Research Article

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Future Medicinal Chemistry

The immunomodulatory activity of caffeic acid phenethyl ester in *Caenorhabditis elegans* is mediated by the CED-10 (Rac-1)/PAK1 pathway

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Aim: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE), the major constituent of propolis, is able to increase the survival of the nematode *Caenorhabditis elegans* after infection with the fungal pathogen *Candida albicans*. **Results:** CAPE increases the expression of several antimicrobial proteins involved in the immune response to *C. albicans*. Structural derivatives of CAPE were synthesized to identify structure–activity relationships and decrease metabolic liability, ultimately leading to a compound that has similar efficacy, but increased *in vivo* stability. The CED-10(Rac-1)/PAK1 pathway was essential for immunomodulation by CAPE and was a critical component involved in the immune response to fungal pathogens. **Conclusion:** *Caenorhabditis elegans* is an efficient heterologous host to evaluate immunomodulatory compounds and identify components of the pathway(s) involved in the mode of action of compounds.

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Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) is a flavonoid-like compound derived from honeybee propolis, a wax-like resinous substance that has been used as a folk medicine since at least 300 BC [1–5]. Since its identification as a major constituent of propolis [6], CAPE has been shown to have a wide range of biological activity, including antiviral, antifungal, antioxidative, antitumor and anti-inflammatory properties [2,7]. The immunomodulatory activity of CAPE has been documented on multiple types of immune cells and, in particular, the peripheral blood mononuclear cells (lymphocytes, monocytes and macrophages) [3]. Overall, the molecular mechanism by which CAPE modulates the immune system remains unknown, although nuclear factor of activated T cells (NFAT) and NF-κB have been proposed as critical mediators of this immune response [8].

CAPE inhibits two key transcription factors involved in T-cell activation, NFAT and NF-κB [8,9]. Inhibition of either of these two transcription factors could lead to the decreased production of cytokines associated with CAPE treatment [3,9,10]. Cytokines produced by Th1 (IL-2) and Th2 (IL-4) type lymphocytes, as well as cytokines produced by of macrophages, monocytes and dendritic cells (IL-1β and IL-12), are transcriptionally repressed in the presence of CAPE [3,9,10]. In contrast to these studies, production of the cytokine TGF-β1 was stimulated in the presence of CAPE, and *in vivo* studies using a murine model indicate $CD4^+$ T-cell populations are increased [3,11]. Moreover, studies using primary splenic lymphocytes exposed indicate that secreted IL-2 and IL-4 cytokine concentrations are significantly increased in the presence of CAPE [11].

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Caenorhabditis elegans has served as a useful *in vivo* heterologous host to identify and evaluate potential therapeutic compounds against several microbes [12] including the clinically relevant yeasts *Candida albicans* [13] and *Cryptococcus neoformans* [14]. Of systemic bloodstream infections, *Candida* spp. are the fourth most common etiological agents responsible for the infection, and within this group of fungi *C. albicans* is the most prevalent [15,16]. In a multistate point-prevalence survey of healthcare-associated infections, *Candida* spp. infections represented 6% of all hospital-acquired infections in the USA in 2011, and ranked first among causes of primary bloodstream infections [17].

Several screens for compounds with efficacy against *C. albicans* using *C. elegans* as a host have been conducted, and have collectively identified 39 compounds with uncharacterized antifungal activity against *Candida* spp. [7,18–20]. One of the identified compounds was CAPE, which was able to significantly increase *C. albicans*-infected nematode survival and disrupt fungal biofilm formation [7]. Importantly, CAPE was able to prolong survival of mice in a candidiasis model of infection [7]. The minimum inhibitory concentration (MIC) of CAPE against *C. albicans* was 64 μg/ml; however, an increase in nematode survival was observed at lower concentrations (4–8 μg/ml) suggesting the survival may also be due to immunomodulatory activity of the compound in the nematode [7].

The innate immune response of *C. elegans* to an intestinal infection of *C. albicans* has indicated that the PMK-1/p38 MAPK cascade is a key component [7,13]. Inactivation of this kinase cascade significantly decreases *C. albicans*-infected nematode survival [7,13]. Global expression studies revealed that 124 and 189 *C. elegans* genes were up- or down-regulated twofold or greater, respectively, after 4 h of infection with the fungus [13]. These genes were enriched for confirmed and putative antimicrobial proteins, and genes encoding proteins involved in detoxification and stress response [13]. Interestingly, unlike mammalian cells, *C. elegans* lacks homologs to NFAT and NF-κB [12] and, therefore, if the increased *C. albicans*-infected nematode survival is due to the immunomodulatory activity of CAPE, the mechanism responsible for conferring the efficacy must be due to other factors.

We demonstrate herein that CAPE demonstrates immunomodulatory activity in *C. elegans* and exploited the use of the nematode as a model host to assess the efficacy of 14 structural analogs of CAPE. A divergent and distinct structure–activity relationship (SAR) within CAPE was developed for the observed antifungal and immunomodulatory activities while, at the same time, reducing the inherent metabolic liability of the parent compound. Further studies of the immunomodulatory activity revealed that the efficacy conferred by the immunomodulating-specific compounds required the CED-10/PAK1 pathway in *C. elegans*. These components were found to be involved in the innate immune response pathway of the nematode against the fungal pathogens *C. albicans* and *C. neoformans*.

Materials & methods/experimental

Caenorhabditis elegans strains & culture conditions

Caenorhabditis elegans N2, *glp-4(bn2); sek-1(km4),* DH26 *fer-15*(b26)II and RB689 *pak-1*(ok448) X were obtained from the *Caenorhabditis* Genetics Center, University of Minnesota. The *fer-15*;*pak-1* double mutant was generated through crosses and is described in detail below. Nematodes were maintained at 15°C, unless otherwise noted and propagated on nematode growth medium seeded with *Escherichia coli* HB101 according to standard techniques [21].

