Bioorganic & Medicinal Chemistry 18 (2010) 5032–5038

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Synthesis of a series of caffeic acid phenethyl amide (CAPA) fluorinated derivatives: Comparison of cytoprotective effects to caffeic acid phenethyl ester (CAPE)

John Yang [a], Gwendolyn A. Marriner [c], Xinyu Wang [a], Phillip D. Bowman [b], Sean M. Kerwin [c,*], Salomon Stavchansky [a,*]

[a] Division of Pharmaceutics, College of Pharmacy, The University of Texas, Austin, TX 78712, USA
[b] US Army Institute of Surgical Research, San Antonio, TX 78234, USA
[c] Division of Medicinal Chemistry and Institute for Cellular and Molecular Biology, The University of Texas, Austin, TX 78712, USA

ARTICLE INFO

*Article history:*
Received 22 March 2010
Revised 27 May 2010
Accepted 31 May 2010
Available online 11 June 2010

*Keywords:*
Oxidative stress
Hydrogen peroxide
Ischemia/reperfusion injury
CAPE

ABSTRACT

A series of catechol ring-fluorinated derivatives of caffeic acid phenethyl amide (CAPA) were synthesized and screened for cytoprotective activity against $H_2O_2$ induced oxidative stress in human umbilical vein endothelial cells (HUVEC). CAPA and three fluorinated analogs were found to be significantly cytoprotective when compared to control, with no significant difference in cytoprotection between caffeic acid phenethyl ester (CAPE) and CAPA.



## 1. Introduction

Oxidative stress plays a significant role in the development of a variety of disease states such as inflammation, ischemia reperfusion injury (I/R), and cardiovascular complications.[1–4] Efforts to ameliorate oxidative stress have led to the discovery of a number of natural and novel compounds that have antioxidative properties or are able to induce genes with the downstream effect of counteracting oxidative stress.[5,6]

Caffeic acid phenethyl ester (CAPE), a plant polyphenolic concentrated in honeybee propolis, has been found to be biologically active in a variety of pathways including cytoprotection against oxidative stress. CAPE has been observed to exhibit anti-inflammatory,[7] anti-viral,[8] anti-carcinogenic,[9] and immunomodulatory[10] effects as well as protection against I/R injury in vivo.[11,12] CAPE has also been investigated for its antioxidative and radical scavenging properties.[13] Previous studies have reported the synthesis and investigation of catechol ring-fluorinated derivatives of CAPE with regard to cytoprotective ability against oxidative stress in vitro.[14] Cytoprotection to oxidative stress by CAPE and analogs has been correlated with their ability to up-regulate heme oxygenase 1 (HMOX 1) gene expression.[15,16] Despite demonstrating significant cytoprotection against oxidative damage both in vivo and in vitro, CAPE is known to be readily hydrolyzed in plasma.[17,18] Studies have suggested that esterase activity in blood and cells is responsible for the rapid degradation of CAPE.[18] Pharmacokinetic studies using a rat model have also shown that CAPE is cleared rapidly after intravenous administration to rats.[19]

The purpose of this study was to synthesize and investigate the cytoprotective activity of a series of amide derivatives of CAPE and previously reported CAPE analogs. Amides are generally associated with higher hydrolytic energies of activation compared to esters. Previous work on CAPA has described its ability to act as an antioxidant against lipid peroxidation[20] as well as a potential anti-inflammatory agent through its inhibition of 5-lipoxygenase.[21] CAPA has also been shown to exhibit significant radical scavenging activity using a 2,2-diphenyl-1-picrylhydrazyl assay.[22] Although various CAPA analogues have been investigated for both radical scavenging activity as well as α-glucosidase inhibition,[23] no catechol ring-fluorinated CAPA analogs have been studied. The cytoprotectant ability of CAPA in vitro has also not been previously addressed. Due to the importance of endothelial cells as a target of oxidative stress in I/R injury and other vascular complications, human umbilical vein endothelial cells (HUVEC) were chosen as

* Corresponding authors. Tel.: +1 512 471 1407; fax: +1 512 471 7474 (S.S.), tel.: +1 512 471 5074; fax: +1 512 232 2606 (S.M.K.).
*E-mail addresses:* skerwin@mail.utexas.edu (S.M. Kerwin), stavchansky@mail.utexas.edu (S. Stavchansky).

