Anandamide prevents the adhesion of filamentous *Candida albicans* to cervical epithelial cells

Ronit Vogt Sionov1*, Mark Feldman1, Reem Smoum2, Raphael Mechoulam2 & Doron Steinberg1

Candidiasis is a fungal infection caused by *Candida* species that have formed a biofilm on epithelial linings of the body. The most frequently affected areas include the vagina, oral cavity and the intestine. In severe cases, the fungi penetrate the epithelium and cause systemic infections. One approach to combat candidiasis is to prevent the adhesion of the fungal hyphae to the epithelium. Here we demonstrate that the endocannabinoid anandamide (AEA) and the endocannabinoid-like N-arachidonoyl serine (AraS) strongly prevent the adherence of *C. albicans* hyphae to cervical epithelial cells, while the endocannabinoid 2-arachidonoylglycerol (2-AG) has only a minor inhibitory effect. In addition, we observed that both AEA and AraS prevent the yeast-hypha transition and perturb hyphal growth. Real-time PCR analysis showed that AEA represses the expression of the *HWP1* and *ALS3* adhesins involved in *Candida* adhesion to epithelial cells and the *HGC1, RAS1, EFG1* and *ZAP1* regulators of hyphal morphogenesis and cell adherence. On the other hand, AEA increased the expression of *NRG1*, a transcriptional repressor of filamentous growth. Altogether, our data show that AEA and AraS have potential anti-fungal activities by inhibiting hyphal growth and preventing hyphal adherence to epithelial cells.

**Abbreviations**

| Acronym | Description |
|---------|-------------|
| AEA     | Anandamide, N-arachidonoyl ethanolamine |
| 2-AG    | 2-Arachidonoylglycerol |
| ALS1    | Agglutinin-like sequence protein 1 |
| ALS3    | Agglutinin-like sequence protein 3 |
| AraS    | N-Arachidonoyl serine |
| BCR1    | Biofilm and cell wall regulator 1 |
| CDC35   | Cell-division-cycle gene 35 |
| CPH1    | *Candida* pseudohyphal regulator 1, a homolog of STE12-like transcription factor |
| CSH1    | Cell surface hydrophobicity associated protein 1 |
| CST20   | *C. albicans* homologous to the p21-activated kinase (PAK) kinase Ste20, a serine/threonine protein kinase |
| CZF1    | *C. albicans* Zinc finger protein, a transcription factor |
| EAP1    | Enhanced adherence to polystyrene 1 |
| ECE1    | Extent of cell elongation protein 1 |
| EED1    | Epithelial escape and dissemination 1, a transcriptional regulator |
| EFB1    | Elongation factor 1-beta. |
| EFG1    | Enhanced filamentous growth protein 1 |
| FKS1    | FKS06 sensitivity, a 1,3-beta-glucan synthase |
| GSP1    | Genetic suppressor of Prp20-1, a GTP-binding nuclear protein |
| HGC1    | Hypha-specific G1 cyclin-related protein 1 |
| HST7    | Homologous to the MAPK kinase (MAPKK) Ste7, a serine/threonine protein kinase |
| HWP1    | Hyphal wall protein 1 |
| MDR1    | Multidrug resistance protein 1 |

1^1^Biofilm Research Laboratory, The Faculty of Dental Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel. 2^2^The Faculty of Medicine, The Institute for Drug Research, The Hebrew University of Jerusalem, Jerusalem, Israel. *^*email: ronit.sionov@mail.huji.ac.il
Candida albicans is a common commensal organism in the genitourinary tracts, the intestine and the oral cavity, but it can also be pathogenic causing infections by invading and damaging epithelial cells, a condition called candidiasis. In addition, C. albicans can cause life-threatening systemic infections by penetrating through the epithelial barriers. C. albicans is a dimorphic fungus that can transform from budding yeast form cells at room temperature to invasive filamentous hyphae at 37 °C, a process vital to pathogenesis. This transition is tightly regulated. The mitogen-activated protein kinase Mk1 that is activated upon physical contact, is required for invasive hyphal growth and normal biofilm development. The transcription factor Aft1 controls the contact-dependent invasive filamentation. Other gene products regulating filamentous growth include the transcriptional regulators Cph1, Ume6, Bcr1, Tec1 and Efg1. Hyphal growth is characterized by the expression of different genes, some of which are involved in adhesion.

Biofilm formation by C. albicans is characterized by four major phases: adherence of yeast cells to a surface; initiation of biofilm formation where the hyphae are formed; maturation into complex, structured biofilm in which the fungi are embedded in an extracellular matrix; and dispersion of yeast cells from the biofilm to initiate biofilms at other sites. The dispersion from biofilms may lead to systemic infections in the bloodstream and dissemination into other tissues. More than 50 interconnected transcriptional regulators are involved in regulating biofilm formation. Initial attachment of C. albicans to a surface appears to involve the glycosylphosphatidylinositol (GPI)-linked cell wall protein Eap1 and the agglutinin-like protein Als3. The agglutinin-like protein Als3 and the hyphal-specific wall protein-1 gene product Hwp1 function as complementary adhesins involved in cell–cell and cell-surface interactions of hyphae. Als3 was found to bind N-Cadherin on endothelial cells and E-Cadherin on epithelial cells. Strains defective in ALS3 can form mycelium normally, but are defective in biofilm formation. HWP1 encodes a cell wall mannose protein essential for normal growth of the mycelium. HWP1 mutant strains could not stably adhere to the epithelial mucosal cells and were more easily engulfed and cleared by the host cells. ALS3, HWP1, HGC1 and ECE1 are upregulated in hyphae. Hyphal G cyclin 1 (Hgc1) is involved in regulating mycelial growth and represses cell separation from hyphae. ECE1 encodes for candidalysin, a peptidase that activates epithelial cells and leads to cytolysis of mononuclear phagocytes, and as such is considered to be a virulence factor. EED1 and PGA34 are dispensable for epithelial invasion, but essential for damage of epithelial cells.