Caenorhabditis elegans-killing assays

To assess the efficacy of compounds to confer an increase in survival to *C. albicans*-infected nematodes, the preinfection liquid killing assay was performed using 30–40 *glp-4(bn2); sek-1(km4)* L4 worms as previously described [22]. Worms that died as a result of adhering to the wall of the plate were not included in the analysis and censored. Each assay was carried out in duplicate and repeated at least twice, and compounds conferring a significant increase in nematode survival were confirmed by repeating the assay using *fer-15*(b26) nematodes. Nematode survival was calculated by the Kaplan–Meier method, and survival differences were tested for significance using the log–rank test. The level of significance was set at $p < 0.05$, as indicated. Representative survival assays are indicated in the figures. Survival was monitored once daily (at ~24-h intervals) and the time points are presented in hours, for accuracy.

Assessment of lifespan in liquid media

Synchronized wild-type N2 nematodes were prepared by egg prep and allowed to hatch overnight. Nematodes were transferred into 2-ml S-basal media with cholesterol in six well plates and fed daily with HB101. Nematode survival was monitored daily throughout the duration of the experiment, and nematodes were moved to new assay plates every other day during egg-laying period. The assay was carried out twice with 35 nematodes per assay condition.

Expression studies

Quantitative reverse transcription PCR analysis of genes was carried out using a Bio-Rad CFX96 real-time PCR system. RNA was obtained from nematodes using a previously described procedure [13] with slight modifications. Briefly, approximately 200 synchronized L4 nematodes were added to 1.9-ml SC-complete media with 2× additives in six-well assay plates. For each well, CAPE was added to a final concentration of 8 µg/ml in 1% DMSO as well as 50 µl of a 5× overnight concentrate of HB101 for the nematodes to feed. Nematodes from a single well were collected at 2 and 4 h post-treatment and total RNA extracted using Tri-Reagent (Molecular Research Center, Inc., OH, USA) and reverse transcribed into cDNA using the Ambion RETROscript reverse transcription kit (Life Technologies, NY, USA). Quantitation was carried using iTaq Universal SYBR green supermix (Bio-Rad, CA, USA) and primers listed in Supplementary Table 2. Previously established primers for *F08G5.8*, *F35E12.5*, *irg-1*, *irg-2*, *irg-3*, *clec-60*, *T24B8.5*, *C17H12.8*, *C32H11.1* and *cyp35B1* were kindly provided by the Ausubel laboratory (Massachusetts General Hospital, MA, USA). C_t-values were normalized using *snb-1* as the reference and calculated using the $2^{-\Delta\Delta Ct}$ method [23].

Assessment of MIC of compounds

The MIC of the compounds was determined using the established procedure in the Clinical and Laboratory Standards Institute M27-A3 guideline [24].

NF-κB luciferase reporter assay with CAPE derivatives

To evaluate the inhibitory effect of the compounds on NF-κB activity, GloResponse™ NF-κB-RE-*luc2P* HEK293 Cell Line (Promega Cat# E8520) was used. All compounds were tested in 12 dilutions with assay concentrations ranging from 33.33 to 0.0163 µM in duplicate plates. A similar dose–response curve was generated using parthenolide and oridonin as positive controls. A total of 5000 NF-κB-RE-*luc2P* HEK293 cells were plated in 384-well plates in duplicate and the next day preincubated with the compounds for 2 h and then stimulated with 20 ng/ml of TNF-α for 5 h and performed Steady-Glo Luciferase assay. Percent of NF-κB activity was calculated by normalizing to treatment with TNFα and solvent alone (DMSO) as 100%.

Phosphate-buffered saline solubility

Solubility was determined in phosphate-buffered saline (PBS) pH 7.4 with 1% DMSO. Each compound was prepared in triplicate at 100 µM in both 100% DMSO and PBS with 1% DMSO. Compounds were allowed to equilibrate at room temperature with a 750-r.p.m. vortex shake for 18 h. After equilibration, samples were analyzed by ultra-performance liquid chromatography–mass spectrometry (Waters, MA, USA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. The DMSO samples were used to create a two-point calibration curve to which the response in PBS was fit.

Plasma stability

Plasma stability was determined at 37°C at 5 h in both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma diluted 50/50 (v/v) with PBS pH 7.4 (0.95% acetonitrile and 0.05% DMSO). Compounds were incubated at 37°C for 5 h with a 350-r.p.m. orbital shake with time points taken at 0 and 5 h. Samples were analyzed by ultra-performance liquid chromatography–mass spectrometry (Waters) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

Generation of *fer-15*(b26);*pak-1*(ok448) nematodes

Nematodes carrying the single mutations (DH26 *fer-15*(b26)II and RB689 *pak-1*(ok448)X) were crossed generating an F1 population of heterozygous nematodes. These worms were allowed to self-fertilize generating an F2 population that includes the desired homozygous *fer-15*;*pak-1* mutant nematodes. The resulting F2 progeny were initially screened for the homozygous *fer-15* mutation by assessment of the temperature-sensitive sterile phenotype conferred by the homozygous *fer-15* mutation. Progeny that were temperature sensitive sterile at 25°C (homozygous *fer-15*) were then subsequently screened for the homozygous *pak-1* mutation by two single-worm PCR assays. As the ok448 *pak-1* mutation is due to a 1425 bp deletion within the coding region, primers that flank the deletion site (5′-GGA CAG AAT GGG AGA AAT TG-3′ and 5′-ATG GTG AAA CTC CTG CTG AT-3′) will generate PCR products of 2033 and 609 bp indicating the *pak-1* wild-type and ok448 alleles, respectively. A second PCR reaction was carried out on the nematodes that indicated the ok448 allele was present using primers within the deletion region (5′-TAC CCA GCT TTC GGT AGT TC-3′ and 5′-ACT GAT GTT GTC ACG GAG TG-3′) to confirm the homozygous *pak-1* mutation.

RNA interference

The *ced-10* RNAi bacterial clone was obtained from the *C. elegans* RNAi library of Source Bioscience (Nottingham, UK). Bacteria were grown in Luria broth (LB) with 100 µg/ml ampicillin and spread

on 10 cm RNAi plates (nematode growth medium + 50 μg/ml ampicillin + 1 mM Isopropyl-β-D-thiogalactopyranoside). Synchronized L1 larvae were placed on each RNAi plate and incubated at 15°C. Their progeny L1 worms were transferred to new RNAi plates and incubated at 25°C for 2 days. Young adult worms were used in killing assays after washing multiple times in M9.

