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1 ***In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of**

2 **vulvovaginal candidiasis**

3

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28

29 Running title: Treatment of vulvovaginal candidiasis with NFAP2

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30 **Abstract**

31 In the consequence of emerging number of vulvovaginitis caused by azole-resistant and
32 biofilm-forming *Candida* species, the fast and efficient treatment of this infection has become
33 challenging. The problem is further exacerbated by the severe side-effects of azoles as long-
34 term use medications in the recurrent form. There is therefore an increasing demand for novel
35 and safely applicable effective antifungal therapeutic strategies. The small, cysteine-rich and
36 cationic antifungal proteins from filamentous ascomycetes are potential candidates as they
37 inhibit the growth of several *Candida* spp. *in vitro*; however no information is available about
38 their *in vivo* antifungal potency against yeasts. In the present study we investigated the
39 possible therapeutic application of one of their representatives in the treatment of
40 vulvovaginal candidiasis, the *Neosartorya fischeri* antifungal protein 2 (NFAP2). NFAP2
41 inhibited the growth of a fluconazole (FLC)-resistant *Candida albicans* strain isolated from
42 vulvovaginal infection, and it was effective against both planktonic cells and biofilm *in vitro*.
43 We observed that the fungal cell killing activity of NFAP2 is connected to its pore-forming
44 ability in the cell membrane. NFAP2 did not exert cytotoxic effects on primary human
45 keratinocytes and dermal fibroblasts at the minimal inhibitory concentration *in vitro*. *In vivo*
46 murine vulvovaginitis model experiments showed that NFAP2 significantly decreases the cell
47 number of the FLC-resistant *C. albicans*, and the combined application with FLC enhances
48 the efficacy. These results suggest that NFAP2 provides a feasible base for the development
49 of a fundamental new, safely applicable mono- or polytherapeutic topical agent in the
50 treatment of superficial candidiasis.

51

52 **Keywords**

53 *Neosartorya fischeri* antifungal protein 2, *Candida albicans*, vulvovaginitis, *in vitro*
54 susceptibility, antifungal mechanism, *in vitro* cytotoxicity, *in vivo* murine model

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55 **Introduction**

56 *Candida* spp. belong to the normal human flora under the control of a sensitive and well-
57 regulated balance mechanism between the fungus and the host-defense system. If this
58 mechanism is disturbed by physiological or non-physiological changes, *Candida* can
59 overgrow the dermal and mucosal surfaces in healthy individuals. One of these symptoms is
60 the vulvovaginal candidiasis (VVC), when *Candida* infects the surface of vaginal and vulvar
61 mucosa (1). VVC is estimated to be the most common fungal infection in a number of
62 countries (2), and has been considered to be an important worldwide public health problem by
63 the World Health Organization (3). VVC affects ~75% of adult women at least once in their
64 lifetime, ~15% of the cases are asymptomatic, and ~10% are recurrent (RVVC) which means
65 more than four infection episodes per year in the absence of predisposing factors. Although
66 VVC is not associated with mortality, it causes discomfort, pain, and social embarrassment
67 which impair sexual and affective relationships, and work performance. Untreated VVC can
68 lead to severe complications, such as vaginitis and penitis if it is transferred to the male
69 partner; and as a consequence pelvic inflammation, infertility, ectopic pregnancy, pelvic
70 abscess, spontaneous abortion and menstrual disorders can occur (1).
71 *Candida albicans* is still the most common VVC associated yeast in most countries. However,
72 epidemiology surveys from the last 15 years have demonstrated an increasing prevalence of
73 non-albicans *Candida* (NAC) species (1). The recommended treatment in the US for
74 uncomplicated *C. albicans* VVC is the vaginal application of nystatin or azole-based topical
75 agents, but considering the personal preference, a single oral dose of 150 mg fluconazole
76 (FLC) is suggested alternatively. For severe acute cases such as RVVC, 150 mg FLC, given
77 every 72 hours for a total of two or three doses, is recommended for six months (4, 5). This
78 long-term FLC use may cause severe side effects in the host (e.g. liver toxicity) and promote
79 the development of a resistance mechanism in the fungus (6). Susceptibility data indicate a

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80 continuous increase in the number of VVC-related and FLC-resistant *C. albicans* isolates (2,
81 7). The development of resistance mechanism is connected to the biofilm-forming ability of
82 the fungus. Namely, *C. albicans* is able to adhere to the surface of vaginal epithelium and
83 form a complex three-dimensional structure of fungal cell agglomerates with reduced
84 susceptibility to azoles and less sensitivity to the killing mechanisms of the host immune
85 system resulting in RVVC frequently (4). Therefore, nowadays the fast and efficient treatment
86 of RVVC becomes more and more challenging, and novel, safely applicable antifungal
87 strategies are needed with high efficiency against *Candida* biofilms.
88 *In vitro* susceptibility data suggest that the small molecular weight, cysteine-rich and cationic
89 antifungal proteins secreted by filamentous ascomycetes (crAFP) are potential therapeutic
90 candidates to fight against *Candida* infections (8-13). In our previous study we already
91 demonstrated that one of their representatives, the *Neosartorya fischeri* antifungal protein 2
92 (NFAP2) effectively inhibits the growth of clinically relevant *Candida* spp. in the
93 standardized clinical susceptibility Clinical and Laboratory Standards Institute (CLSI) M27-
94 A3 testing method, and interacts synergistically with FLC *in vitro* (12). These observations
95 propose the *in vivo* efficacy and potential applicability of NFAP2 as mono- or polytherapeutic
96 agent in anti-*Candida* therapy.
97 To prove this assumption, in the present study we investigated the *in vivo* applicability of
98 NFAP2 in the treatment of VVC. First of all, we determined the *in vitro* cell-killing efficacy
99 and antifungal mechanism of NFAP2 against a FLC-resistant and biofilm-forming *C. albicans*
100 strain isolated from human VVC, before testing the *in vitro* cytotoxicity of NFAP2 on primary
101 human keratinocytes (HKC) and dermal fibroblasts (HDF). Based on the promising *in vitro*
102 results, we successfully applied NFAP2 alone and in combination with FLC in an *in vivo*
103 murine VVC model system.
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105 **Results**

106 ***In vitro* susceptibility.** In our previous work we observed that the antifungal efficacy and the
107 minimal inhibitory concentration (MIC) of NFAP2 depend on the applied test medium and the
108 investigated *Candida* strain (10, 12). One of the major virulence factors of *C. albicans* is the
109 ability to form a biofilm, which shows less susceptibility or intrinsic resistance to
110 conventional antifungal agents. Furthermore, the formation of biofilm plays a role in the
111 colonization of mucosal surfaces (14). Hence, we determined the exact MICs of FLC and
112 NFAP2 for planktonic and sessile biofilm cells of *C. albicans* 27700 in RPMI 1640 medium
113 simulating the human extracellular environment in composition. MIC values of FLC proved
114 to be 16 µg/ml and 512 µg/ml for planktonic and sessile cell population, respectively.
115 According to susceptibility breakpoints (15), *C. albicans* 27700 is resistant to FLC. Both cell
116 types showed the same susceptibility to NFAP2 with MICs of 800 µg/ml. It is noteworthy,
117 that 400 µg/ml NFAP2 already caused >50% decrease in turbidity and metabolic activity for
118 planktonic cells. At this concentration NFAP2 was inactive against the biofilm, significant
119 decrease in turbidity and in metabolic activity was not observed.