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doi:10.1016/j.bmc.2010.05.080

# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **01 JUL 2010** | **N/A** | **-** |

| 4. TITLE AND SUBTITLE | 5a. CONTRACT NUMBER |
|---|---|
| **Synthesis of a series of caffeic acid phenethyl amide (CAPA) fluorinated derivatives: comparison of cytoprotective effects to caffeic acid phenethyl ester (CAPE)** | 5b. GRANT NUMBER |
| | 5c. PROGRAM ELEMENT NUMBER |
| 6. AUTHOR(S) | 5d. PROJECT NUMBER |
| **Yang J., Marriner G. A., Wang X., Bowman P. D., Kerwin S. M., Stavchansky S.,** | 5e. TASK NUMBER |
| | 5f. WORK UNIT NUMBER |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| **United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234** | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | 10. SPONSOR/MONITOR'S ACRONYM(S) |
| | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

12. DISTRIBUTION/AVAILABILITY STATEMENT
**Approved for public release, distribution unlimited**

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | |
| **unclassified** | **unclassified** | **unclassified** | **UU** | **7** | |

the model in which to study the cytoprotective effects of these compounds against hydrogen peroxide induced oxidative stress.

## 2. Results

### 2.1. Synthesis

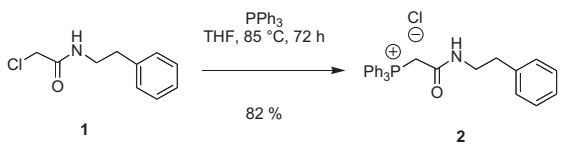
CAPA and five additional fluorinated amide analogues of CAPE were prepared using a Wittig coupling approach. The known chloroacetamide **1**,[24,25] was reacted with triphenyl phosphine to give the phosphonium chloride **2** (Scheme 1). Wittig coupling of **2** with unprotected hydroxybenzaldehydes **3a–e** proved problematic, in contrast to previous studies employing the analogous ester phosphonium chloride.[14] Thus, the hydroxybenzaldehydes **3a–e**, which were either commercially available or obtained via demethylation of the corresponding methoxybenzaldehydes with boron tribromide, were first transiently protected as the *t*-butyldimethylsilyl ethers by treatment with *t*-butyldimethylsilyl chloride and imidazole prior to Wittig coupling (Table 1). The resulting α,β-unsaturated amides were subjected to deprotection with TBAF, to afford CAPA and the desired amides **4b–e** in modest overall yields (Table 1). For aldehydes that do not require transient protection of the catechol functionality, the Wittig coupling proceeds in higher yield. The dimethoxybenzaldehyde **3f** was used directly in the Wittig coupling to afford **4f** in reasonable yield. With the exception of amide **4e** which was isolated as a ~3:1 mixture of (*E*)-/(*Z*)-isomers after column chromatography, the amides **4** were obtained as >90% pure (*E*)-isomers after column chromatography and, for **4a–c** and **4f**, recrystallization.

### 2.2. Cytotoxicity of amide derivatives compared to CAPE in HUVEC

CAPE and certain catechol ring-fluorinated CAPE analogs have been reported to be cytotoxic to HUVEC at higher concentrations.[14] CAPA and the CAPA derivatives were screened along with CAPE for toxicity in HUVEC. Each of the compounds were incubated with HUVEC at 10, 20, 40, and 60 μM concentrations for 24 h at 37 °C. Cell viability was measured using the CellTiter-Blue® assay and compared to a vehicle control. Cell viability less than 90% of control was considered toxic. The results, shown in Figure 1 demonstrate that CAPA and amides **4b** and **4d–f** showed no toxicity at any of the tested concentrations. CAPE exhibited cytotoxicity at 40 and 60 μM. The amide **4c** showed cytotoxicity at all concentrations.

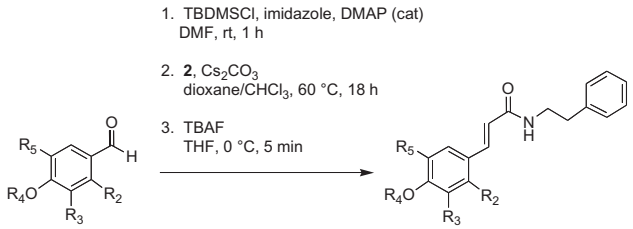
### 2.3. Cytotoxicity of $H_2O_2$ in HUVEC