Results & discussion

Efficacy of CAPE to increase survival of *Candida albicans*-infected nematodes is due to immunomodulatory activity

In a previous study, CAPE was shown to have direct antifungal activity with an MIC of 225 μM (64 μg/ml in 2% DMSO); a concentration that was higher than necessary to confer an increase in nematode survival (14–28 μM; 4–8 μg/ml) leading to the speculation that the efficacy may be due to immunomodulatory activity [7]. The alternate possibility for the increased survival of *C. elegans* is that CAPE is able to prolong the overall lifespan of the nematode. In order to assess if the increased survival is due to alteration in life expectancy, we evaluated the nematode lifespan under various conditions in the presence and absence of CAPE to determine if the compound has an influence. Contrary to the increase in survival observed for *C. albicans*-infected nematodes, there was a significant decrease in survival ($p = 0.0015$) when the nematodes were assayed in a liquid assay in the presence of 35 μM (10 μg/ml) of CAPE at 20°C (Supplementary Figure 1A). When the temperature for the lifespan assay was increased to the same temperature used in the *C. albicans*-killing assays (25°C), there was no significant difference in the lifespan of the nematodes ($p = 0.2333$; Supplementary Figure 1B). The inability of CAPE to alter the nematode lifespan under the same environmental conditions used as the *C. albicans* survival assay supports the hypothesis that the increased *C. albicans*-nematode survival could be due to immunomodulatory activity of the compound.

In order to confirm that CAPE elicits immunomodulatory activity in *C. elegans*, we evaluated the induction of genes known to be involved in the immune response. As noted above, our group has investigated the immune response of *C. elegans* challenged with *C. albicans* and identified several proteins with putative antimicrobial and pathogen-related functions [13]. The transcription of 8 genes (from a total of 33 genes significantly upregulated ≥3.0-fold) that were induced upon feeding on *C. albicans* was monitored after treatment with 28 μM (8 μg/ml) CAPE by quantitative PCR (qPCR). These genes were selected on the basis of their increased expression in response to *C. albicans* infection and their presumed function, and include the antimicrobial proteins *abf-2*, *fipr-22,23*, *cnc-4* and *cnc-7*; the chitinases *cht-1* and *T19H5.1*; the ferritin homolog *ftn-1*; and the predicted lipase *Y46H3A.4*. The expression of another 11 genes that have differential expression in the presence of bacterial pathogens and are believed to act as part of the immune response or detoxification was also monitored after treatment with CAPE. Of the 19 total genes that were analyzed, 3 were induced by ≥3.0-fold in 4 h after treatment with 28 μM (8 μg/ml) of CAPE (Table 1). These three genes included the antimicrobial proteins encoded by *fipr-22,23* and *cnc-4* and the chitinase encoded by *cht-1*. All three of these genes were also increased in expression after treatment with *C. albicans*, suggesting they may be partially responsible for the increased survival of CAPE-treated nematodes when challenged with the fungus. Of note, the expression of the putative antimicrobial proteins, encoded by *fipr-22,23* and *cnc-4*, with CAPE was at a higher level when compared at the same time point (4 h) after feeding on *C. albicans*. None of the genes included in the assay were found to be repressed by exposure to CAPE.

Increased *Candida albicans*-infected nematode survival following CAPE treatment is not observed with its metabolite caffeic acid

Metabolism studies have shown that CAPE can be readily hydrolyzed into the major metabolite, caffeic acid (Supplementary Material & Figure 1) [25]. Accordingly, the ability of caffeic acid to confer an increase in survival to *C. albicans*-infected nematodes was assessed (Figure 2). At a concentration of 14 μM (4 μg/ml), CAPE was able to confer a significant increase in survival to nematodes infected with *C. albicans* ($p = 0.0158$), while an increase in survival was not observed when treated with concentrations of caffeic acid ranging from 22–177 μM (4–32 μg/ml; $p = 0.5625$ at 22 μM). The inability of caffeic acid to confer an increase in *C. albicans*-infected nematode survival demonstrates that the intact parent compound is necessary for the immunomodulatory activity and the activity is not due to active CAPE metabolites. However, it should be noted that it is possible that other metabolites are produced, but their quantity would most likely be negligible.

SAR assessment of CAPE analogs to identify moieties involved in immunomodulatory activity & metabolic liabilities

The studies detailed in the previous sections established the impact of CAPE in the *C. elegans*–*C. albicans* model and provided us with a unique whole-

Table 1. Genes that were expressed threefold or greater after treatment with 28 μM (8 μg/ml) caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester.

Gene	Function	Average fold increase after 2 h	Average fold increase after 4 h
fipr-22,23	Putative antimicrobial peptide	5.26 ± 1.94	22.98 ± 1.80
cnc-4	Putative caenacin antimicrobial peptide	2.90 ± 2.68	31.19 ± 1.30
cht-1	Chitinase	1.26 ± 0.66	3.50 ± 0.21

animal model system to study the immune effects of this compound. In the next series of studies, we leveraged the *C. elegans*–*C. albicans* system in a series of SAR studies of CAPE. In order to assess potential sites of metabolic liability in CAPE, while tuning the immunomodulatory activity and/or antifungal activity of its analogs, modifications to four structural elements of CAPE were designed and tested: the catechol moiety and the cinnamyl linkage, which are both potential sites of conjugative (glucuronidation) and/or oxidative metabolism; the ester linkage, which undergoes hydrolysis to generate caffeic acid as a metabolite; and the phenethyl ring, which is susceptible to oxidative processes.