120 **Anti-*Candida* mechanism.** Our previous observations applying the membrane impermeant,
121 red-fluorescent nuclear and chromosome stain propidium-iodide (PI) already suggested the
122 prompt plasma membrane disruption ability of NFAP2 on yeast cells as the key factor of the
123 antifungal effect (10, 12), but the exact mechanism for the membrane disruption has not been
124 investigated yet. First, we quantified the number of disrupted cells by fluorescence-activated
125 cell sorting (FACS) analysis. It revealed that 38.20±3.12% ($p = 0.00007$) of the FLC-resistant
126 *C. albicans* 27700 cells have a PI-positive phenotype after 24 hours of NFAP2-treatment at
127 the MIC compared to the untreated control (3.26±1.72%) (Fig. S1 in the supplemental
128 material). Scanning electron microscopy (SEM) images showed that NFAP2 forms pores in
129 the plasma membrane, causing the loss of cell content which finally results in cell death (Fig.

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130 1). Several different molecular mechanisms of membrane disruption were proposed for
131 antimicrobial peptides and proteins previously. Many of such mechanisms (including pore
132 formation) involve significant conformational changes and/or oligomerization of the
133 membrane-acting proteins (16-18). This conformational change can be detected by electronic
134 circular dichroism spectroscopy (ECD) (19). We observed that the ECD spectrum of NFAP2
135 in the presence of yeast cells is similar to that of the pure aqueous NFAP2 solution and
136 demonstrates previously described spectral contributions emerging from β-conformation (200
137 nm, 212 nm) and disulfide bridges (228 nm) (Fig. 2) (12). The presence of *C. albicans* 27700
138 cells did not induce any change in the secondary structure of the protein within 24 hours of
139 incubation. However, the number of colony forming units (CFU) decreased significantly (p =
140 000062), from $6.10 \pm 0.54 \times 10^6$ cells/ml to $2.49 \pm 0.34 \times 10^6$ cells/ml in the samples, during the
141 24 hours time frame of ECD measurements. This suggests that while 100 mg/ml NFAP2
142 exposure results in notable cell death, mechanisms of action accompanied by large scale
143 structural changes can be ruled out for NFAP2.

144 ***In vitro* cytotoxicity.** *In silico* prediction showed high binding affinity of NFAP2 to the
145 human serum albumin (HSA) (ΔG = -12.16 kcal/mol, Kd = 1.21e-09 M) (20), hence its
146 systemic application as antifungal drug is debatable. However, NFAP2 is considered as a
147 potential candidate for a novel topical antifungal agent, and the most possible therapeutic
148 application is the treatment of superficial candidiasis (12). To verify this suggestion, it is
149 necessary to elucidate the cytotoxic potential of the protein on HKC and HDF as the
150 predominant cell type in the epidermis, and the most common cells of connective tissue
151 synthesizing the extracellular matrix and collagen, respectively. *In vitro* viability staining of
152 primary HKCs and HDFs with PI after exposure to NFAP2 for 24 hours revealed no change
153 in the number of PI-positive cells even after treatment with twice the MIC (Fig. S2 in the
154 supplemental material).

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155 ***In vivo* application.** Based on the observed *in vitro* MIC values, NFAP2 is considered as a
156 monotherapeutic agent in the treatment of VVC caused by FLC-resistant strains. *In vitro* data
157 already suggested that NFAP2 could interact synergistically with FLC against *C. albicans*
158 (12), hence the *in vivo* antifungal effect of NFAP2-FLC combination was also investigated to
159 reveal a possible FLC-resistance reversion. Results of the *in vivo* experiments are shown in
160 Fig. 3. The single 35 mg/kg and the daily 5 mg/kg doses of FLC could not reduce
161 significantly ($p > 0.05$) the vaginal fungal burden compared to untreated mice. In comparison
162 with the untreated group of animals, 800 μg/ml/day NFAP2 regimens alone or in combination
163 with 5 mg/kg/day FLC caused significant reduction ($p \leq 0.05$) in the number of living *C.*
164 *albicans* cells from vaginal tissue. This reduction was more prominent when NFAP2 was
165 applied in combination with FLC ($p = 0.0017$) than as a monotherapeutic agent ($p = 0.0177$).
166 Furthermore, the yeast cell number decreasing activity of NFAP2-FLC combination proved to
167 be significantly more effective than that of FLC alone ($p = 0.0001$ and $p = 0.0084$ compared
168 to 35 mg/kg single and 5 mg/kg daily dose, respectively). All significance values are indicated
169 in Table S2 in the supplemental material.
170 **Histology**. Grocott-Gömöri methenamine-silver nitrate (GMS) staining revealed the presence
171 of yeast and pseudohyphal form of *Candida* cells in the vaginal tissues of infected mice (Fig.
172 4A-D). However, decrease in the fungal cell number was observable when the animal was
173 treated with NFAP2 or NFAP2-FLC combination (Fig. 4C and D) in comparison with the
174 untreated and FLC-treated groups (Fig. 4A and B). Inflammatory reaction indicated by
175 neutrophilic granulocytes was observable in all samples stained with hematoxylin-eosin
176 (H&E) (Fig. 4), but it was more moderate in NFAP2 and NFAP2+FLC treated animals (Fig.
177 4C and D) than in untreated and FLC-treated groups (Fig. 4A and B). The vaginal
178 inflammation detected in uninfected mice could have been the consequence of the prior
179 estradiol-valerate treatment (Fig. 4E) (21).