Hydrogen peroxide is one of the principle reactive oxygen species produced in various vascular complications including ischemia reperfusion injury,[26] and has also been used in other in vitro models as an inducer of oxidative stress in endothelial cells.[27,28] To determine a suitable dose, HUVEC were treated with $H_2O_2$ at concentrations ranging from 0.01 to 5 mM for 1 h. Following the 1 h period the culture media was replaced and the cells were allowed to recover for 18 h. Cell viability was assessed with CellTiter-Blue® following the 18 h period. The target dosage was one that reduced




**Scheme 1.**

**Table 1**
Synthesis of CAPA and fluorinated CAPA analogues




| Compound | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Yield[a] (%) |
|---|---|---|---|---|---|
| **a** (CAPA) | H | H | H | OH | 14 |
| **b** | F | H | H | OH | 7 |
| **c** | H | OH | H | F | 15 |
| **d** | H | OMe | H | F | 22 |
| **e** | H | H | H | F | 8[b] |
| **f** | F | H | Me | OMe | 63[c] |

[a] Isolated overall yield from benzaldehyde **3** after column chromatography and recrystallization.
[b] Isolated as ~3:1 mixture of (*E*)-/(*Z*)-isomers.
[c] Step 2 only.

cell viability to approximately 20% of control, which was provided by 2 mM $H_2O_2$. The results are shown in Figure 2.

### 2.4. Cytoprotection against $H_2O_2$ induced oxidative stress in HUVEC

To evaluate oxidative stress in vitro, we employed a model using $H_2O_2$ as the inducer of oxidative damage. HUVEC were treated with CAPE or the amide derivatives **4a–f** at 20 μM concentration for 5 h. The cells were rinsed and then treated with 2 mM $H_2O_2$. After 1 h, the $H_2O_2$ containing medium was replaced with cell culture media and the cells were allowed to recover for 18 h. At the end of the 18 h period, cell viability was assessed with the CellTiter-Blue® Cell Viability assay and compared to cells treated only with vehicle and $H_2O_2$, as well as with those that were not exposed to $H_2O_2$. The results are shown in Figure 3. CAPA and compounds **4b**, **4c**, and **4e** exhibited significant cytoprotection against $H_2O_2$ when compared to vehicle only pre-treatment. CAPE was also significantly cytoprotective against $H_2O_2$. There was no significant difference in cytoprotective activity between CAPE and CAPA ($P$ >0.05).

In a dose dependent cytoprotection assay, HUVEC were treated with CAPE and CAPA at 1, 5, 20, 40, and 60 μM concentrations prior to the induction of oxidative stress with $H_2O_2$. The results are shown in Figure 4. The $EC_{50}$ was calculated for both compounds by linear regression using the first three data points. The $EC_{50}$ was found to be 8 μM for CAPE and 2 μM for CAPA.

## 3. Discussion

Introducing a fluorine group on the catechol ring increases the electronic density of the conjugated system, can decrease the interaction with catechol methyltransferase, and may also have a significant effect on receptor binding or selectivity.[29] The hydroxyl groups on the CAPA catechol may contribute to the antioxidative activity of the compound. We were interested in seeing the effect of replacing one of these hydroxyls with a fluorine, hydrogen or methoxy group on the cytoprotective activity of the compound.

Prior to evaluating the cytoprotective activity of CAPE and the CAPA derivatives, each compound was screened for toxicity in HUVEC. CAPE was found to be toxic at 40 and 60 μM, in accord with