One approach to prevent systemic candidiasis is to prevent the adherence of the filamentous fungi to epithelial cells. Here we have studied the ability of the endocannabinoids anandamide (N-arachidonoyl ethanolamine; AEA) and 2-arachidonoylglycerol (2-AG), and the endocannabinoid-like compound N-arachidonoyl serine (AraS) to prevent the interaction between C. albicans hyphae and the epithelial cells. Endocannabinoids are endogenous bioactive lipids derived from arachidonic acid which is produced by hydrolysis of membrane phospholipids. The endocannabinoid system affects multiple functions including feeding, pain, learning and memory. In addition, AEA has been shown to exert anti-inflammatory activities attenuating the development of inflammation in a mouse model of ulcerative colitis. In human and rodents, AEA acts as an endogenous agonist of the cannabinoid CB1 and CB2 receptors, and can also activate the vanilloid receptor TRPV1 resulting in transient calcium influx. Also 2-AG may act on other receptors besides CB1 and CB2, including GABA, PPARγ, TRPV1 and GPR55. AraS binds weakly to the CB1, CB2 and TRPV1 receptors, but seems to act on GPR55 to stimulate angiogenesis and endothelial wound healing.

We have previously shown that both AEA and AraS reduce biofilm formation of methicillin-resistant Staphylococcus aureus (MRSA) and sensitize MRSA to antibiotics. Both compounds reduced the metabolic activity and spreading ability of these bacteria. So far, endocannabinoids have not been tested for their activity on Candida, and the aim of the present research was to study this issue. We show here that treatment of C. albicans with either AEA or AraS strongly reduced the interaction between C. albicans hyphae and cervical epithelial cells. In addition, we observed that AEA and AraS prevented yeast-hypha transition and the growth of preformed hyphae. Gene expression studies showed down-regulation of the adhesins HWP1 and ALS3, the transcriptional regulators EFG1 and ZAP1, and the hyphal morphogenesis regulators HGCl and RAS1. On the other hand, NRG1, a repressor of filamentous growth, was upregulated.

Results
Anandamide and N-arachidonoyl serine prevent the yeast-hypha transition of Candida albicans. It is well known that the virulence of Candida depends on its transition from the yeast form to filamentous hyphae. It was therefore important to study the effect of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the endocannabinoid-like N-arachidonoyl serine (AraS) on this transition. To this end, we exposed GFP-expressing Candida albicans in the yeast form to various concentrations of AEA,
Anandamide impairs the further growth of preformed hyphae. Since AEA prevented the morphogenetic switch from yeast to hyphae, we wondered whether AEA could affect the hyphae after being formed. For this purpose, we allowed the Candida to form hyphae by an overnight incubation at 37 °C, and then exposed the hyphae to various concentrations of AEA for 1 h. The majority of the hyphae length in the control and 10 μg/ml AEA-treated samples ranged between 25–60 μm (Fig. 2a,b). Most of the C. albicans hyphae treated with 50, 125 and 250 μg/ml AEA for 1 h showed shorter hyphae length ranging from 15–30 μm (Fig. 2c–e). Occasionally, some hyphae with exceptional long length (90–100 μm) appeared in both the control and treated samples (Fig. 2). We next wanted to know whether the shorter hyphae observed in the AEA-treated samples are due to an inhibition of hyphal growth. To study this possibility, C. albicans in its yeast form was first allowed to form hyphae by incubating them 4 h at 37 °C, and then subjected to a 3 h time-lapse microscopy at 37 °C in the absence or presence of 125 μg/ml AEA. As expected, the hyphae continued to grow in the control samples (Fig. 3a and Suppl. Figure 3a—time-lapse video). However, most of the hyphae ceased growing after being exposed to AEA (Fig. 3b and Suppl. Figure 3b—time-lapse video), suggesting that AEA interferes with hyphal morphogenesis. Similar inhibition of hyphal growth was observed when exposing the hyphae to 50 μg/ml AEA (data not shown).

Anandamide (AEA) prevents the adherence of Candida albicans to cervical epithelial cells. Next we studied the effect of AEA on C. albicans adherence to cervical epithelial cells. GFP-expressing C. albicans were allowed to form hyphae by an overnight incubation at 37 °C. The hyphae were pretreated with various concentrations of AEA for 1 h, and then co-cultured on confluent HeLa cervical epithelial cells for another hour (Fig. 4a–e). In parallel, the same fungi samples were incubated on tissue culture plastic plates as controls that reflect the inputs (Fig. 5a–e). The morphology of the whole fungal population prior to incubation with HeLa or on plastic is shown in Fig. 2. Pretreatment of C. albicans hyphae with 50 μg/ml and 125 μg/ml AEA (Fig. 4c,d) reduced their adherence to the epithelial cells by 40 ± 8% and 62 ± 4%, respectively, with a statistical significance of p < 0.001 compared to control (Fig. 4f). Increasing the AEA concentration to 250 μg/ml (Fig. 4e) did only cause a slightly higher inhibition of 72 ± 2% (Fig. 4f), suggesting that a plateau effect is observed at 125 μg/ml. Of note, AEA-treated C. albicans hyphae that were able to bind to the epithelial cells, showed 3–fivefold shorter hyphae (Fig. 4d,e) compared to control fungi (Fig. 4a) with a p < 0.001 for 50–250 μg/ml AEA (Fig. 4g). A maximal effect on hyphae length was observed at 50 μg/ml (Fig. 4g). At a concentration of 10 μg/ml, AEA had no significant effect on the hyphae length (Fig. 4b). The hyphae that adhered to HeLa cells were also thinner and several fungi appeared without hyphae at all when using 50–250 μg/ml AEA. The appearance of shorter hyphae adherent to HeLa cells following AEA treatment is a direct consequence of the AEA effect on hyphal growth as described above (Figs. 2, 3). Of particular importance is the preferential inhibition of hyphal adhesion to HeLa cells in comparison to plastic as shown by the relative reduction in the ratio of HeLa-adherent hyphal growth versus plastic-adherent hyphal (Suppl. Figure 4). Pretreatment of HeLa cells with AEA prior to addition of C. albicans didn’t alter the attachment of the fungi to the cells (data not shown). It should be noted that normal untreated hyphal of different lengths exhibited similar ability to adhere to HeLa cells (Suppl. Figure 5), meaning that the preferential appearance of shorter adherent hyphae after exposure to AEA is a direct consequence of the treatment.