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181 **Discussion**

182 crAFPs (such as the NFAP2-related *Aspergillus giganteu*s antifungal protein, AFP; and
183 *Penicillium chrysogenum* antifungal protein, PAF) are of particular interest in the fight against
184 fungal infections as they show *in vitro* growth inhibitory activity against fungal pathogens,
185 and they are non-toxic to mammalian cells (22, 23). However, their *in silico* predicted strong
186 binding ability to HSA (ΔG = -13.52 kcal/mol, Kd = 1.22e-10 M for AFP, and ΔG = -11.09
187 kcal/mol, Kd = 7.33e-09 M for PAF) diminishes the expectations for systemic application
188 (20). In this study we provide for the first time information about the *in vivo* antifungal
189 efficacy of a crAFP as a topical agent in the treatment of mucosal infection caused by *C.*
190 *albicans*; an opportunistic human pathogenic yeast.

191 NFAP2 represents a novel, phylogenetically distinct group of crAFPs, and shows a unique
192 high anti-yeast activity *in vitro* (10, 12). The *in vivo* animal model experiments in our study
193 required the determination of the *in vitro* MIC of NFAP2 against the applied microorganism
194 for the infection, and the investigation of the cell-killing ability under clinically approved test
195 conditions. Previous studies demonstrated that *in vitro* antifungal efficacy of crAFPs highly
196 depends on the ion strength of the test medium (24, 25). According to this, NFAP2 shows
197 higher MICs on the same *Candida* strain in the highly cationic RPMI 1640 than in a low
198 cationic medium (12). This feature is not exclusive to NFAP2; relative high MICs were
199 observed for PAF (26) and NFAP (27), when their activity was tested against different human
200 pathogenic filamentous fungi in RPMI 1640. RPMI 1640 is a standard medium recommended
201 by CLSI for clinical susceptibility tests, and it simulates the composition of human
202 extracellular environment. Our results showed that both planktonic and sessile biofilm cells of
203 the tested FLC-resistant *C. albicans* isolated from human VVC are susceptible to NFAP2 in
204 this medium. Biofilm formation of *C. albicans* isolates from hospitalized patients is directly

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205 related to the virulence. *C. albicans* is more tolerant to antifungal drugs in this form than the
206 planktonic cells, contributing to the pathogenesis of superficial and systematic candidiasis
207 (28). Parallel to this observation, the sessile biofilm cells of the involved *C. albicans* isolate
208 were less susceptible to FLC and NFAP2 than the planktonic cells. The applied CLSI M27-
209 A3 method recommends 10^3 cells/ml as inoculum for the MIC determination. However, the
210 detected MIC based on this method does not guarantee the same inhibitory efficacy against
211 higher cell numbers (29). After 24 hours of incubation, around one-third of the yeast cells
212 were killed when the MIC of NFAP2 was applied against 10^7 cells/ml (Fig. S1 in the
213 supplemental material). This amount represents the yeast cell number that was used for the
214 vaginal infection in the *in vivo* animal model experiments.
215 The potential *in vivo* application of a drug candidate in the treatment of mycotic infections
216 highly depends on its fungal selectivity, namely the exerted antifungal mechanism on the
217 pathogenic fungi, and the cytotoxic effects on the host cells. Antifungal plant defensins with
218 similar features to crAFPs (such as disulfide-bond stabilized tertiary structure, positive net
219 charge, and amphipathic surface) are non-toxic to human cells, and they bind to specific
220 fungal membrane components of yeast cell causing membrane permeabilization and/or
221 disruption (30). These actions may require the conformational change of the antifungal plant
222 defensin (31). Our results show that the yeast cell killing activity of NFAP2 is realized by
223 pore formation in the fungal plasma membrane without any changes in the secondary
224 structure (Fig. 1 and Fig. 2). These observations together with the lack of *in vitro* toxicity
225 (even at twice the MIC, Fig. S2 in the supplemental material) on primary HKCs and HDFs
226 suggest the fungal selectivity of NFAP2 to yeast cells. Furthermore, based on the reported
227 antifungal mode of action of membrane destructive plant defensins (30), we hypothesize that
228 the presence of a fungal-specific plasma membrane target may be involved in the antifungal
229 mechanism of NFAP2. To reveal the nature of this target awaits further investigations.

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230 Membrane disrupting antifungal peptides are considered as a potential new class of
231 antifungals to treat FLC-resistant VVC, however, their *in vivo* antifungal potency in this
232 infection and their impact on the host body have not been tested yet (32, 33). Our above
233 discussed *in vitro* results proposed the *in vivo* therapeutic potency of NFAP2 as a topical
234 agent in the treatment of VVC caused by FLC-resistant *C. albicans*. Considering the fact that
235 biofilm formation is involved in the *C. albicans* colonization of mucosal surfaces (14), one
236 dosage of NFAP2 in the *in vivo* murine VVC model corresponded to the determined *in vitro*
237 MIC. However, total recovery from the infection was not reached at this dosage (Fig. 4C).
238 Instead, the daily application of NFAP2 significantly decreased the cell number of the FLC-
239 resistant *C. albicans* strain in the vagina in contrast to FLC (Fig. 3). This result proves the
240 potential effectiveness of NFAP2 monotherapy in the treatment of superficial yeast
241 infections. Until today the *in vivo* applicability of crAFPs as antifungal agents was
242 investigated only with PAF (34, 35). Since PAF effectively inhibits the growth of human
243 pathogenic filamentous fungi (23), its therapeutic potential was tested by Palicz et al. (2016)
244 in a murine pulmonary aspergillosis model (35). Twice a day intraperitoneal application of
245 PAF was not able to overcome the fungal invasion finally, however, it could prevent the
246 spread of *Aspergillus fumigatus* in the lung tissue in the first days and prolonged the survival
247 of the animals with one day (35).
248 Before the present study, the described *in vitro* synergistic interaction between NFAP2 and
249 FLC against *Candida* isolates already suggested the polytherapeutic potential of the protein
250 (12). Our results from *in vivo* murine VVC model experiments clearly corroborates that the
251 combined application of NFAP2 and FLC is more effective against the involved FLC-
252 resistant *C. albicans* isolate than the treatment with the two compounds alone (Fig. 3). This
253 result suggests a positive *in vivo* interaction between them in the vaginal tissue and the
254 reversion of FLC-resistance. Similarly to our findings a better outcome was observed in a