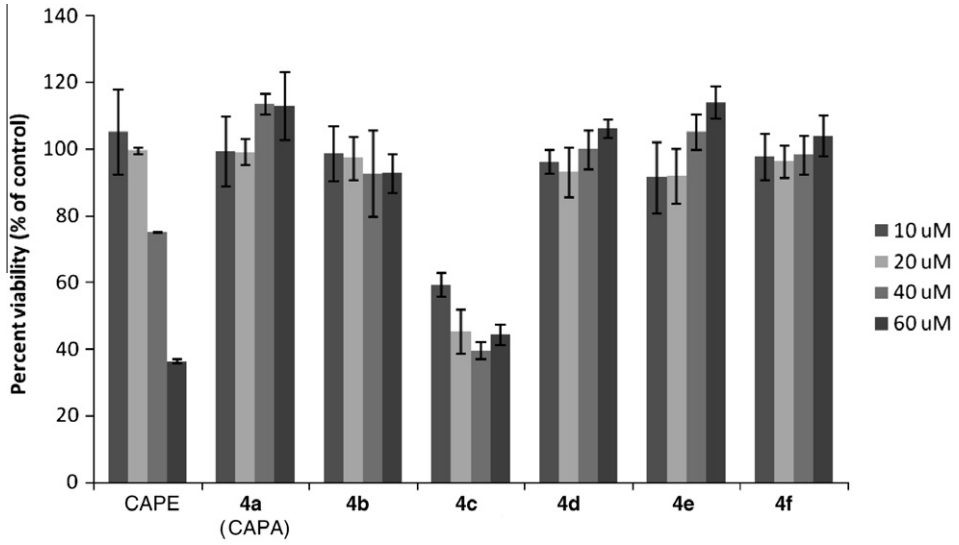



**Figure 1.** Toxicity of CAPE, CAPA, and CAPA derivatives toward HUVEC. Compounds were incubated in HUVEC for 24 h at 37 °C. Cell viability was determined by the Alamar Blue assay. Values are reported as a percentage of the vehicle control (0.1% DMSO).

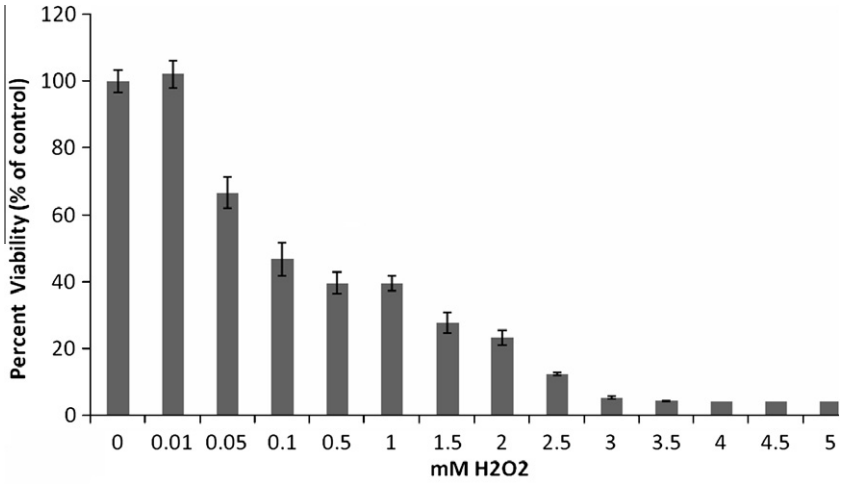



**Figure 2.** Toxicity of $H_2O_2$ in HUVEC. HUVEC were incubated in culture media containing the indicated concentration of $H_2O_2$ for 1 h at 37 °C. The culture media was replaced and cells were allowed 18 h to recover, then were assessed for viability with the CellTiter-Blue®.

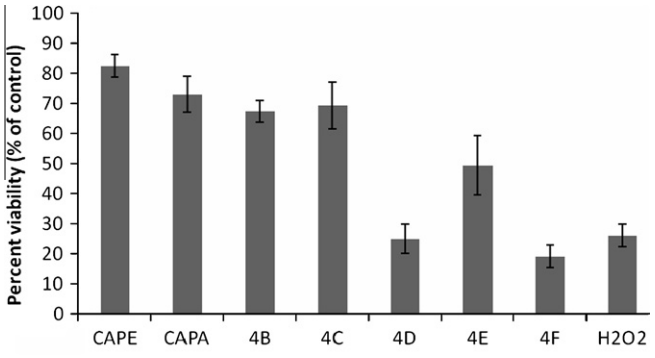



**Figure 3.** Cytoprotection of HUVEC against 2 mM $H_2O_2$ by CAPE, CAPA, and CAPA analogues. All compound concentrations were at 20 μM. CAPE, CAPA, 4B, 4C and 4E all showed significant cytoprotection when compared to untreated ($H_2O_2$ only) ($P$ <0.05). CAPA derivatives 4D and 4F provided no cytoprotection.

previous studies.[14] There are interesting differences in cytotoxicity of the certain amide derivatives when compared to their corresponding ester analogues. Amides **4e** and **4f** were not cytotoxic at any concentration up to 60 μM, the highest concentration examined. The corresponding ester derivatives were similarly reported to be non-cytotoxic at concentrations up to 15 μg/mL (ca. 50 μM). Amide **4c** was toxic at all the concentrations tested; similar to the corresponding ester analogue.[14] However, whereas CAPE and the esters corresponding to **4b** and **4d** are cytotoxic at concentrations greater than 40 μM, CAPA and the amides **4b** and **4d** are not cytotoxic even at concentrations as high as 60 μM. The origin of this difference in cytotoxicity between CAPE and certain fluorinated CAPE analogues versus CAPA and the corresponding fluorinated CAPA analogues is not clear.