In contrast to the reduced adherence of AEA-treated C. albicans to HeLa cells, the AEA-treated fungi adhered even better to polystyrene tissue culture plates in comparison to untreated fungi (Fig. 5a–e) with statistical significance in the concentration range of 50–250 μg/ml AEA (Fig. 5f; p < 0.05). This accords with the higher amount of hyphae observed in AEA-treated samples in comparison to control samples (Fig. 5). The hyphae length of AEA-treated C. albicans that bound to plastic were about twofold shorter on average in comparison to control (Fig. 5g) with statistical significance in the concentration range of 50–250 μg/ml AEA (p < 0.05). When incubating the C. albicans with AEA for 24 h, there was no significant alteration in the biofilm mass formed on plastic (Suppl. Figure 6). However, both 2-AG and AraS significantly reduced the biofilm mass on plastic in a dose-dependent manner with a p < 0.05 (Suppl. Figure 6).

N-Arachidonoyl serine (AraS), but not 2-arachidonoylglycerol (2-AG), prevents the adherence of Candida albicans to cervical epithelial cells. We next analyzed the effect of AraS and 2-AG on the ability of C. albicans to adhere to cervical epithelial cells and compared their effect with that of AEA (Fig. 6a–h). Pretreatment of C. albicans with 125 μg/ml and 250 μg/ml AraS for 1 h (Fig. 6c,g) reduced adherence to the epithelial cells by 30 ± 5% and 63 ± 5%, respectively, with a statistical significance of p < 0.05 compared to control (Fig. 6i). The hyphae of the fungi that adhered to the epithelial cells following AraS treatment (Fig. 6g) were shorter and thinner in comparison to untreated fungi (Fig. 6a,e). However, treatment of C. albicans with 125 μg/ml and 250 μg/ml 2-AG (Fig. 6b,f) didn’t interfere with their adherence to HeLa cells (Fig. 6i). Both AraS and 2-AG treated C. albicans adhered well to polystyrene plastic within 1 h (Fig. 7). This is in contrast to the reduced biofilm mass formation on plastic after 24 h incubation in the presence of the compounds (Suppl. Figure 6).

Anandamide (AEA) prevents the adherence of Candida albicans to cervical epithelial cells.
Figure 1. Anandamide prevents yeast-hypha transition. GFP-expressing *Candida albicans* taken from PDA plates in its yeast form was incubated with different concentrations of AEA at 37 °C for 4 h, and the morphology studied by confocal microscopy. (a, c, e, g) Bright field. (b, d, f, h, j) Green fluorescence.
Figure 2. AEA treatment of *C. albicans* hyphae leads to shorter hyphae than control. (a–e) Confocal microscopy of control and AEA-treated *C. albicans*. GFP-expressing *C. albicans* taken from PDA plates in its yeast form was incubated in RPMI at 37 °C for 16 h, and then exposed to different concentrations of AEA for 1 h and the morphology studied by confocal microscopy. The merged bright fields and green fluorescence are shown. (f) The average hyphae lengths of untreated and AEA-treated *C. albicans*. Number of hyphae measured for each sample was 90–110 from 4–5 different fields. **p < 0.001.
Figure 3. AEA inhibits hyphal growth. (a) Spinning scan microscopy of control hyphae at time 0, 15, 30, 45, 60 and 90 min. (b) Spinning scan microscopy of hyphae at time 0, 15, 30, 45, 60 and 90 min in the presence of 125 μg/ml AEA. C. albicans in yeast form was allowed to form hyphae by incubating them 4 h at 37 °C, and thereafter a time-lapse study was performed using a Nikon spinning scan microscopy in the absence (a) or presence of 125 μg/ml AEA (b). Time 0 is 30 min after adding AEA.
Figure 4. AEA treatment of *C. albicans* reduced their adherence to HeLa cervical epithelial cells. (a–e) Confocal microscopy of co-cultures of control and AEA-treated *C. albicans* on confluent HeLa cells. The fungi were pretreated with the indicated concentrations of AEA for 1 h prior to co-incubation with HeLa cells for an additional 1 h. The fungi express GFP. (f) Quantification of the relative adherence of untreated and AEA-treated *C. albicans* to HeLa cells. 8–10 images taken from 3 independent experiments were analyzed for each treatment. (g) The average hyphae lengths of untreated and AEA-treated *C. albicans* that were able to adhere to HeLa cells. The number of hyphae measured for each treatment was 50–75. **p < 0.001.
Figure 5. *C. albicans* treated with AEA adhered to polystyrene plastic surface even better than untreated fungi. (a–e) Confocal microscopy of control and AEA-treated *C. albicans* on tissue culture plates. The fungi were pretreated with the indicated concentrations of AEA for 1 h prior to incubation on polystyrene surfaces for an additional 1 h. (f) Quantification of the relative adherence of untreated and AEA-treated *C. albicans* to plastic. 8–10 images taken from 3 independent experiments were analyzed for each treatment. (g) The hyphae lengths of untreated and AEA-treated *C. albicans* that were able to adhere to plastic. The number of hyphae measured for each treatment was 50–75. *p < 0.05.*
AEA altered the expression of genes involved in adhesion and hyphal morphogenesis. In order to understand the anti-adhesive effects of AEA on C. albicans interaction with epithelial cells, we exposed the fungi to various concentrations of AEA for 2 h, followed by gene expression analysis of genes relevant for