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255 murine pulmonary aspergillosis model when PAF was combined with amphotericin B
256 (AMB), namely the PAF-AMB combination prolonged the survival of the animals and
257 decreased the lung injury score compared to their monotherapeutic application (35).
258 Intranasal application of PAF in mice did not alter the important physiological parameters of
259 the animals and did not cause morphological changes in the affected organs. Furthermore
260 inflammatory response of the skin following PAF application was not observed (34). Based
261 on these and other *in vivo* toxicity results PAF is considered as a safely applicable antifungal
262 compound (34, 35). Our histological examinations signed that NFAP2 could also be safely
263 used in topical therapy since it did not cause morphological alterations and serious
264 pathological reactions of the vaginal and vulvar tissues (Fig. 4), and did not change the
265 macromorphology of the affected organs (data not shown). The presence of neutrophilic
266 granulocytes after NFAP2 application indicates that they are recruited to the site of the
267 infection to kill the fungal pathogen (Fig. 4C and D), and NFAP2 does not inhibit this
268 process. However, the fungal infection was still present in the vagina after treatment with
269 NFAP2 or NFAP2-FLC combination (Fig. 4C and D); significant decrease in the viable *C.*
270 *albicans* cell number was observed in comparison with the untreated group of animals (Fig.
271 3). As NFAP2 did not show any cytotoxic effects even at twice the MIC (Fig. S2 in the
272 supplemental material), the protein should be administered in higher doses than the *in vitro*
273 MIC dose applied in our experiments to reach the full recovery from the infection.
274 Considering our *in vivo* results presented in this study and the fact that recombinant NFAP2
275 can be produced in high amount by the GRAS microorganism *P. chrysogenum* (12), this
276 protein provides a feasible base to develop a novel topical agent in the treatment of superficial
277 candidiasis caused by drug-resistant *Candida* strains.
278
279 **Materials and methods**

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280 **Strains and media.** The previously well-characterized FLC-resistant and biofilm-forming *C.*
281 *albicans* 27700 strain isolated from human vulvovaginal candidiasis was used in the
282 experiments (36). It was maintained on yeast extract glucose agar slants with KH_2PO_4
283 (YEGK) at 4 °C. Primary HKC and HDF cells were isolated and grown in CellnTec basal
284 (CnT-BM.1; CellnTec, Bern, Switzerland) and R10 medium, respectively, as described
285 previously (37). CFU was determined on yeast extract peptone dextrose (YPD) and
286 Sabouraud dextrose (SD) agar plates. *In vitro* antifungal susceptibility tests were performed in
287 RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 0.03% (w/v)
288 L-glutamine and buffered to pH 7.0 with 0.165 M 4-morpholinopropanesulfonic acid (Sigma-
289 Aldrich, St Louis, MO, USA). Media compositions are listed in Table S1 in the supplemental
290 material.

291 **Protein production and purification.** Recombinant NFAP2 was produced by *Penicillium*
292 *chrysogenum* and purified by cation-exchange chromatography as described before (12). To
293 exclude the effects of any contaminating compounds during the experiments, NFAP2 was
294 further purified by semipreparative reversed-phase high performance liquid chromatography
295 (RP-HPLC) on a Shimadzu-Knauer apparatus (Kyoto, Japan) to reach 100% purity (Fig. S3 in
296 the supplemental material). The following solvent system was applied: (A) 0.1% (v/v)
297 trifluoroacetic acid (TFA), (B) 80% (v/v) acetonitrile, 0.1% (v/v) TFA. Linear gradient from 0
298 to 30% (v/v) solvent (B) over 60 min was used at the flow rate of 4 ml/min. Peaks were
299 detected at 220 nm. Purity of the NFAP2 was checked by analytical RP-HPLC on an Agilent
300 1200 Series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) using the same
301 solvent system as for purification from 15 to 30% (v/v) solvent (B) over 15 min at 1 ml/min
302 flow rate.

303 ***In vitro* susceptibility testing.** Susceptibility testing of *C. albicans* 27700 planktonic cells to
304 FLC and NFAP2 was performed using the broth microdilution method in accordance with the

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305 CLSI approved standard M27-A3 protocol (38). The final drug concentrations ranged from 25
306 to 1600 µg/ml and from 2 to 1024 µg/ml for NFAP2 and FLC (Sigma-Aldrich, St Louis, MO,
307 USA), respectively. Susceptibility of sessile biofilm *C. albicans* 27700 cells to FLC and
308 NFAP2 was determined by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-
309 carboxanilide (XTT) reduction assay following the protocol described in Pierce et al. (2008)
310 (39) with slight modifications. Briefly, aliquots of 100 µl of standardized *C. albicans* 27700
311 suspension (1×10^6 CFU/ml) in RPMI 1640 were inoculated in wells of polystyrene flat-
312 bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland) and incubated statically for
313 24 hours at 37 °C to allow the biofilm-formation. The one-day-old biofilms were washed
314 three times with 200 µl saline in order to remove the non-attached fungal cells, then the final
315 concentration of NFAP2 (25-1600 µg/ml), and FLC (8-512 µg/ml) was pipetted onto them.
316 After 24 hours incubation at 37 °C, metabolic activity was quantified. Briefly, wells were
317 filled with 100 µl of 0.5 mg/ml XTT / 1 µM menadione solution (both from Sigma-Aldrich, St
318 Louis, MO, USA), and then the plates were covered with aluminum foil and incubated for 2
319 hours at 37°C. After this incubation period, the absorbance (A_{492}) of 80 µl supernatant was
320 measured in flat-bottom 96-well microtiter plates. MIC for planktonic and sessile biofilm cells
321 was defined as the lowest protein or drug concentration at which ≥90% reduction was
322 detected in turbidity and metabolic activity in comparison with the untreated control. The
323 percentage change in turbidity and metabolic activity was calculated on the basis of
324 absorbance (A_{492}) as $100\% \times (A_{well} - A_{background})/(A_{drug\text{-}free\ well} - A_{background})$. $A_{background}$
325 corresponds to the absorbance of fungal-free and drug-free wells. Susceptibility of *C. albicans*
326 27700 was tested in three independent experiments.
327 **FACS.** FACS, SEM and ECD investigations (later) were performed on mid-log phase *C.*
328 *albicans* 27700 cells grown up in RPMI-1640 medium at 30 °C under continuous shaking at
329 160 rpm. The proportion of the dead cells after NFAP2-treatment was determined by applying