First, modifications to the catechol moiety (compounds **1–6**; Table 2) were assessed. The phenolic moieties of CAPE were modified via alkylation in a benzodioxole (compound **1**) or benzodioxane (compound **2**) ring system. By incorporating the phenols into these constrained ring systems, we evaluated the necessity of hydrogen-bond donors at these sites while maintaining the electronic nature of the catechol moiety and mitigating potential conjugative metabolism. In a nematode survival assay, compound **1** showed no change in host survival when challenged with *C. albicans*. However, compound **2** retained efficacy at a concentration ranging from 13 to 51 μM (4–16 μg/ml), which is similar to the range observed for CAPE (14–28 μM; 4–8 μg/ml). Both compounds had no antifungal activity as measured by MIC against *C. albicans*. The electronic and/or hydrogen-bond donor/acceptor effects of the individual phenols were further probed by removing one or the other (compound **3**) or examining regioisomeric effects (compound **4**). Additionally, fused-ring system isosteres for either the electronic (compound **5**) or hydrogen-bond acceptor properties (compound **6**) were also examined. Compounds **3** and **4** possess a single phenol at the meta or ortho position, respectively. Both compounds showed complete loss of antifungal activity (MIC >256 μg/ml) and had no effect in the nematode survival assay. Similarly, loss of activity was observed in both assays when the catechol moiety was replaced with either an electron-rich indole (compound **5**) or an electron-poor benzoxazole (compound **6**) ring. The ability of the phenols or the benzodioxane group to act as hydrogen-bond acceptors appears to play a key role in the activity of the compound in a host survival assay. On the other hand, the electronics of the ring system, whether electron-rich or electron-poor does not seem to have an effect.

The catechol group (free phenols) appears critical for the minimal direct antifungal activity of CAPE, but was not necessary for the immunomodulatory effects of the compounds as measured via the nematode survival assay (Table 2). MIC studies with other commercially available flavonoids (resveratrol, apigenin and myricetin) demonstrated that highly hydroxylated aromatics also display antifungal activity suggesting a general mechanism, probably driven by oxidative potentials (Supplementary Table 1). Overall, modifica-

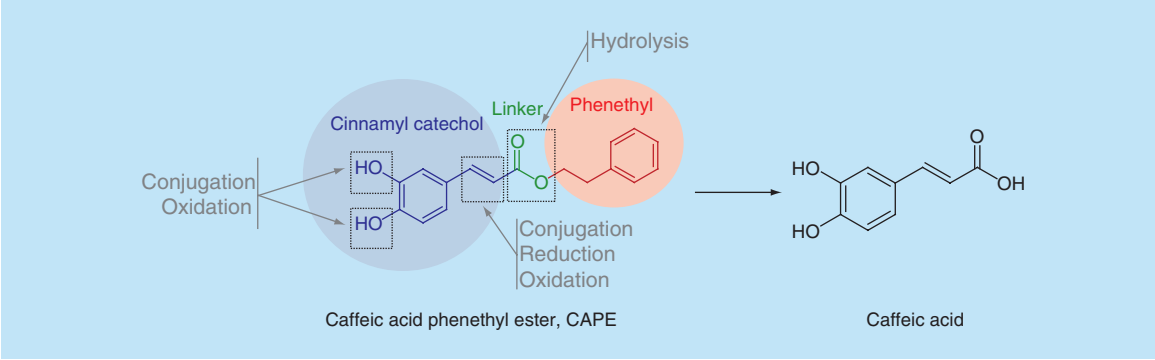


Figure 1. Key pharmacophoric elements of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester and associated metabolites.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester.

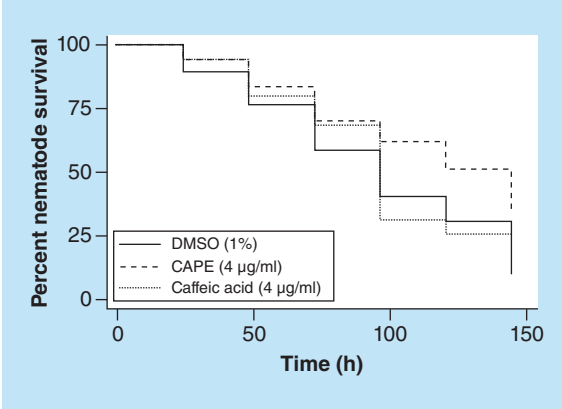


Figure 2. Survival assay of *Caenorhabditis elegans* infected with *Candida albicans* and treated with caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester or caffeic acid. Infected nematodes live significantly longer when treated with CAPE (p = 0.0158) while there is no significant difference in survival when infected nematodes are treated with caffeic acid (p = 0.5625). CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester.

tions to the catechol moiety led to compound **2** which retained similar efficacy to CAPE in a host survival assay, but demonstrated no inherent antifungal activity. However, these catecholic capping modifications did not lead to improved metabolic stability in rodents and displayed poor solubility (Table 2); therefore, this compound was not tested further.

In order to improve the metabolic stability of compound **2**, we examined the cinnamyl moiety within CAPE which may be susceptible to reductive, oxidative and/or conjugative metabolic processes. The reduction of the conjugated double bond to a saturated ethylene linker would eliminate this potential metabolic liability, but also drastically change the conformational flexibility of the small molecule by increasing the number of rotatable bonds. The reduction of the cinnamyl moiety in CAPE provided compound **7** which displayed improved antifungal activity (MIC: 56 µM [16 µg/ml]) and similar *C. albicans* infected-nematode survival efficacy (14–28 µM; 4–8 µg/ml) compared with its parent CAPE. Additionally, compound **7** demonstrated improved physicochemical properties, as well improved metabolic properties (a PBS solubility of 246 µM and a mouse plasma stability of 100%; Table 2). Notably, the difference between MIC and survival efficacy is only about twofold, and, although this difference was consistent in numerous assays, no final conclusions in regard to antifungal activity or nematode survival are based on this marginal difference.

