defects in acidic or alkaline pH (**Fig. 3**). These results suggest that alkaline pH homeostasis is controlled by the

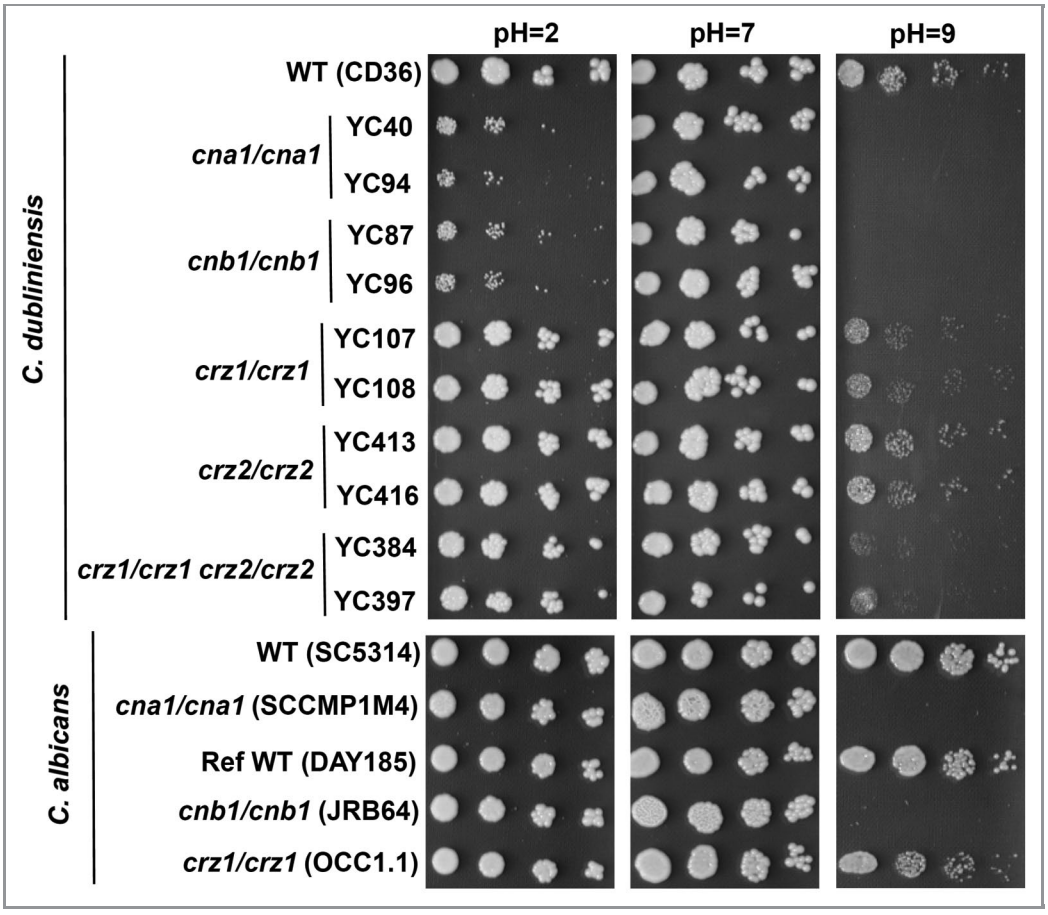

Figure 3. Calcineurin plays a greater role in controlling acidic pH response in *C. dubliniensis*. Calcineurin is essential for growth at alkaline conditions (pH = 9) in *C. dubliniensis* and *C. albicans*. While *C. dubliniensis* calcineurin mutants had attenuated growth at acidic pH, the *C. albicans* calcineurin mutants did not. Cells were grown overnight in YPD medium at 24°C, washed twice in dH_2O, 5-fold serially diluted, and spotted onto YPD medium containing 150 mM HEPES buffered at pH 2 or 7. For pH 9 medium, YPD was buffered with 150 mM pH 14 HEPES (85 ml of YPD plus 15 ml of 1M HEPES at pH 14). The pHs of solid media were confirmed with pH indicator strips (Sigma-Aldrich, Z134147). All strains were spotted on the same media.