CAPE was significantly cytoprotective against $H_2O_2$ induced oxidative stress in HUVEC. This was also demonstrated previously in a similar model,[30] as well as a study which used menadione to generate an oxidative stress.[14] Four of the amide derivatives of CAPE were also found to be significantly cytoprotective. These four compounds all contained either one or two hydroxyl groups on the cinnamic acid phenyl ring. Although compound **4c** proved to be very cytotoxic in HUVEC over a 24 h period, the toxicity is less apparent over a 5 h incubation time, as the compound was found to be significantly

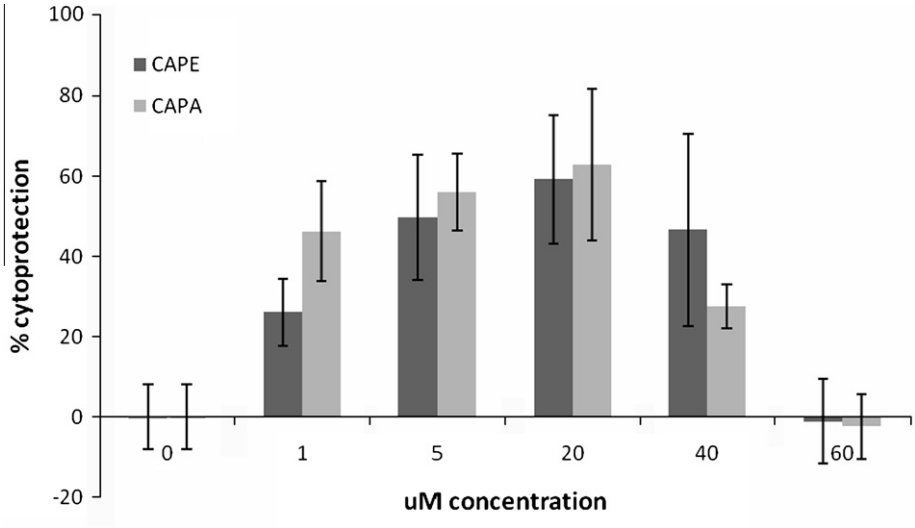



**Figure 4.** Dose response cytoprotection relationship of CAPE and CAPA against 2 mM $H_2O_2$. Concentrations above 40 μM are cytotoxic for both CAPE and CAPA and gave lower cell viability than untreated HUVEC as shown. CAPE and CAPA showed significant cytoprotection at concentrations from 1 through 40 μM.

cytoprotective against $H_2O_2$, and exhibited significantly higher cell viability over the vehicle control. While the mechanism behind this cytoprotective activity is not completely known, it is suggested that the antioxidative and radical scavenging properties of the catechol group are correlated with the protection against $H_2O_2$. The catechols CAPE, CAPA, **4b**, and **4c** all display cytoprotective effects; whereas, the monomethylated and dimethylated analogues **4d** and **4f**, respectively, were not cytoprotective. The *ortho*-fluorophenol **4e** demonstrated intermediate cytoprotection, which may be due to the ability of the *ortho*-fluorine substituent to stabilize the phenol radical formed upon hydrogen atom donation.[31] Interestingly, the cytoprotective activity for these amides is quite different from that reported for the corresponding esters.[14] The ester corresponding to **4c** is not cytoprotective, despite the presence of the catechol functionality, and the ester corresponding to **4e** is cytoprotective, despite the lack of any free phenolic hydroxyl groups. In the dose dependent cytoprotection assay, a biphasic response was observed for both CAPE and CAPA. The cytoprotection percentage increases from 1 up to 20 μM of CAPE and CAPA, then starts to decline at 40 μM. The drop off in cytoprotection at higher CAPE concentration had been attributed to CAPE's cytotoxicity above 20 μM,[13] as indicated in Figure 1. However, a similar effect is observed for CAPA, even though it is not cytotoxic at 40 μM. It is unclear as to why this phenomenon occurs.