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330 the membrane impermeant, red-fluorescent nuclear and chromosome stain PI (Sigma-Aldrich,
331 St Louis, MO, USA). The yeast cells (1×10^7 cells) were incubated in the presence of NFAP2
332 at MIC (800 μg/ml) in RPMI 1640 for 24 hours at 30 °C with continuous shaking at 160 rpm.
333 After incubation, cells were collected by centrifugation (17,000 × g, 2 min) and washed with
334 PBS (pH 7.4), then stained with 5 μg/ml PI for 10 min at room temperature in the dark, and
335 finally washed again with PBS (pH 7.4), before resuspending them in PBS (pH 7.4). The
336 number of PI-positive cells was counted and analyzed using FlowSight Imaging Flow
337 Cytometer (Amins, Merck Millipore, Billerica, MA, USA) and the related Image Data
338 Exploration and Analysis Software (IDEAS, Amins, Millipore, Billerica, MA, USA). Twenty
339 thousand cells were screened, and the FACS analysis was repeated in three independent
340 experiments. Cells treated with 70% (v/v) ethanol for 10 min at 4 °C were used as positive
341 staining control. Untreated cells (RPMI 1640 without NFAP2) were used as natural death
342 control. FACS analyses were achieved in three independent experiments.
343 **SEM.** *C. albicans* 27700 cells (1×10^7 cells) were treated with MIC of NFAP2 (800 μg/ml) as
344 described before for the FACS analysis. Untreated cells served as positive phenotype control.
345 Eight microliters of the cell suspensions in PBS were spotted on a silicon disc coated with
346 0.01% Poly-L-Lysine (Merck Millipore, Billerica, MA, USA), then the cells were fixed by
347 gently adding 2.5% (v/v) glutaraldehyde and 0.05 M cacodylate buffer (pH 7.2) in PBS (pH
348 7.4) for one hour. After that, the discs were washed twice with PBS (pH 7.4) and dehydrated
349 with a graded ethanol series (30%, 50%, 70%, 80%, and 100% (v/v) ethanol, each for 15 min
350 at room temperature). The samples were dried with Quorum K850 critical point dryer
351 (Quorum Technologies, Laughton, East Sussex, UK), followed by 12 nm gold coating and
352 observed under a JEOL JSM-7100F/LV scanning electron microscope (JEOL Ltd, Tokyo,
353 Japan).

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354 **ECD spectroscopy.** *C. albicans* 27700 cells were washed two times and resuspended in
355 ddH_2O or in aqueous solution of NFAP2 (100 µg/ml) in a final concentration of 10^7 cells/ml.
356 ECD spectroscopic measurements of these samples and an aqueous solution of NFAP2 (100
357 µg/ml) were performed in the 185-260 nm wavelength range using a Jasco-J815
358 spectropolarimeter (JASCO, Tokyo, Japan). Spectra were collected at 25 °C with a scan speed
359 of 100 nm/s using a 0.1 cm pathlength quartz cuvette. Spectra presented are accumulations of
360 10 scans for each sample. Spectrum acquisitions were done after 0 and 24 hours of incubation
361 of the samples at 30 °C under continuous shaking at 160 rpm. After the spectroscopic
362 measurements, CFU of the NFAP2-treated and untreated samples was determined. This
363 experiment was repeated twice.
364 **Determination of CFU.** Following ECD measurements, cells were collected by
365 centrifugation (17,000 × g, 2 min) and washed two times with YPD medium then ten-fold
366 serial dilutions were prepared in five steps in one milliliter YPD. 100 µl cell suspensions from
367 the last three steps were spread on YPD agar plates in three replicates. Colony number was
368 counted after incubation for 24 hours at 30 °C.
369 ***In vitro* cytotoxicity assay.** Fluorescence viability staining was performed on primary HKC
370 and HDF cells grown in chambered cell culture slides (Falcon, Corning Life Sciences,
371 Tewksbury, MA, USA). The cells (4×10^3 cells/well) were seeded and grown until they
372 reached 70-80% confluence at 37 °C and 5% CO_2, then NFAP2 in the concentration range
373 between 400-1600 µg/ml was added and the plates were incubated for 24 hours under the
374 same conditions. After the incubation period, the cells were washed with phosphate buffered
375 saline (PBS, pH 7.4) and the fluorescent dye PI (1 µg/ml) and 2′-(4-hydroxyphenyl)-5-(4-
376 methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate (Hoechst, 1 µg/ml;
377 Sigma-Aldrich, St Louis, MO, USA) were added for 10 minutes in the dark. Untreated cells
378 were used as living controls, and 50% ethanol-treated (for 10 minutes) as dead control. The

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379 cells were washed three times with PBS (pH 7.4) and observed with a Zeiss Axioplan
380 fluorescence microscope (Zeiss, Oberkochen, Germany), equipped with an Axiocam mono
381 microscope digital camera (Zeiss, Oberkochen, Germany), excitation/emission filters 365/420
382 nm for blue fluorescence and 546/590 or 565/620 nm for red fluorescence. Image acquisition
383 and editing was done with ZEN 2 (blue edition) microscope software (Zeiss, Oberkochen,
384 Germany) and GIMP 2 (GNU Image Manipulation Program, version 2.8.10). The study with
385 primary HKC and HDF was carried out in accordance with the recommendations of the Ethics
386 Committee of the Medical University of Innsbruck (Innsbruck, Austria). The protocol was
387 approved from the Ethics Committee of the Medical University of Innsbruck. All subjects
388 gave written informed consent in accordance with the Declaration of Helsinki. The *in vitro*
389 cytotoxicity assay was repeated twice.

390 ***In vivo* murine vulvovaginitis model.** Groups of ten BALB/c immunocompetent female
391 mice (weight: 20-22 g) were used in this study. The animals were maintained in accordance
392 with the Guidelines for the Care and Use of Laboratory Animals (40); experiments were
393 approved by the Animal Care Committee of the University of Debrecen (permission no.:
394 12/2014). Mice were administered 50 µl subcutaneous estradiol-valerate (10 mg/ml prepared
395 in sesame seed oil) 72 hours prior to infection to establish the VVC (41, 42). In accordance
396 with our previous studies, mice were challenged intravaginally with 1-1.2 $\times$ 10^7 CFU of *C.*
397 *albicans* 27700 in final volume of 25 µl (36, 42). Mice were divided into the following five
398 groups: i) untreated control, ii) 800 µg/ml/day NFAP2, iii) 35 mg/kg/once FLC which
399 corresponds to the normal human dose of 150 mg based on 24h-AUC value (43), iv)
400 5mg/kg/day FLC, and v) 800 µg/ml/day NFAP2 + 5 mg/kg/day FLC. All treatments were
401 started after 24 hours of the infection when the presence of *C. albicans* biofilm had become
402 evident on the murine vaginal mucosa (44). FLC treatment was given intraperitoneally at a
403 volume of 0.5 ml, while NFAP2 was administered intravaginally at a volume of 25 µl and one

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404 hour after the FLC treatment when it was applied in combination with FLC. Untreated control
405 mice were given 0.5 ml and/or 25 µl physiological saline for intraperitoneally and
406 intravaginally, respectively. At four days postinfection, fungal vagina burden was determined
407 after sacrificing of animals. Whole vaginae were excised, weighed and homogenized in one
408 milliliter saline. Aliquots of 100 µl of the undiluted and diluted (1:10) homogenates were
409 plated onto SD agar plates. The plates were incubated for 48 hours at 35 °C, and then the
410 CFUs were determined. The lower limit of detection was 50 CFU/g/tissue. All animal
411 experiments were repeated two times, and five animals were involved in each group in each
412 treatment.