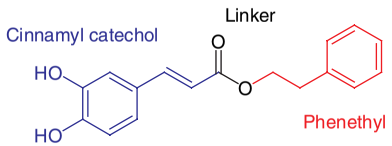
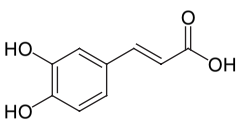
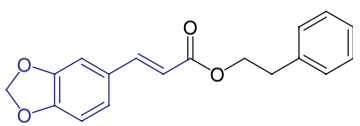
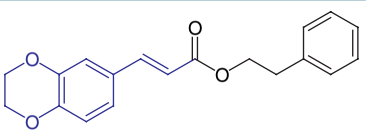
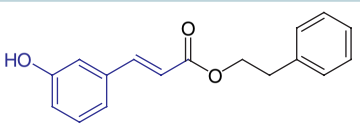
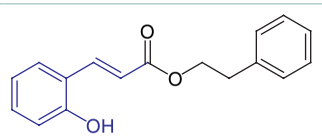
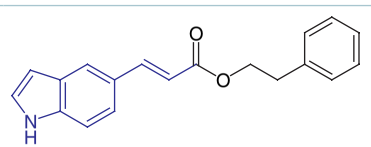
Next, a series of simple analogs modifying the phenethyl moiety were designed (compounds **8–10**; Table 2) to establish and define the SARs for this portion of the molecule. Compounds with a shorter linker length (benzyl, compound **8**), as well as more hydrophilic substitutions (the 4-pyridyl, compound **9**; the morpholine, compound **10**), were evaluated for their antifungal and nematode survival activities. With the exception of compound **10**, which displayed moderate activity in the nematode survival assay, these modifications did not provide improved activity relative to the parent compound CAPE. For this reason, we chose to retain the phenethyl moiety in subsequent analogs exploring alternative linking motifs.

Changes to the ester linkage in CAPE were next assessed (Table 2) in order to modify its propensity for hydrolysis. Although the carbon replacement keto analog (compound **11**) and NH–amide analog (compound **12**) displayed a complete loss of efficacy in the nematode survival assay, both compounds retained antifungal activity on par with CAPE. Methylation of the amide in compound **12** provided compound **13** which demonstrated good efficacy in both the nematode survival assay and the antifungal assay (13–107 µM; 4–32 µg/ml), demonstrating that the ester linkage in CAPE does not contribute to either of these biological activities. Compound **13** also displayed improved solubility (78 µM) and plasma stability (91% remaining) relative to CAPE, providing more stable linkage alternatives.

Based on the combined SARs defined above and the observed effects on stability, compound **14** was designed to incorporate the optimal modifications of each CAPE substructure into a single hybrid molecule. We predicted this hybrid molecule would retain the ability of CAPE to enhance nematode survival exclusively through immunomodulatory activity and demonstrate improved metabolic stability. The hybrid compound **14** possesses the benzodioxane moiety of compound **2**, the saturated ethyl linkage in compound **7** and the *N*-methyl amide linker of compound **13**. These combined structural modifications in compound **14** conferred an increase in survival to *C. albicans*-infected nematodes with effective concentration ranges of 6–49 µM (2–16 µg/ml; Figure 3A & Table 2). Moreover, compound **14** displayed improved solubility (236 µM) and mouse plasma stability (99% remaining after 1 h), compared with its parent compound CAPE (2% remaining after 1 h; Table 2) and also displayed complete stability in rat plasma (102%).

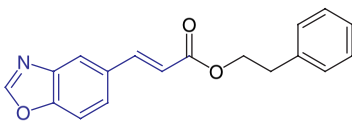
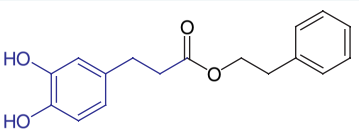
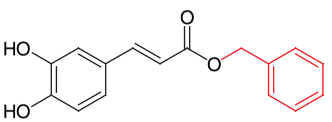
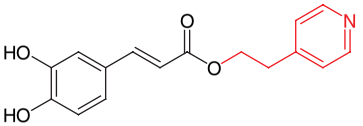
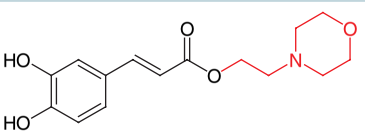
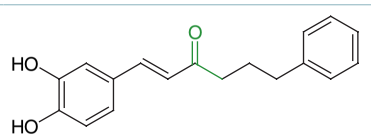
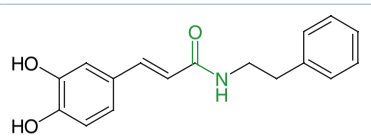
As observed with CAPE, at a concentration of 31 µM (10 µg/ml) of compound **14** in a liquid assay at 20°C, the lifespan of *C. elegans* was decreased (p = 0.0773; Supplementary Figure 2A); whereas when the lifespan was assessed at an assay temperature of

Table 2. Structure of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester derivatives and selected physiochemical and biological properties.

Compound	Structure	Nematode survival (μg/ml)	NF-κB inhibition†	MIC (μg/ml)	Solubility in PBS (μM)	Plasma stability (% remaining)		
						Human	Mouse	Rat
CAPE		4–8	66	32–64	18	97	2	ND
Caffeic acid		>32	ND	>256				
1		None	86	>256				
2		4–16	44	>256	2	93	1	ND
3		None	ND	128–256				
4		None	ND	>256				
5		32	75	>256				

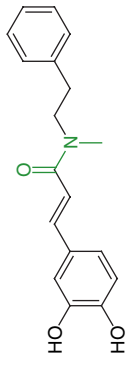
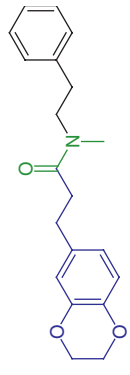
†Percent of NF-κB activity was calculated by normalizing to treatment with TNF-α and solvent alone (DMSO) as 100%.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester; MIC: Minimum inhibitory concentration; PBS: Phosphate-buffered saline.

Table 2. Structure of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester derivatives and selected physiochemical and biological properties (cont.).