It is not well understood how cytoprotection is provided by pretreatment with these cytoprotective agents. Cytoprotection probably involves an interplay between direct anti-oxidant activity and indirect anti-oxidant activity through effects on the transcription of anti-oxidant genes.[32] Studies previously performed in our group have shown that the cytoprotective activity of CAPE was correlated with the levels of the heme oxygenase 1 (HMOX 1) gene expression. CAPE is a potent inducer of the HMOX 1 gene transcription and has been shown to up-regulate it as much as eightfold over control.[15] Studies have also shown that when heme oxygenase activity is inhibited, the cytoprotective effect of CAPE against menadione induced oxidative stress is abolished.[15] Within a series of catechol ring-fluorinated CAPE analogs, cytoprotection is correlated with up-regulation of HMOX 1, but not direct anti-oxidant activity.[16] However, for CAPA and the CAPA analogs examined here, it is not clear how the balance of direct and indirect anti-oxidant effects contribute to cytoprotection. Work is currently being done to evaluate the role of HMOX 1 and to determine if CAPA exhibits similar response in regards to activation of HMOX 1.

The findings demonstrate that CAPA is less toxic than CAPE, and that there is no significant difference in cytoprotection between the two when tested at 5 and 20 μM concentrations against 2 mM hydrogen peroxide. It is anticipated that CAPA is more stable than CAPE in plasma. Previous studies have shown that ester compounds are very prone to hydrolysis via esterases, and that CAPE exhibits a short half life in plasma.[16] Plasma stability is an important issue in the drug development process, as it determines how much of the initial dose actually reaches the target site. Cinnamic acid-derived amides are known to be particularly stable to hydrolysis.[33] In preliminary studies, the expected increased stability of CAPA has been confirmed. CAPA is surprising stable to acid (0.1 M HCl, pH ~1), although it undergoes hydrolysis readily at pH ~10. While incubation of CAPE (100 μM) in rat plasma for 18 h at 37 °C results in complete hydrolysis, CAPA remains largely intact under these conditions (see Supplementary data).

## 4. Conclusions

CAPA and catechol ring-fluorinated derivatives of CAPA were synthesized and investigated for cytoprotective activity against hydrogen peroxide induced oxidative stress in HUVEC. All but one of the CAPA derivatives synthesized were non-toxic up to the maximally tested concentration, with **4c** being toxic at all tested concentrations. The results here also show that CAPA, **4b**, **4c**, and **4e** are all significantly cytoprotective in this model. The only two analogs which were not cytoprotective were the methylated compounds **4d** and **4f**. Although the mechanism of cytoprotection for these amides is not well understood, cytoprotection is correlated with the presence of free catechol hydroxyl groups in the analogs examined. CAPA was less toxic in HUVEC when compared to CAPE, however, there was no significant difference found in cytoprotection between the two compounds. This is significant as CAPA retains CAPE's cytoprotective activity yet is more stable in plasma.

## 5. Experimental

### 5.1. Materials and apparatus

The reagents chloroacetyl chloride, phenethylamine, chloro-*tert*-butyldimethylsilane (TBDMSCl), 3,4-dihydroxybenzaldehyde, 2-fluoro-4,5-dimethoxybenzaldehyde, 3-fluoro-4-methoxybenzal-

dehyde, 3-fluoro-4-hydroxy-5-methoxybenzaldehyde, tetra-butylammonium fluoride (TBAF), hydrogen peroxide, and boron tribromide were purchased from Sigma Aldrich (St Louis, MO) and used without further purification. All solvents were distilled prior to use. Nuclear magnetic resonance (NMR) spectroscopy was performed with a Varian Unity + 300 (300 MHz). Melting points were obtained using a Buchi B-540 apparatus and are uncorrected. Mass spectrometry services were provided by the Mass Spectrometry Facility at the University of Texas at Austin. Carbon, hydrogen, and nitrogen (CHN) elemental analysis was conducted by Quantitative Technologies Inc. (Whitehouse, NJ). HPLC was performed on a Varian Prostar 320 system. Purity of the final compounds was assessed by both normal (Si) and reverse phase ($C_8$) HPLC.