413 **Histology.** Vaginae of different but identically treated mice were involved in histological
414 investigations as those described above. The histopathological examination and histochemical
415 staining were performed on routine formalin fixed, paraffin embedded, mouse vaginal tissues.
416 Serial 4 µm thick sections were cut from paraffin blocks and routine GMS and H&E stains
417 were performed (45).

418 ***In silico* analysis.** The binding ability of NFAP2 to HSA (UniProt IDs: A0A1D0CRT2 and
419 P02768, respectively; 46) was predicted by the PPA-Pred2 (Protein-Protein Affinity
420 Predictor) server (20).

421 **Statistical analyses.** CFU data after ECD experiments were analyzed using Microsoft Excel
422 2010 software (Microsoft,Edmond, WA, USA), and the two sample t-test was used to
423 determine the significance values. Vaginal burden was analyzed using Kruskal-Wallis test
424 with Dunn's post-test for multiple comparisons using the software GraphPad Prism version
425 6.05 (GraphPad Software, San Diego, CA, USA). Significance was defined as $p < 0.05$, based
426 on the followings: * : $p \leq 0.05$, ** : $p \leq 0.005$, *** : $p \leq 0.0001$.

427

428 **Supplemental material**

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429 Supplemental material for this article may be found at

430 **SUPPLEMENTAL FILE 1,** PDF file, 0.7 MB**.**

431

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442

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579 **Figure legends**

580

581 **FIG 1** Scanning electron microscopy of *C. albicans* 27700 cells after incubation in (A and B)
582 RPMI 1640 medium, and (C and D) in RPMI 1640 medium supplemented with 800 µg/ml
583 NFAP2 for 24 hours at 30 °C with continuous shaking at 160 rpm. Framed regions in (A and
584 C) are shown at higher magnification in (B and D) respectively. Arrows indicate the pore
585 formation in the cell envelop and the loss of cell content after exposure to NFAP2. Scale bars,
586 1 µm.

587

588 **FIG 2** ECD spectra of NFAP2 in ddH$_2$O (blue), and in the presence of *C. albicans* cells
589 immediately after exposure (red) to, and after 24 hours of incubation (green) with 100 µg/ml
590 NFAP2 at 30 °C with continuous shaking at 160 rpm.

591

592 **FIG 3** *In vivo* efficacy of NFAP2, FLC and their combination in murine vulvovaginitis
593 model. The bars represent the mean ± SEM (standard error of mean) of the vaginal tissue
594 burden of BALB/c mice intravaginally infected with FLC-resistant *C. albicans* 27700 isolate.
595 Significant differences (p-values) between the CFU numbers were determined based on the
596 comparison with the untreated control. Other significance values existing between the
597 different treatments are presented in Table S2 in the supplemental material. Level of
598 significant differences are indicated at $p \leq 0.05$ (*), $p \leq 0.005$ (**).

599

600 **FIG 4** Histological investigation of vaginal tissue from mice suffering from vulvovaginal
601 candidiasis (A) without and with topical (B) 5 mg/kg/day FLC, (C) 800 µg/ml NFAP2, and
602 (D) combined 5 mg/kg/day FLC + 800 µg/ml NFAP2 treatments. (E) Vaginal tissue of
603 uninfected mice. Vaginal tissues were stained with GMS (left) and H&E (right). Blue arrows

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604 indicate the presence of *C. albicans* 27700 cells (left images) and neutrophilic granulocytes

605 (right images). Scale bars, 50 µm.

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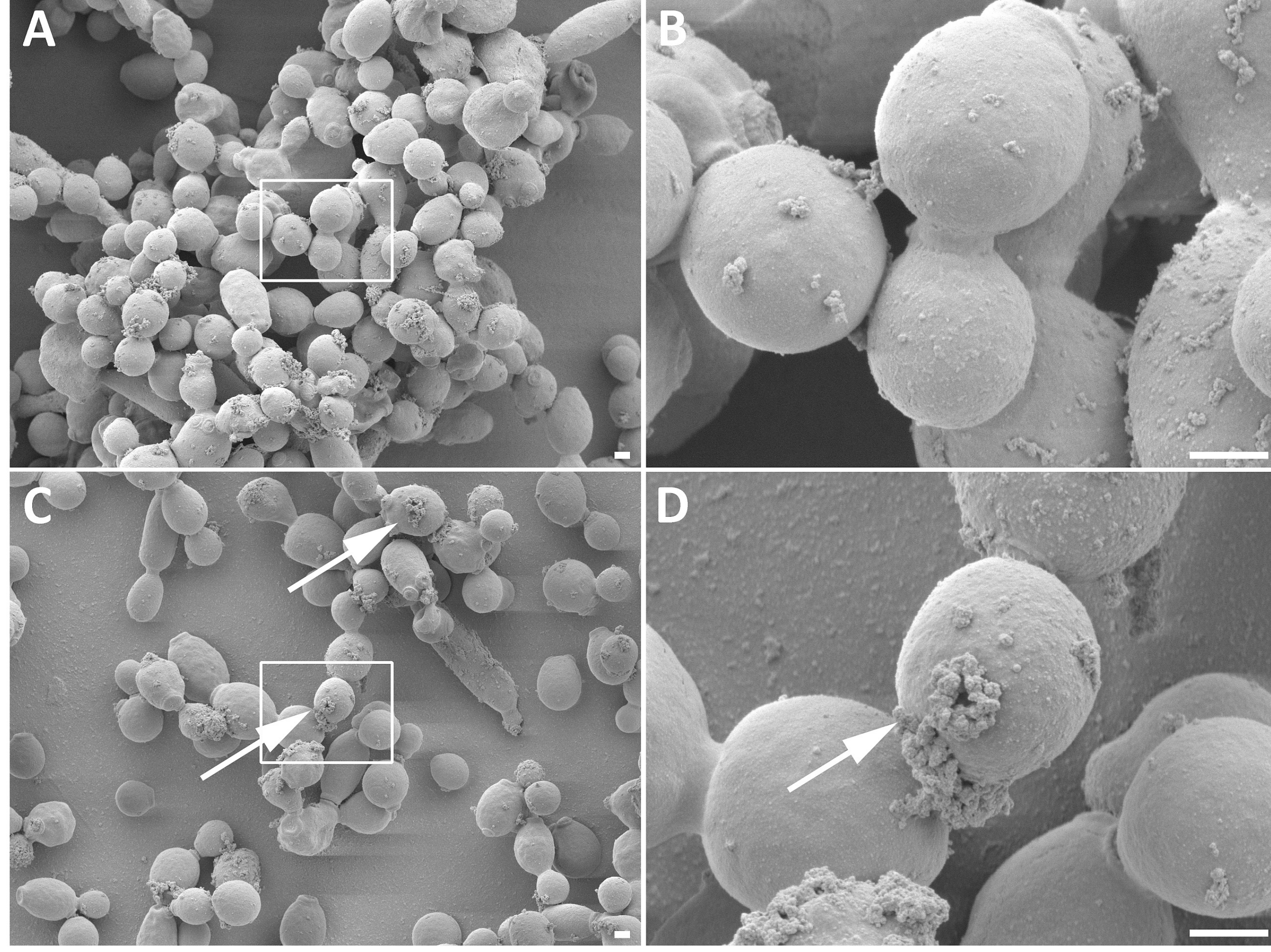