Compound	Structure	Nematode survival (μg/ml)	NF-κB inhibition†	MIC (μg/ml)	Solubility in PBS (μM)	Plasma stability (% remaining)		
						Human	Mouse	Rat
6		>256	143	>256				
7		4–8	49	16	246	100	95	ND
8		64	68	32				
9		64	99	>256				
10		4–16	113	>256				
11		>256	42	32–64				
12		>256	94	32–64				

†Percent of NF-κB activity was calculated by normalizing to treatment with TNF-α and solvent alone (DMSO) as 100%.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester; MIC: Minimum inhibitory concentration; PBS: Phosphate-buffered saline.

Table 2. Structure of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester derivatives and selected physiochemical and biological properties (cont.).

Compound	Structure	Nematode survival (μg/ml)	NF-κB inhibition†	MIC (μg/ml)	Solubility in PBS (μM)	Plasma stability (% remaining)		
						Human	Mouse	Rat
13		4–32	50	32–64	78	93	91	ND
14		2–16	92	>256	236	97	99	102

†Percent of NF-κB activity was calculated by normalizing to treatment with TNF-α and solvent alone (DMSO) as 100%.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester; MIC: Minimum inhibitory concentration; PBS: Phosphate-buffered saline.

25°C, there was no significant difference in lifespan when compared with solvent alone ($p = 0.8544$; Supplementary Figure 2B), indicating that the increase in *C. albicans*-infected nematode survival is due to immunomodulatory activity of compound **14**. A correlation between the efficacy of compound **14** to confer nematode survival and expression of fungal immune response genes was found. Compound **14** was able to induce higher expression of *fipr-22,23* and *cnc-4* in less time than CAPE (Table 3).

In mammalian cells, CAPE has previously been demonstrated to have inhibitory activity against the NF-κB transcriptional protein complex [8,9]. As *C. elegans* lacks a homolog of NF-κB, we evaluated the inhibitory activity of the synthesized CAPE derivatives against this regulator of the immune response. A luciferase reporter assay was used to assess the ability of the CAPE analogs to inhibit NF-κB activity. Of the 14 CAPE analogs, 4 compounds were able to inhibit NF-κB activity at a concentration of 33 μM to a greater extent than CAPE (65.9% NF-κB activity compared with solvent alone; Table 2). The four compounds that were able to inhibit NF-κB were compound **2** (44.4%), compound **7** (49.0%), compound **11** (42.1%) and compound **13** (50.2%; Table 2). Although there was a correlation between NF-κB inhibitory activity and *C. albicans*-infected *C. elegans* survival, the only exception was compound **14** which had no inhibitory activity on NF-κB (Table 2).

Overall, as structural modifications were made to CAPE and evaluated in *C. albicans*-infected *C. elegans*, an SAR between antifungal and immunomodulatory activity became apparent. Modification of the free phenolic groups on the catechol moiety abrogated antifungal activity (compounds **1–6**; Table 2), but retained enhanced nematode survivability (compounds **2**, **5** and **14**; Table 2) through immunomodulatory effects. Systematic structural modifications allowed us to dissect the multiple biological effects exhibited by CAPE and resolve the multiple inherent metabolic liabilities in the parent compound. Modifications to each of the metabolic hot spots of CAPE, such as saturation of the cinnamyl motif and modification of the ester linkage, greatly increased plasma stability of the compounds across multiple species and improved physicochemical properties (Table 2).

The increased *Candida albicans*-infected nematode survival of CAPE-treated *Caenorhabditis elegans* requires CED-10 & p21-activated kinase-1

Previous reports have suggested that the GTPase Rac-1 is a molecular target of CAPE in mammalian cells [26]; however, this hypothesis is based on the structural similarity to caffeic acid [27] which, as we have shown above, is unable to promote survival of infected nema-