Figure 6. C. albicans treated with AraS showed strong reduction in their adherence to cervical epithelial cells. (a–h) Confocal microscopy of co-cultures of control and AEA-treated C. albicans on confluent HeLa cells. The fungi were pretreated with the indicated concentrations of 2-AG, AraS or AEA for 1 h prior to co-incubation with HeLa cells for an additional 1 h. (i) Quantification of the relative adherence of untreated and treated C. albicans to HeLa cells. 5–7 images were analyzed for each treatment. *p < 0.05, **p < 0.001.
Figure 7. *C. albicans* treated with 2-AG, AraS or AEA retained binding capacity to polystyrene surface. (a–h) Confocal microscopy of control and treated *C. albicans* bound to polystyrene plastic surface. The fungi were pretreated with the indicated concentrations of 2-AG, AraS or AEA for 1 h prior to incubation in tissue culture plates for an additional 1 h. (i) Quantification of the relative adherence of untreated and treated *C. albicans* to plastic. 5–7 images were analyzed for each treatment. *p < 0.05, **p < 0.001.
biofilm formation, adherence and hyphal morphogenesis (Tables 1, 2 and 3). Some genes were upregulated including the ALS1 adhesion molecule, the transcription factor TEC1 involved in hyphal development and the transcriptional repressor NRG1 that prevents filamentous growth (Table 1). ALS1 and TEC1 were mainly upregulated at the lower concentrations (10 and 50 μg/ml AEA), while NRG1 was upregulated at the higher concentrations (50–250 μg/ml AEA). Of note, the three multidrug efflux transporters MDR1, CDR1 and CDR2 were strongly upregulated (Table 1). On the other hand, AEA repressed the expression of the adhesins HWP1 and ALS3, the cell elongation protein ECE1, the signal transduction regulators HGC1 and RAS1 and the transcription regulators EFG1 and ZAP1 (Table 2). Also the cell hydrophobicity-associated protein CSH1 was strongly repressed as well as the virulence factor phospholipase D1 (PLD1) (Table 2). There were also several genes that were not significantly affected by AEA including the cell wall adhesion EAP1 and the anti-adhesive protein YWP1 (Table 3). Altogether, these alterations in gene expression may explain, at least in part, the inhibition of hyphal growth by AEA and the reduced adherence of AEA-treated hyphae to epithelial cells.

**Discussion**

Candidiasis is a major health problem where Candida species forms biofilm on endothelial and epithelial cells. In immunosuppressed people it can lead to systemic infection and even death\(^6\). The oral cavity, the genitourinary tract and the intestine are the most frequent infection sites. It is important to find treatments that can interfere with the early adhesion of the fungi to the host cells. Here we have shown that treatment of C. albicans with either AEA or AraS strongly reduced their adherence to cervical epithelial cells, making them potential drugs in the co-treatment of this infectious disease. Not only do these compounds affect the hyphal attachment to epithelial cells, but they also lead to a strong reduction in the hyphal length in comparison to control. The appearance of shorter hyphae in the AEA and AraS-treated samples is a direct result of their inhibitory effect on hyphal growth. These compounds were also shown to prevent the yeast-hypha transition. Since the hyphae are associated with higher infectivity than the yeast form\(^6\),\(^22\), the perturbation of hyphal growth by AEA and AraS might be beneficial in reducing the virulence of C. albicans. Of note, AEA didn’t affect the biofilm formation on polystyrene plastic surface, suggesting different requirements for the two modes of adhesion.

In order to gain better insight into the action mechanism of AEA, we undertook a gene expression study focusing on genes relevant to adhesion, biofilm formation and hyphal morphogenesis. We found genes that were upregulated by AEA, others that were down-regulated and even others that were not significantly affected. Of the genes whose expression was altered by AEA, the upregulation of ALS1, TEC1 and NRG1 and the downregulation of HWP1, ALS3, HGC1, RAS1, ZAP1, CSH1, ECE1 and PLD1 were the most outstanding. Als1, Als3 and Hwp1 are adhesins that are involved in the attachment of C. albicans to epithelial cells,\(^7\),\(^12\),\(^23\)–\(^26\). Eap1 and Als1 are important for the initial attachment to a surface, while Hpw1 and Als3 are important for the stable attachment

### Table 1.

| Gene     | AEA conc (μg/ml) | Fold change | Function                                                                 |
|----------|------------------|-------------|--------------------------------------------------------------------------|
| ALS1     | 10               | +1.9 ± 0.5* | Major cell surface adhesion protein which mediates yeast-to-host tissue adherence and yeast aggregation |
|          | 50               | +4.1 ± 1.3* |                                                                          |
|          | 125              | n.s         |                                                                          |
|          | 250              | n.s         |                                                                          |
| TEC1     | 10               | +2.1 ± 0.4* | Transcription factor of the TEA/ATTS family which regulates genes involved in hyphal development, cell adhesion, biofilm development, and virulence |
|          | 50               | +3.9 ± 1.3* |                                                                          |
|          | 125              | n.s         |                                                                          |
|          | 250              | n.s         |                                                                          |
| NRG1     | 10               | n.s         | Transcriptional repressor of filamentous growth                           |
|          | 50               | +2.2 ± 0.7* |                                                                          |
|          | 125              | +2.6 ± 1.0* |                                                                          |
|          | 250              | +2.0 ± 0.3* |                                                                          |
| MDR1     | 10               | −3.0 ± 0.3* | Multidrug resistance protein 1                                            |
|          | 50               | n.s         |                                                                          |
|          | 125              | +2.5 ± 1.1* |                                                                          |
|          | 250              | n.s         |                                                                          |
| CDR1     | 10               | +3.0 ± 0.3* | Pleiotropic ABC efflux transporter of multiple drugs                      |
|          | 50               | +4.8 ± 1.6* |                                                                          |
|          | 125              | +1.6 ± 0.4* |                                                                          |
|          | 250              | n.s         |                                                                          |
| CDR2     | 10               | +5.7 ± 0.6* | Multidrug efflux transporter                                               |
|          | 50               | +8.5 ± 2.9* |                                                                          |
|          | 125              | +5.2 ± 2.4* |                                                                          |
|          | 250              | +3.4 ± 0.1* |                                                                          |

* \( p < 0.05 \).
to epithelial cells\textsuperscript{24}. \textit{EAPI} expression was unaffected by AEA, while \textit{ALS1} was only upregulated at the lower AEA concentrations (10–50 μg/ml). The upregulation of \textit{ALS1} showed a similar pattern to that of the transcription factor \textit{TEC1}, which is known to regulate \textit{ALS1} expression\textsuperscript{6}. In contrast, \textit{HWP1} and \textit{ALS3} were downregulated at the higher AEA concentrations (50–250 μg/ml), an effect that seems to outweigh the upregulation of \textit{ALS1}.