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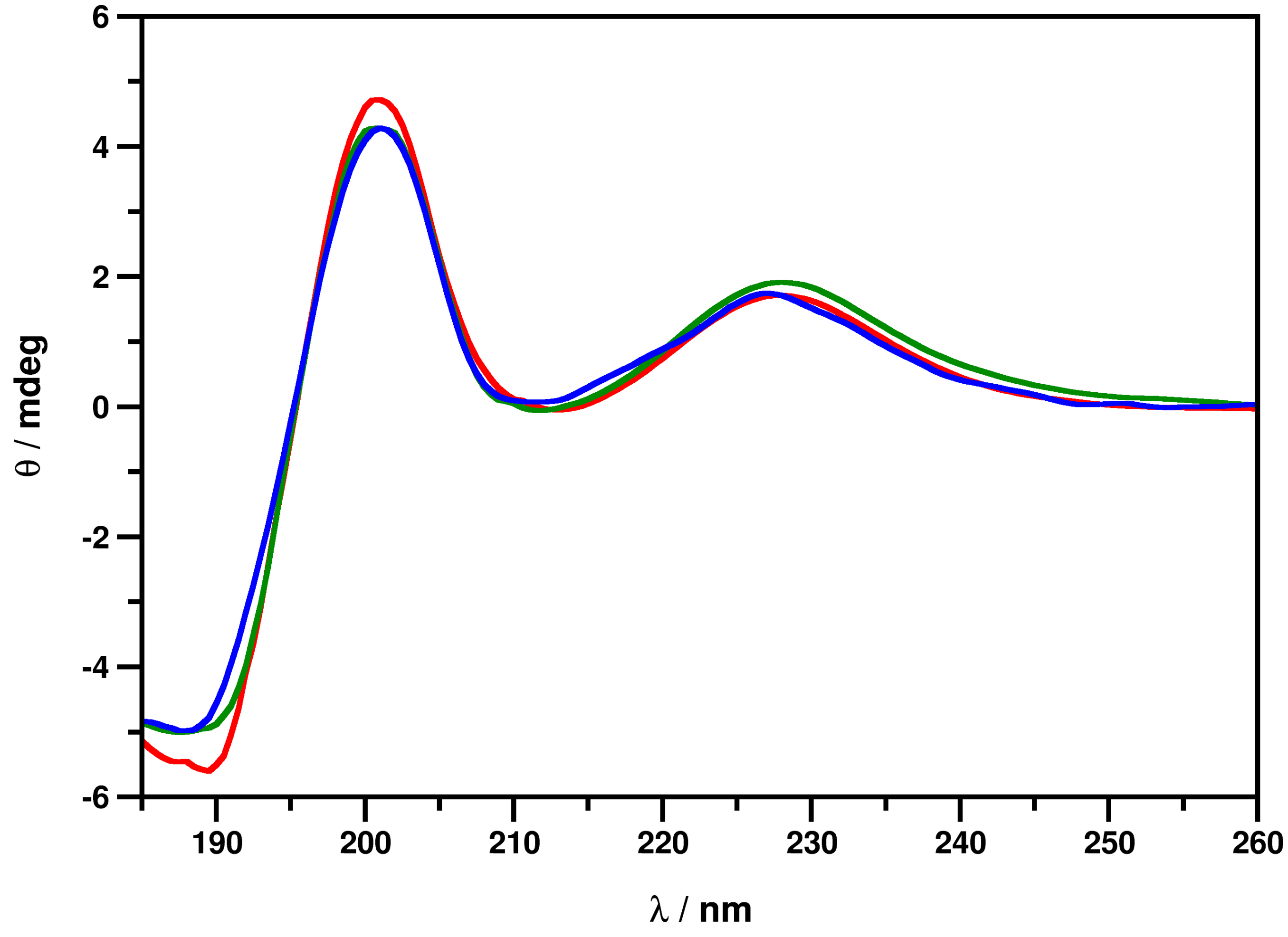