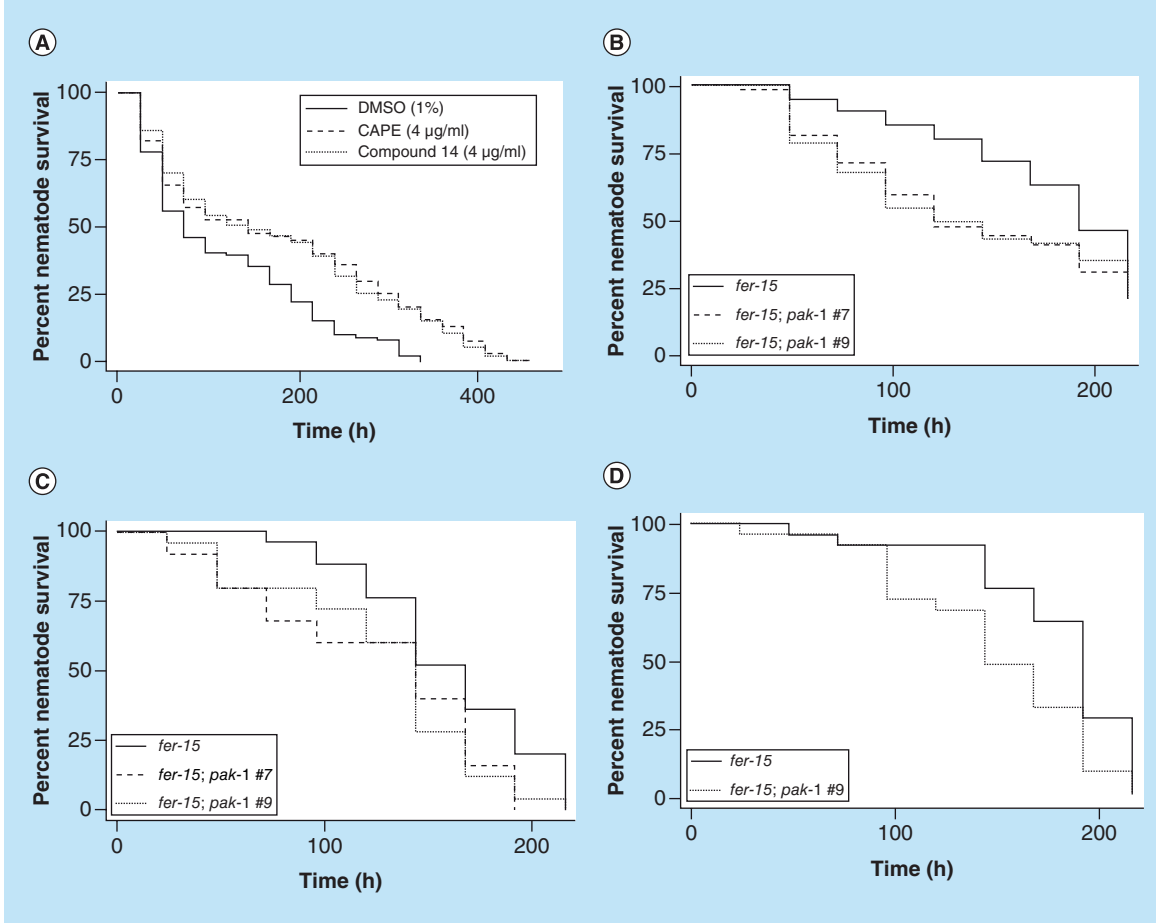


Figure 3. *Caenorhabditis elegans* survival assays after infection with fungal pathogens. (A) CAPE and compound **14** are capable of significantly increasing *Candida albicans*-infected nematode survival. **(B)** *pak-1* is involved in the immune response to *C. albicans* in a liquid-killing assay. Two independently generated *fer-15;pak-1* nematodes were significantly more susceptible to *C. albicans* infection when compared with *fer-15* nematodes. **(C)** *pak-1* is involved in the immune response to *C. albicans* in a solid killing assay. Two independently generated *fer-15;pak-1* nematodes were significantly more susceptible to *C. albicans* infection when compared with *fer-15* nematodes. **(D)** *pak-1* is involved in the immune response to *Cryptococcus neoformans*. *fer-15;pak-1* nematodes were significantly more susceptible to *C. neoformans* infection when compared with *fer-15* nematodes.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester.

todes (Figure 2). In mammalian cells, Rac-1 activates the p21-activated kinase-1 (PAK1), which is involved in a number of immune responses, including the p38 MAPK cascade and NF-κB [28–31]. These reports prompted investigation into the potential of PAK1 involvement in the *C. elegans* immune response.

A *C. elegans* strain harboring the *pak-1* mutation in the temperature sterile *fer-15* background was generated to aid in these studies to limit progeny production during the killing assays. Two independently generated progeny were obtained that had the desired genotype of *fer-15*(b26);*pak-1*(ok448). When both progeny of the *fer-15;pak-1* nematodes were challenged with *C. albicans* in a liquid killing assay, both strains were more susceptible to the fungal infection and had a significant reduction in survival (Figure 3B). A similar correlation was found when the mutants were challenged with *C. albicans* in a solid media killing assay (Figure 3C). There was no significant difference in survival in both the liquid and solid killing assays between the two *fer-15;pak-1* nematode strains ($p = 0.7515$ for liquid assay and $p = 0.8752$ for solid assay), and therefore only one strain was used in subsequent experiments.

In order to assess if the involvement of *pak-1* in the immune response is specific for *C. albicans*, killing assays were conducted using the *pak-1* mutant nematodes and the fungus *C. neoformans*. As observed when challenged with *C. albicans*, *pak-1* mutant nematodes are more susceptible to infection by *C. neoformans* and survival is significantly reduced ($p = 0.0119$; Figure 3D). Collectively the data demonstrate that PAK1 is important in the immune response to pathogenic fungi.

The ability of CAPE to confer an increase in *C. albicans*-infected nematode survival was evalu-

Table 3. Genes that were expressed threefold or greater after treatment with 8 μg/ml of compound 14.

Gene	Function	Average fold increase after 2 h	Average fold increase after 4 h
fipr-22,23	Putative antimicrobial peptide	23.02 ± 12.86	36.02 ± 1.76
cnc-4	Putative caenacin antimicrobial peptide	6.70 ± 3.16	24.57 ± 4.37

ated using the *pak-1* mutant nematodes. When *pak-1* was disrupted, an increase in nematode survival was not observed and the survival was significantly deceased compared with control nematodes ($p \leq 0.0001$) and they were not significantly different to untreated CAPE nematodes (*pak-1* vs *pak-1* and 14 μM [4 μg/ml] CAPE $p = 0.2193$; *ced-10* RNAi vs *ced-10* RNAi and 14 μM [4 μg/ml] CAPE $p = 0.6607$; Figure 4A)

As the Rho GTPase Rac-1 activates PAK1 in mammalian cells, the involvement of the *rac-1* ortholog, *ced-10*, in the *C. elegans* immune response during infection of *C. albicans* was investigated. Nematodes that were suppressed in transcription of *ced-10* by RNAi were more susceptible to infection by *C. albicans* and had a significantly shorter survival time when compared with control nematodes ($p = 0.0005$; Figure 4B). As with the *pak-1* mutant nematodes, when the *C. albicans*-infected *ced-10* RNAi nematodes were treated with CAPE, there was no significant increase in survival and was decreased in comparison to control nematodes ($p = 0.0001$) and they were not significantly different than untreated CAPE nematodes (*ced-10* RNAi vs *ced-10* RNAi and 14 μM [4 μg/ml] CAPE; $p = 0.6607$; Figure 4B).

A similar trend was observed when compound **14** was assayed. Nematodes lived significantly longer when treated with 12 μM (4 μg/ml) of compound **14** (*fer-15*, 2% DMSO vs *fer-15*, compound **14**; $p = 3 \times 10^{-6}$) which was absent in nematodes lacking *pak-1* (*fer-15;pak-1*, 2% DMSO vs *fer-15;pak-1*, compound **14**; $p = 0.459$). Collectively, these studies support that the immunomodulatory activity of CAPE and compound **14** both have the same mode of action and that this immunomodulatory activity is mediated by the PAK1 kinase.

Although several pathways have been characterized in *C. elegans* that are involved in immune response signaling, the involvement of the CED-10/PAK1 pathway in the innate immune response had not been previously known. Importantly, this study demonstrates that by taking advantage of *C. elegans* as an alternative model host, immunomodulatory compounds can be further developed. In particular, the lack of a homolog to NF-κB in the nematode allowed identification of additional molecular targets that might have been unnoticed in the presence of the transcription factor complex.