This conclusion is based on the observation that \textit{C. albicans} strains lacking \textit{HWP1} are unable to form stable attachments to human buccal epithelial cells\textsuperscript{23} and specific antibodies to \textit{Als3} blocks \textit{C. albicans} adhesion to vascular endothelial cells and buccal epithelial cells\textsuperscript{24}. The downregulation of \textit{HWP1} and \textit{ALS3} together with the simultaneous downregulation of \textit{ECE1}, which is also known to support adhesion\textsuperscript{26}, might explain, at least partly, the reduced adhesion of AEA-treated \textit{C. albicans} to epithelial cells.

Interesting is the AEA-mediated downregulation of \textit{RAS1}, an upstream regulator of the Cdc35/cAMP/PKA/Efg1 and the Cdc24/Cst20/Hst7/Cph1 MAPK signal transduction pathways that regulate hyphal morphogenesis\textsuperscript{6,22}. \textit{Ras1} is considered a master hyphal regulator and mutant \textit{RAS1} strains show severe defects in hyphal growth\textsuperscript{27} and reduced adherence to epithelial cells\textsuperscript{12}. Alteration in \textit{RAS1} levels by AEA has thereby direct influence on hyphae formation and adhesion to epithelial cells. In addition, AEA reduced the gene expression of hyphal-promoting transcription factor \textit{EFG1} whose activity is affected by the Ras1/Cdc35/cAMP/PKA pathway, while it had no significant effect on the expression of the hyphal-promoting transcription factor \textit{CPH1} that is affected by the Ras1/Cdc24/Cst20/Hst7 MAPK pathway. \textit{Efg1} has been shown to be required for the adhesion of fungi to both reconstituted human epidermis and reconstituted intestinal epithelium\textsuperscript{28} and the cAMP/PKA/Efg1

Table 2. Genes that are downregulated in \textit{C. albicans} after a 2 h-incubation with AEA. n.s not significant. \( ^* p < 0.05 \).
signal transduction pathway has been demonstrated to be necessary for all stages of oral *C. albicans* infection. Efg1 is an upstream regulator of the adhesins *ALS1*, *ALS3*, *ECE1* and *HWPI*. The gene expression of the intermediate mediators *CDC35*, *CST20*, *HST7* were, however, unaffected by AEA. Since the signals transmitted by Ras1 are reduced by AEA, the activity of these intermediate mediators as well as the activity of the transcription factors Efg1 and Chp1 will consequently be dampened, resulting in retarded hyphal morphogenesis. The retardation of hyphal growth might further be effectuated by the prominent downregulation of the hypha-specific G1 cyclin-related protein 1 (HGC1) that regulates the Cdc28 kinase during hyphal growth. On top of these effects, the AEA-mediated upregulation of *NRG1*, a transcriptional repressor of filamentous growth, may further contribute to the observed reduction in hyphal length and size. Nrg3 has been shown to repress the expression of *ALS3*, *ECE1* and *HWPI*. Thus the upregulation of *NRG1* together with the downregulation of *EFG1* may fortify the repression of the adhesin genes. Moreover, the AEA-mediated downregulation of the zinc-responsive transcription factor *ZAP1* that is known to be required for efficient hyphae formation, might have an additional impact. Altogether, the combined alterations in gene expression caused by AEA might explain both the AEA-induced inhibition of hyphal growth and the reduced adherence of AEA-treated hyphae to epithelial cells.

**Table 3.** Genes that are not significantly affected in *C. albicans* after a 2 h incubation with AEA.

| Function                                      | Gene |
|-----------------------------------------------|------|
| Cell wall adhesin EAP1, Cell wall protein which mediates cell–cell and cell-substrate adhesion. Required for biofilm formation and plays a role in virulence | EAP1 |
| Yeast-form wall Protein 1, Cell wall protein which plays an anti-adhesive role and promotes dispersal of yeast forms, which allows the organism to seek new sites for colonization | YWP1 |
| Beta-1,3-glucan synthase catalytic subunit 1 | FK51 |
| GTP-binding nuclear protein GSP1/ran         | GSP1 |
| Adenylyl cyclase                             | CDC35|
| Serine/threonine-protein kinase, MAP4K, required for hyphal formation and virulence | CST20|
| Serine/threonine-protein kinase              | HST7 |
| Transcription factor involved in the formation of pseudohyphae and hyphae | HST7 |
| Elongation factor 1-beta                     | EFB1 |
| Filamentous growth                          | UM66 |
| Transcription factor regulating filamentous growth | TUP1 |
| A key regulator of hyphal maintenance       | EED1 |

In conclusion, AEA and AraS prevent yeast-hyphae transition, inhibit hyphal growth and reduce the ability of *C. albicans* hyphae to adhere to epithelial cells. This is, among others, achieved by altered expression of genes involved in cell–cell interaction and of genes regulating hyphal morphogenesis.

**Material and methods Chemicals.** Anandamide (AEA), N-arachidonoyl serine (AraS) and 2-arachidonoylglycerol (2-AG) were synthesized as described and dissolved in ethanol. We also purchased anandamide from Sigma (St. Louis, MO), and N-arachidonoyl serine (AraS) and 2-arachidonoylglycerol (2-AG) from Cayman Chemical.

**Cell lines.** HeLa cervical carcinoma cells were cultivated in DMEM (Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal calf serum (FCS; Biological Industries, Beth HaEmek, Israel), 2 mM L-glutamine and 1 mM sodium pyruvate, and incubated at 37 °C in a humidified atmosphere containing 5% CO2.