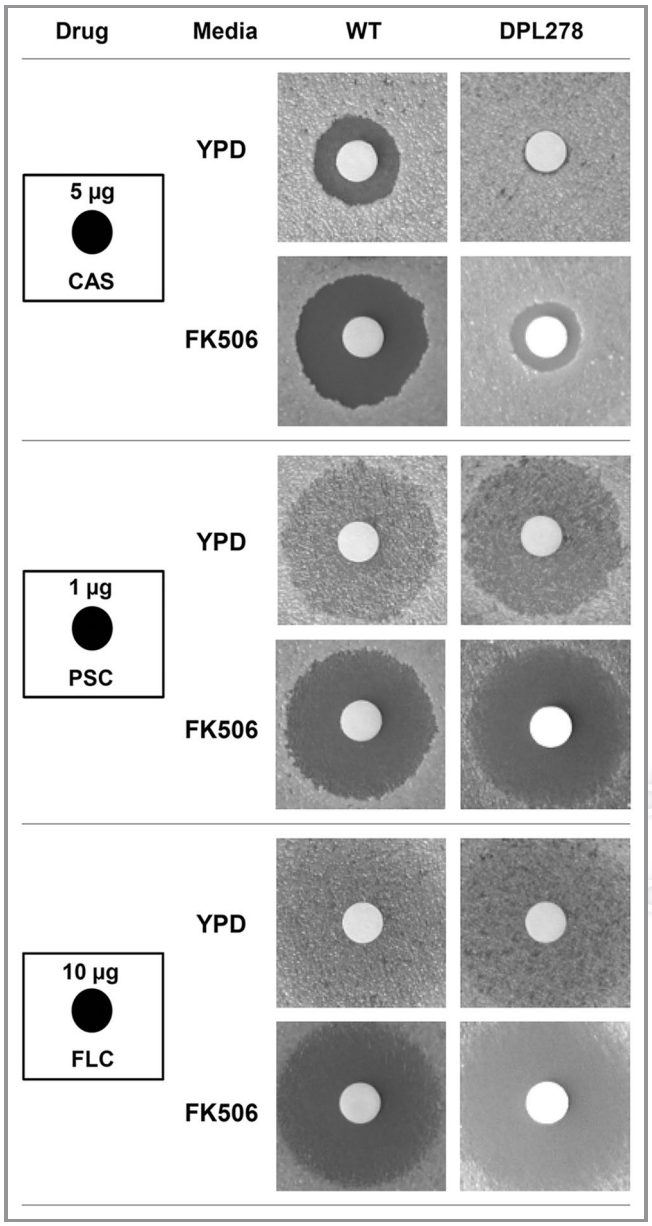

Figure 4. Calcineurin inhibitor exhibits synergism with caspofungin against wild-type and echinocandin-resistant *C. dubliniensis* strain. Disk diffusion assays were used to determine echinocandin and azole susceptibility of a wild-type (CD36) and an echinocandin-resistant strain (DPL278). Cells were grown overnight at 30°C, and cells at a density of 0.1 OD_{600} (in 100 μl) were spread on the surface of YPD medium in the absence or presence of FK506 (1 μg/ml). The disk (6 mm) containing 5 μl of caspofungin (CAS, 5 μg), posaconazole (PSC, 1 μg), or fluconazole (FLC, 10 μg) was placed on the surface of the plates, and incubated at 30°C for 48 h.

Crz1-dependent calcineurin pathway in *C. dubliniensis* and *C. albicans*, whereas acidic pH homeostasis is governed by a Crz1-independent calcineurin pathway in *C. dubliniensis*.

Calcineurin Inhibitor Exhibits Synergism with Caspofungin against *C. dubliniensis* Echinocandin-resistant Strain

To determine if the calcineurin inhibitor FK506 exhibits synergism with caspofungin against the echinocandin-resistant strain DPL278,[22] we performed disk diffusion and checkerboard assays following the procedures described by the Clinical and Laboratory Standards Institute (CLSI) protocol M27-A3. The DPL278 strain is resistant to caspofungin but susceptible to azoles (**Fig. 4 and Table 1**). We further found that the resistance to caspofungin of the DPL278 strain was reversed by supplementation with FK506 (**Fig. 4 and Table 1**). Meanwhile, we demonstrated that FK506 exhibits synergism with caspofungin, fluconazole, or posaconazole against *C. dubliniensis* wild-type and echinocandin-resistant strain [fractional inhibitory concentrations (FIC) < 0.5, **Table 1**], suggesting that these drug combinations have therapeutic potential for *C. dubliniensis* infections.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Cecelia Shertz and Joanne Kingsbury for edits and comments on the manuscript, and David Perlin and Ana Alastruey-Izquierdo at the Public Health Research Institute Center of UMDNJ-New Jersey Medical School for the *C. dubliniensis* echinocandin- resistant strain DPL278. These studies were funded by a Duke University Chemistry Department Undergraduate Research Fellowship (J.Z.), the Center for AIDS Research (CFAR grant, 2P30 AI064518–06 to Y.-L.C.), NIH/NIAID R01 grant AI50438 (J.H.), and pilot funds from Merck and Co. Inc. and Astellas Pharma Inc. (J.H. and Y.-L.C.).

Table 1. FK506 exhibits synergism with azole or echinocandin drugs against *C. dubliniensis* wild-type and echinocandin-resistant strains

Strain	MIC_{50} alone (μg/ml)[a]				FIC[b]		
	CAS	FLC	PSC	FK506	CAS + FK506	FLC + FK506	PSC + FK506
WT (CD36)	0.5	0.125	0.0078	> 4.0	0.0176	0.375	0.0197
DPL278	4.0	0.125	0.0156	> 4.0	0.0781	0.125	0.1289

[a]One wild-type *C. dubliniensis* strain (CD36) and one echinocandin-resistant strain (DPL278) were grown overnight with shaking at 30°C and washed twice in dH_2O. The OD_{600} was taken of the cultures with a spectrophotometer and diluted to 0.01 OD_{600}/ml in RPMI-1640 medium (Sigma R6504, 8.4 g in 1 L dH_2O buffered to pH = 7 with sodium hydroxide pellets). Minimum inhibitory concentrations (MIC) of each drug alone and fractional inhibitory concentrations of the drugs in combination were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol M27-A3. Final concentrations of fluconazole (FLC) and caspofungin (CAS) ranged from 16 to 0.0312 μg/ml. FK506 concentrations ranged from 4.0 to 0.063 μg/ml while posaconazole (PSC) concentrations ranged from 2.0 to 0.0039 μg/ml. [b]FIC index = ($MIC_{combined}$ drug 1/MIC_{alone} drug 1) + ($MIC_{combined}$ drug 2/MIC_{alone} drug 2) FIC $\leq$ 0.5 (synergy), $>$ 0.5 but $<$ 1.0 (additive), $>$ 1.0 but $\leq$ 2.0 (no interaction), $>$ 2.0 (antagonism).

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