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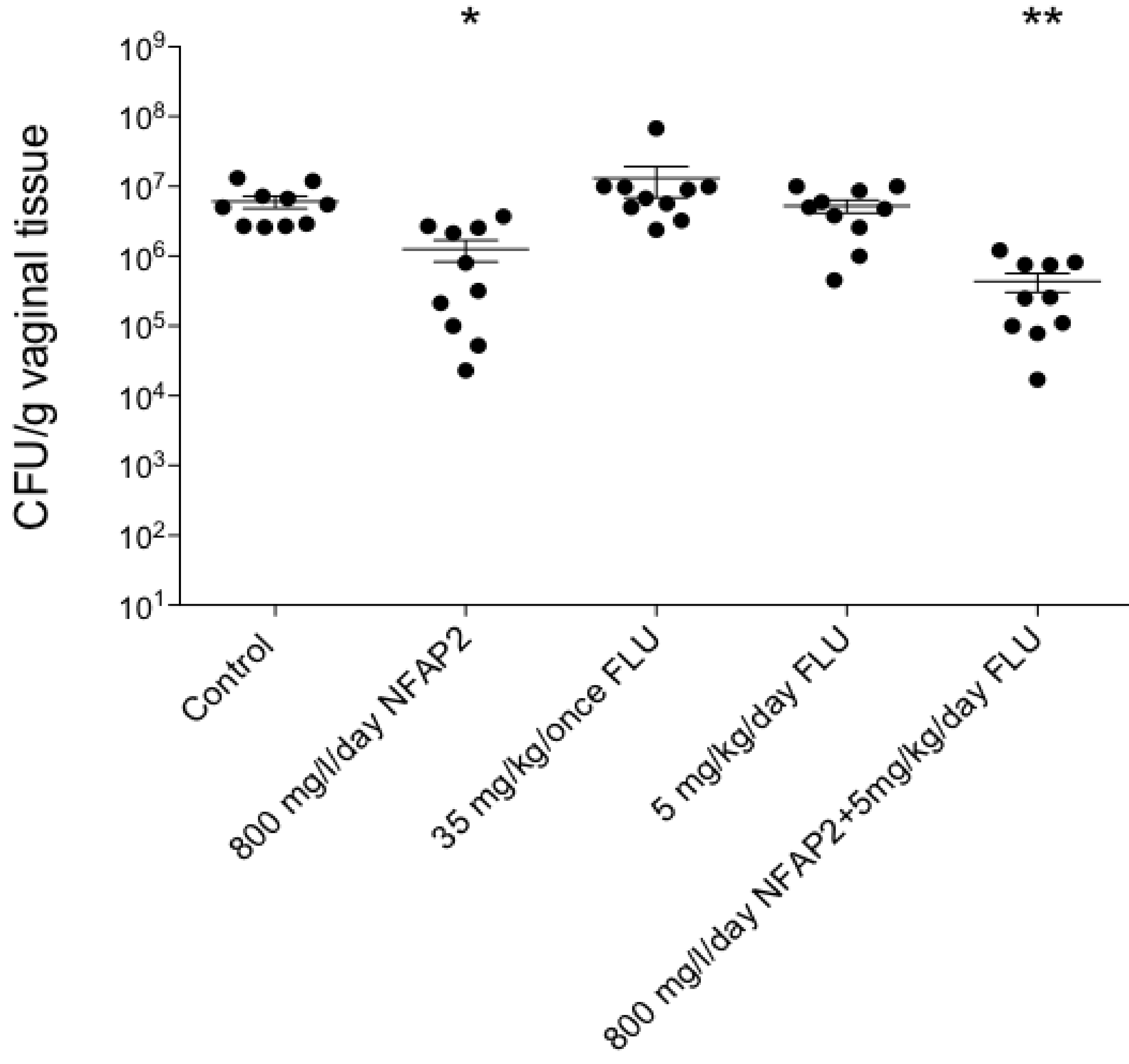

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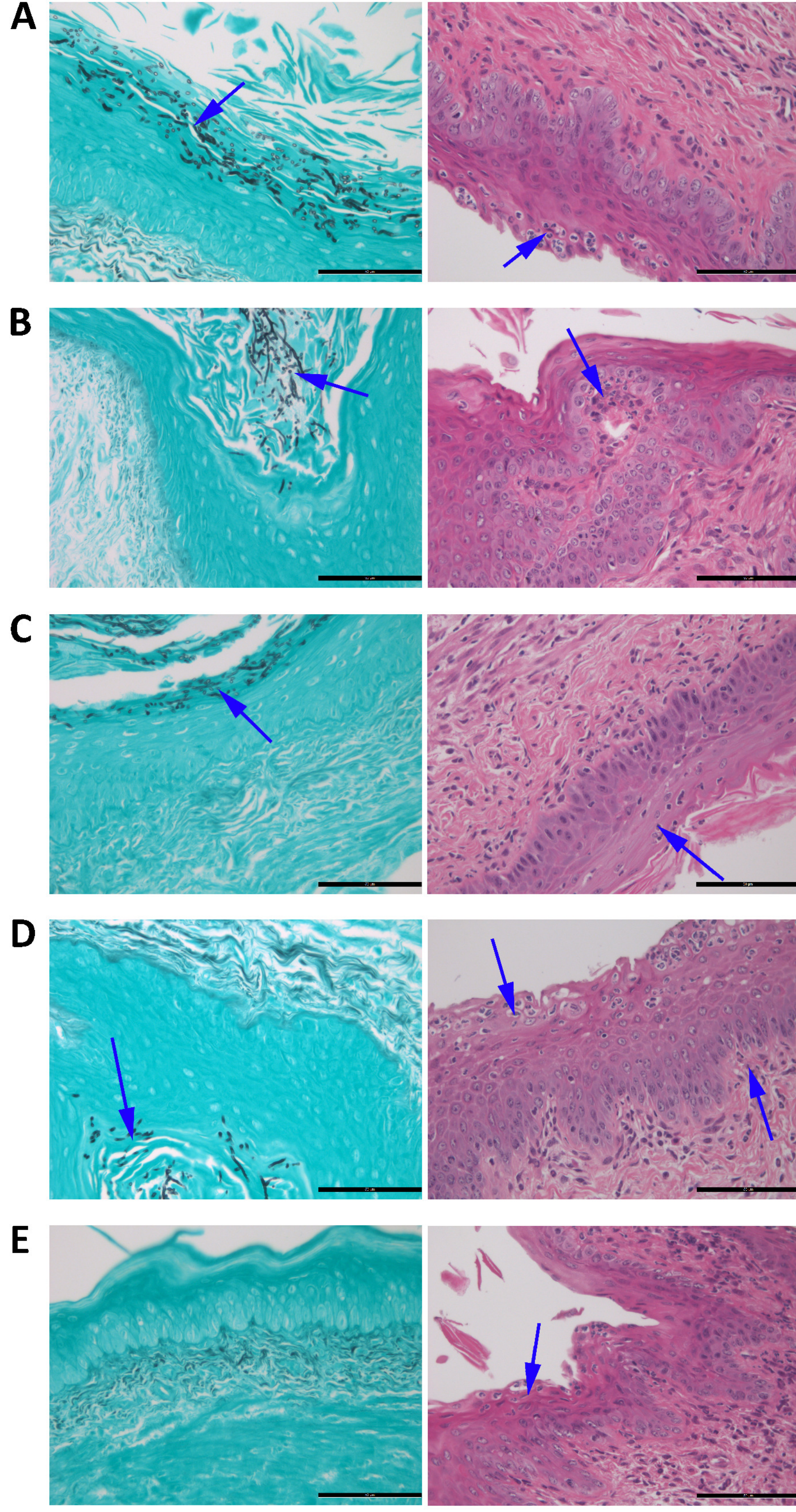

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