**Fungal strain and growth conditions.** *C. albicans* SC5314 that has the GFP gene integrated within the ENO1 genomic locus, was kindly provided by Prof. J. Berman (Tel Aviv University, Israel). The fungi were first seeded on potato-dextrose agar plates (Acumedia, Neogen, Lansing, MI) at room temperature where they grow in the yeast form, and then inoculated in RPMI (Sigma, St. Louis, MO) for a 16–18 h incubation at 37 °C to let them form hyphae. For the yeast-hypha transition assay, colonies of *C. albicans* in yeast form were incubated in RPMI at an OD600nm of 0.5 and incubated with different concentrations of AEA, AraS and 2-AG at 37 °C for 4 h. For time-lapse microscopy, hyphae that has been formed after 4 h incubation of *C. albicans* in yeast form (OD600nm of 0.25) at 37 °C, were incubated in 300 μl RPMI in a μ-slide 8 well chambered coverslip (ibidi GmbH, Martinsried, Germany) in the absence or presence of 125 μg/ml AEA. The Okolab incubation chamber was used to maintain the temperature at 37 °C. Images were captured each 5 min for 3 h using the Nikon spinning disk.
microscope (Yokogawa W1), the ×20 CFI PLAN APO VC objective and the SCMOS ZYLA camera. The images were processed using the NIS-Element AR program.

**Biofilm formation on plastic culture plates.** *C. albicans* that have been cultivated overnight in RPMI at 37 °C, were resuspended and diluted to an OD\textsubscript{600nm} of 0.25 in RPMI and then seeded in 96-flat bottomed microplates (Corning, NY) in 200 μl RPMI with different concentrations of test compounds per well. Following an overnight incubation at 37 °C, the biofilms formed in the wells were washed twice with PBS and stained for 20 min with 1% crystal violet. The stained biofilms were washed twice with DDW and after drying, the stain was dissolved in 200 μl of 33% acetic acid and the absorbance read in a Tecan M200 microplate reader at 595 nm\textsuperscript{20}.

**C. albicans adherence to HeLa cells.** The adherence assay was performed by a slight modification of Feldman et al.\textsuperscript{43}. Two hundred and fifty thousand HeLa cells were seeded in 1 ml DMEM supplemented with 8% FCS in 24 well tissue culture plate (Corning, NY) the day before assay. At the following morning, the medium was changed to 1 ml of RPMI supplemented with 1% FCS. *C. albicans* that have grown for 16–18 h at 37 °C, were resuspended in fresh RPMI to an OD of 1.0, and then exposed to different concentrations of AEA, 2-AG, AraS or corresponding concentrations of ethanol for 1 h serving as controls. Thereafter, 100 μl of the pretreated *C. albicans* were added to the 1 ml HeLa cell cultures, and the co-culture incubated for 1 h at 37 °C. At the end of incubation, the cells were washed twice with 1 ml PBS and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 30 min. The co-cultures were visualized using NIKON confocal microscope and the NIS-Element AR software. Quantitative analysis of GFP was done using the ImageJ software. For each sample 8–10 images were taken and each image was analyzed for the amount of GFP. The percentage adherence was calculated according to relative GFP staining. In addition, the length of the hyphae was measured using the Adobe Photoshop software. Between 50 and 100 fungi were measured per sample.

**Real-time quantitative PCR.** *C. albicans* that have been grown overnight in RPMI at 37 °C were treated for 2 h with various concentrations of AEA or corresponding concentrations of ethanol. At the end of incubation, the RNA was extracted from the fungi using TRI-Reagent (Sigma, St. Louis, MO)\textsuperscript{46}. Cell disruption was done in 1 ml TRI-Reagent in the presence of 200 μl 1 mm acid-washed glass beads (Sigma, St. Louis, MO) with

| Gene    | Forward primer       | Reverse primer       |
|---------|----------------------|----------------------|
| 18S rRNA| TCTTCTCTGATTGGTTGCTGG | TCGATAGGCTCTCTAGAAAGG |
| ACT1    | ATGAGAAGTGGTGGTCTAGGGA | ATGAGAAGTGGTGGTCTAGGGA |
| ALS1    | TATGGGTTGCTTGGAGATCCG | ATGAGAAGTGGTGGTCTAGGGA |
| ALS3    | CAGGCAATGGATGTTGATTCG | ATGAGAAGTGGTGGTCTAGGGA |
| CDC35   | TTATCAAGGCTGTTTCTGCT | ATGAGAAGTGGTGGTCTAGGGA |
| CDR1    | GTATCTATCCACCTAGACCATCT | ATGAGAAGTGGTGGTCTAGGGA |
| CDR2    | TGGCGAAGGAGAGAGCTGAGTT | ATGAGAAGTGGTGGTCTAGGGA |
| CPH1    | ATGACCACTTATATATACCTC | ATGAGAAGTGGTGGTCTAGGGA |
| CSH1    | CTCAGTGGCTAGGAGGATGAT | ATGAGAAGTGGTGGTCTAGGGA |
| CST20   | TTCAGCTATTCCAAAAGGAAGA | ATGAGAAGTGGTGGTCTAGGGA |
| EAP1    | AGGAGGAAGGTTGGCTATACG | ATGAGAAGTGGTGGTCTAGGGA |
| ECE1    | GCCTGGTATCCAGTATTGAT | ATGAGAAGTGGTGGTCTAGGGA |
| EED1    | AGGAGGACCATCCAAAAGGA | ATGAGAAGTGGTGGTCTAGGGA |
| EBR1    | GCCTGCTAAAGGCTCCAAAAAC | ATGAGAAGTGGTGGTCTAGGGA |
| EFG1    | TATGCCACACCACAAAGACT | ATGAGAAGTGGTGGTCTAGGGA |
| FKS1    | CTTTGAGATCATGCTGCTAC | ATGAGAAGTGGTGGTCTAGGGA |
| GSP1    | TGAGGTCATCCATCCATTGAGAT | ATGAGAAGTGGTGGTCTAGGGA |
| HGC1    | AATGAGGACCATCCAAAAGGA | ATGAGAAGTGGTGGTCTAGGGA |
| HST7    | ACTCCACAATCCAATAATACA | ATGAGAAGTGGTGGTCTAGGGA |
| HWP1    | CAGAGGTAGAGGCTGACAGAGT | ATGAGAAGTGGTGGTCTAGGGA |
| MDR1    | TCCAGTCGATGCAGAAATGTC | ATGAGAAGTGGTGGTCTAGGGA |
| NRG1    | CCAGCTTCACTCCACAGCAT | ATGAGAAGTGGTGGTCTAGGGA |
| PLD1    | GCCAAGAGGCAAGGCTGAGG | ATGAGAAGTGGTGGTCTAGGGA |
| RAS1    | GCCGCTAGAGAACAATATA | ATGAGAAGTGGTGGTCTAGGGA |
| TEC1    | AGGTCCCTCCTGTTAAAGTG | ATGAGAAGTGGTGGTCTAGGGA |
| TUP1    | CTGGGAGTGGCCCATAGA | ATGAGAAGTGGTGGTCTAGGGA |
| UME6    | AGCAGCCTTGGCCTGATGAGT | ATGAGAAGTGGTGGTCTAGGGA |
| WYP1    | GCAGCTGCTAGCTTGGCTA | ATGAGAAGTGGTGGTCTAGGGA |