The MAPK cascade composed of NSY-1/SEK-1/PMK-1 is an integral signaling component of the innate immune response of *C. elegans* to several fungal pathogens [7,13,32,33], and the expression of *fipr-22,23*, *cnc-4* and *cnc-7* requires PMK-1 [13]. Expression studies with CAPE and compound **14** demonstrated that *fipr-22,23* and *cnc-4* were significantly upregulated after 4 h of treatment with either of the compounds

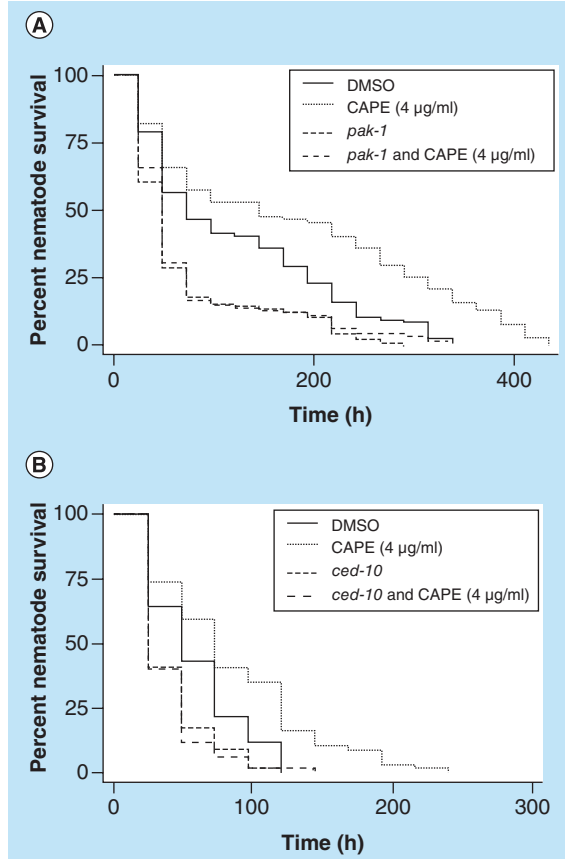


Figure 4. PAK1 and CED-10 is involved in the immune response to *Candida albicans*. **(A)** *pak-1* is involved in conferring the efficacy of CAPE against *Candida albicans*. *pak-1*-mutant nematodes do not respond to treatment with CAPE and the survival of CAPE-treated nematodes is not significantly different than nematodes treated with solvent alone. **(B)** *ced-10* is involved in conferring the efficacy of CAPE against *C. albicans*. Nematodes suppressed by RNAi for *ced-10* do not respond to treatment with CAPE and the survival of CAPE-treated nematodes is not significantly different than nematodes treated with solvent alone.
CAPE: Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester.

(Tables 1 & 3), suggesting that CED-10/PAK1 and the PMK-1 MAPK cascade are in the same pathway. In mammalian cells, RAC1 and PAK1 function upstream of several signaling pathways including the p38 MAPK cascade [28,34]. The involvement of PAK1 and PMK-1 in the same pathway in *C. elegans* suggests that there exists a commonality between the pathways of the nematode and mammalian cells. Additionally, other known PMK-1-regulated genes that are differentially transcribed in response to bacterial pathogens (*C17H12.8*, *C32H11.12*, *F08G5.6*, *F35E12.5* and *T24B8.5*) [35] were not altered in gene expression upon treatment with CAPE, suggesting other factors, which are dependent on the pathogen, may be involved.

In summary, the diverse biological activity of CAPE is mediated by the RAC1/PAK1 pathway. PAK1 is responsible for upstream phosphorylation of several important pathways in mammalian cells. In addition to the p38 MAPK cascade, PAK1 is responsible for activation of pathways involving ERK, c-Jun N-terminal kinase and NF-κB [30,31]. The previously described inhibitory activity of CAPE on NF-κB activity could actually be the result of inhibitory activity on PAK1, as opposed to NF-κB activity. Stimulation of NF-κB activity for various types of immune cells requires PAK1 and a constitutively active PAK1 is capable of stimulating NF-κB activity [30]. Furthermore, PAK1 is implicated in the development of various tumor types and the inhibition of PAK1 could be responsible for conferring the antitumor activity of CAPE [31]. Therefore, the development of the metabolically stable CAPE analogs that retain activity, such as compound **14**, could have far reaching implications, as it could potentially be further developed for multiple clinical applications.

Conclusion

The immunomodulatory activity of CAPE is responsible for the increased nematode survival when challenged with the clinically relevant fungus *C. albicans*. Structural analogs of CAPE have been synthesized that have increased plasma stability, which do not have decreased potency. CAPE and analogs of the compound require the CED-10/PAK1 pathway in *C. elegans*. This pathway is important for conferring an innate immune response in the nematode.

Future perspective

The further development of immunomodulatory compounds may provide a means for clinical treatment against many pathogenic microbes and potentially autoimmune diseases.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.future-science.com/doi/full/10.4155/fmc-2016-0085

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Executive summary

Immunomodulatory activity

- Caffeic acid(3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) has immunomodulatory activity in the nematode *Caenorhabditis elegans*.
- Several genes implicated in the innate immune response to *Candida albicans* are upregulated after treatment with CAPE including putative antimicrobial peptides.

Structural analogs of CAPE

- The level of hydroxylation on the cinnamyl moiety of CAPE is responsible for the antifungal activity of the compound.
- Modification of the ester and double bond of CAPE increases the plasma stability of the molecule, without decreasing the immunomodulatory activity.

Immune response in *Caenorhabditis elegans*

- The CED-10(Rac-1)/PAK1 pathway in *C. elegans* is involved in the innate immune response to the pathogenic fungi *C. albicans* and *Cryptococcus neoformans*.
- CED-10/PAK1 is required for the immunomodulatory activity of CAPE in the nematode.

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