| Table 4. Primers used for real-time PCR. |
Received: 10 April 2020; Accepted: 31 July 2020
Published online: 13 August 2020

References
1. Kim, J. & Sudbery, P. Candida albicans, a major human fungal pathogen. J. Microbiol. 49, 171–177. https://doi.org/10.1007/s12275-011-1064-7 (2011).
2. Fan, Y., He, H., Dong, Y. & Pan, H. Hypha-specific genes HGC1, ALS3, HWP1, and ECE1 and relevant signaling pathways in Candida albicans. Mycopathologia 176, 329–335. https://doi.org/10.1007/s11046-013-9684-6 (2013).
3. Kumamoto, C. A. A contact-activated kinase signals Candida albicans invasion, growth and biofilm development. Proc. Natl. Acad. Sci. USA 102, 5576–5581. https://doi.org/10.1073/pnas.0407097102 (2005).
4. Guissani, A. D., Vinces, M. & Kumamoto, C. A. Invasive filamentous growth of Candida albicans is promoted by Cz1p-dependent relief of Epf1p-mediated repression. Genetics 160, 1749–1753 (2002).
5. Lobue, M. B., Gulati, M., Johnson, A. D. & Noble, C. J. Development and regulation of single- and multi-species Candida albicans biofilms. Nat. Rev. Microbiol. 16, 19–31. https://doi.org/10.1038/nrmicro.2017.107 (2018).
6. Finkel, J. S. & Mitchell, A. P. Genetic control of Candida albicans biofilm development. Nat. Rev. Microbiol. 9, 109–118. https://doi.org/10.1038/nrmicro2475 (2011).
7. Phan, Q. T. et al. Alc3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. PLoS. Biol. 5, e64. https://doi.org/10.1371/journal.pbio.0050064 (2007).
8. Cleary, I. A. et al. Candida albicans adhesin Als3p is dispensable for virulence in the mouse model of disseminated candidiasis. Microbiology 157, 1806–1815. https://doi.org/10.1099/mic.0.046326-0 (2011).
9. Tsuchimori, N. et al. Reduced virulence of HWPI-deficient mutants of Candida albicans and their interactions with host cells. Infect. Immun. 68, 1997–2002. https://doi.org/10.1128/iai.68.4.1997-2002.2000 (2000).
10. Moyes, D. L. et al. Candidylisin is a fungal peptide toxin critical for mucosal infection. Nature 532, 64–68. https://doi.org/10.1038/nature16725 (2016).
11. Kasper, L. et al. The fungal peptide toxin Candidyalisin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes. Nat. Commun. 9, 4260. https://doi.org/10.1038/s41467-018-06607-1 (2018).
12. Wächter, B., Wilson, D., Haedicke, K., Dalle, F. & Hube, B. From attachment to damage: defined genes of Candida albicans mediate adhesion, invasion and damage during interaction with oral epithelial cells. PLoS ONE 6, e17046. https://doi.org/10.1371/journal.pone.0017046 (2011).
13. Freitas, H. R. et al. Polyunsaturated fatty acids and endocannabinoids in health and disease. Nutr. Neurosci. 21, 695–714. https://doi.org/10.1080/1028415X.2017.1347373 (2018).
14. Murillo-Rodriguez, E., Pastrana-Trejo, J. C., Salas-Crisostomo, M. & de-la-Cruz, M. The endocannabinoid system modulating levels of consciousness, emotions and likely dream contents. CNS Neurol. Disord. Drug Targets 16, 370–379. https://doi.org/10.2174/1871127217623161908 (2017).
15. Engel, M. A. et al. Uckercollit in AKR mice is attenuated by intraperitoneal administered anandamide. J. Physiol. Pharmacol. 59, 673–689 (2008).
16. Muller, C., Morales, P. & Reggio, P. H. Cannabinoid ligands targeting TRP channels. Front. Mol. Neurosci. 11, 487. https://doi.org/10.3389/fmoln.2018.00487 (2018).
17. Pacher, P., Kogan, N. M. & Mechoulam, R. Beyond THC and endocannabinoids. Annu. Rev. Pharmacol. Toxicol. 60, 637–659. https://doi.org/10.1146/annurev-pharmtox-010818-012441 (2020).
18. Baggelaar, M. P., Maccarrone, M. & van der Stelt, M. Z. Arachidonoylglycerol: a signaling lipid with manifold actions in the brain. Prog. Lipid. Res. 71, 1–17. https://doi.org/10.1016/j.plipres.2018.05.002 (2018).
19. Zhang, X., Maor, Y., Wang, J. F., Kunos, G. & Groopman, J. E. Endocannabinoid-like N-arachidonyl serine is a novel angio- genic mediator. Br. J. Pharmacol. 160, 1583–1594. https://doi.org/10.1111/1476-5381.12841.60.498141 (2010).
20. Feldman, M., Smoum, R., Mechoulam, R. & Steinberg, D. Antimicrobial potential of endocannabinoid and endocannabinoid-like compounds against methicillin-resistant Staphylococcus aureus. Sci. Rep. 8, 17696. https://doi.org/10.1038/s41598-018-35793-7 (2018).
21. Feldman, M., Smoum, R., Mechoulam, R. & Steinberg, D. Potential combinations of endocannabinoid/endocannabinoid-like compounds and antibiotics against methicillin-resistant Staphylococcus aureus. PLoS ONE 15, e0231583 (2020).
22. Sudbery, P. E. Growth of Candida albicans hyphae. Nat. Rev. Microbiol. 9, 737–748. https://doi.org/10.1038/nrmicro2636 (2011).
23. Staab, J. F., Bradway, S. D., Fidel, P. L. & Sundstrom, P. Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science 283, 1535–1538. https://doi.org/10.1126/science.283.5407.1535 (1999).
24. Coleman, D. A. et al. Monoclonal antibodies specific for Candida albicans Alc3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces. J. Microbiol. Methods 78, 71–78. https://doi.org/10.1016/j.mimet.2009.05.002 (2009).
25. Fu, Y. et al. Expression of the Candida albicans gene ALS1 in Saccharomyces cerevisiae induces adherence to endothelial and epithelial cells. Infect. Immun. 66, 1783–1786 (1998).
26. Noble, C. J. et al. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. 2, e63. https://doi.org/10.1371/journal.ppat.0020063 (2006).
27. Feng, Q., Summers, E., Guo, B. & Fink, G. Ras signaling is required for serum-induced hyphal differentiation in Candida albicans. J. Bacteriol. 181, 6339–6346 (1999).
28. Dieterich, C. et al. In vitro reconstructed human epithelia reveal contributions of Candida albicans EFG1 and CPH1 to adhesion and invasion. Microbiology 148, 497–506. https://doi.org/10.1099/00221287-148-2-497 (2002).
30. Bishop, A.  

31. Braun, B. R., Kadosh, D. & Johnson, A. D. NRG1, a repressor of filamentous growth in C. albicans, is down-regulated during filament induction. *EMBO J.*, 20, 4753–4761. https://doi.org/10.1093/emboj/20.17.4753 (2001).

32. Kim, M. J., Kil, M., Jung, J. H. & Kim, J. Roles of Zinc-responsive transcription factor Csr1 in filamentous growth of the pathogenic Yeast *Candida albicans*. *J. Microbiol. Biotechnol.*, 18, 242–247 (2008).

33. Nobile, C. J. et al. Biofilm matrix regulation by *Candida albicans* Zap1. *PloS Biol.*, 7, e1000133. https://doi.org/10.1371/journal.pbio.1000133 (2009).

34. Mechoulam, R., Hanus, L. O., Pertwee, R. & Howlett, A. C. Early phytocannabinoid chemistry to endocannabinoids and beyond. *Nat. Rev. Neurosci.*, 15, 757–764. https://doi.org/10.1038/nrn3811[pii] (2014).

35. Radhakrishnan, R. & Ross, D. A. From, “Azalla” to anandamide: Distilling the therapeutic potential of cannabinoids. *Biol. Psychiatry* 83, e27–e29. https://doi.org/10.1016/j.biopsych.2017.11.017 (2018).

36. Maccarrone, M. et al. Endocannabinoid signaling at the periphery: 50 years after THC. *Trends Pharmacol. Sci.* 36, 277–296. https://doi.org/10.1016/j.tips.2015.02.008S0165-6147(15)00034-6 (2015).

37. Di Scala, C., Fantini, J., Yahi, N., Barrantes, F. J. & Chahinian, H. Anandamide revisited: how cholesterol and ceramides control receptor-dependent and receptor-independent signal transmission pathways of a lipid neurotransmitter. *Biomolecules* https://doi.org/10.3390/biom8020031 (2018).

38. Fenwick, A. J. et al. Direct anandamide activation of TRPV1 produces divergent calcium and current responses. *Front. Mol. Neurosci.*, 10, 200. https://doi.org/10.3389/fnmol.2017.00200 (2017).

39. Devane, W. A. et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949. https://doi.org/10.1126/science.1470919 (1992).

40. Milman, G. et al. N-arachidonoyl L-serine, an endocannabinoid-like brain constituent with vasodilatory properties. *Proc. Natl. Acad. Sci. USA* 103, 2428–2433. https://doi.org/10.1073/pnas.0510676103 (2006).

41. Mechoulam, R. et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochim. Biophys. Acta.* 50, 83–90. https://doi.org/10.1016/0005-2950(95)00109-d (1995).

42. Gerami-Nejad, M., Zarchi, I. F., McClellan, M., Matter, K. & Berman, J. Shuttle vectors for facile gap repair cloning and integration into a neutral locus in *Candida albicans*. *Microbiology* 159, 565–579. https://doi.org/10.1099/mic.0.064097-0 (2013).

43. Feldman, M., Tanabe, S., Howell, A. & Grenier, D. Cranberry proanthocyanidins inhibit the adherence properties of *Candida albicans* and cytokine secretion by oral epithelial cells. *BMC Complement. Altern. Med.* 12, 6. https://doi.org/10.1186/1472-6882-12-4 (2012).

44. Feldman, M., Al-Quntar, A., Polacheck, I., Friedman, M. & Steinberg, D. Therapeutic potential of thiazolidinedione-8 as an antibiofilm agent against *Candida albicans*. *PloS ONE* 9, e93225. https://doi.org/10.1371/journal.pone.0093225 (2014).

Acknowledgements
This study was partially supported by Agriculture Ministry of Israel. We are grateful to Dr. Yael Feinstein-Rotkopf for operating the Nikon spinning scan microscope at our Interdepartment Core Research Facility. We thank Muna Aqawi and Sarah Gingichashvili for their support.

Author contributions
Conceived and designed the experiments: R.S.V., M.F. and D.S. Performed the experiments: R.S.V. Analysed the data: R.S.V. Synthesized the reagents: R.S. and R.M. Wrote and reviewed the paper: R.S.V., M.F., R.S., R.M. and D.S.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-70650-6.

Correspondence
and requests for materials should be addressed to R.V.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020