### 5.2. Preparation of Wittig reagent

#### 5.2.1. 2-Chloro-*N*-phenethylacetamide (1)[24,25]

To a solution of phenethylamine (20 mmol, 2.52 mL) in $CH_2Cl_2$ (40 mL) was added $K_2CO_3$ (24 mmol, 3.32 g). Chloroacetylchloride (22 mmol, 1.75 mL) was slowly added to the reaction mixture. The reaction mixture was stirred at 45 °C under argon for 18 h. The mixture was diluted with $CH_2Cl_2$, washed with water and brine, and then dried over $Na_2SO_4$. The resulting solution was concentrated under a rotary evaporator and the resulting solid filtered to give 2.93 g of white crystals (74% yield); mp 63.6–64.6 °C (lit,[25] 65 °C); $^1$H NMR spectrum matches literature.[25]

#### 5.2.2. Phenethylcarbamoylmethyl-triphenylphosphonium chloride (2)

To a solution of triphenylphosphine (18.9 mmol, 4.96 g) in THF (50 mL) was added 2-chloro-*N*-phenethylacetamide (**1**) (12.6 mmol, 2.5 g). The mixture was stirred at 85 °C for 72 h under argon. The reaction mixture was diluted with diethyl ether and filtered, giving 4.75 g of white solid (82% yield); mp 220.9–222.8 °C; $^1$H NMR ($CDCl_3$) $\delta$: 2.67 (2H, t, $J$ = 8.1 Hz), 3.31 (2H, q), 5.05 (2H, d, $J$ = 14.4 Hz), 7.15–7.25 (5H, m), 7.59–7.68 (5H, m), 7.72–7.88 (10H, m). $^{13}$C NMR ($CDCl_3$) $\delta$: 32.30 ($J_{C-P}$ = 54.94 Hz), 35.35, 41.73, 118.63 ($J_{C-P}$ = 88.45 Hz), 126.33, 128.73 ($J_{C-P}$ = 30.79 Hz), 130.31 ($J_{C-P}$ = 12.68 Hz), 134.24 ($J_{C-P}$ = 10.49 Hz), 135.16 ($J_{C-P}$ = 2.72 Hz), 139.17, 162.661 ($J_{C-P}$ = 4.98 Hz); CI-MS $m/z$ 424 ($M-Cl^-$, 100). HRCI-MS: Calcd for $C_{28}H_{27}NOP$: 424.1830. Found: 424.1825.

### 5.3. General procedure for the demethylation of benzaldehydes

#### 5.3.1. 2-Fluoro-4,5-dihydroxybenzaldehyde

2-Fluoro-4,5-dimethoxybenzaldehyde (4 mmol, 736 mg) was dissolved in 10 mL of $CH_2Cl_2$. The mixture was placed in a −78 °C acetone and dry ice bath and 10 mL of a 1 M solution of $BBr_3$ in $CH_2Cl_2$ was added slowly under argon. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. Methanol was added to the resulting mixture, and the solvent evaporated. This process was repeated three times. Column chromatography (5:1 $CH_2Cl_2$/EtOAc) afforded 590 mg (94.5% yield) of 2-fluoro-4,5-dihydroxybenzaldehyde as a white solid which was carried forward without further purification.

### 5.4. General procedure for the Wittig reaction

#### 5.4.1. 3-(3,4-Dihydroxyphenyl)-*N*-phenethylacrylamide (4a, CAPA)

A mixture of 3,4-dihydroxybenzaldehyde (3 mmol, 414 mg), imidazole (9 mmol, 612 mg), TBDMSCl (9 mmol, 1356 mg), and DMAP (0.3 mmol, 36 mg) were dissolved in 5 mL of DMF and allowed to react at room temperature under argon for 1 h. The reaction mixture was extracted with diethyl ether, washed with deionized water, and then dried over $Na_2SO_4$. Column chromatography (2% EtOAc in hexane) of the residue after evaporation of the solvent afforded 540 mg of the protected benzaldehyde, which was combined with the phosphonium chloride (**2**) (1.8 mmol, 828 mg) and $Cs_2CO_3$ (3.9 mmol, 1651 mg) and then 5 mL of dioxane and 5 mL of $CHCl_3$. The resulting mixture was heated to 60 °C for 18 h. The reaction solution was separated, and the solid washed with $CHCl_3$. The combined organics were washed with water, dried over $Na_2SO_4$, and evaporated. Column chromatography (3:1 hexane/EtOAc) of the residue gave 550 mg of yellow oil. The oil was dissolved in 5 mL of THF and TBAF (2.5 mL, 1 M in THF) was then added and the mixture was stirred for 5 min at 0 °C. The reaction mixture was concentrated on a rotary evaporator and subjected to chromatography on a silica gel column (4:3 EtOAc/hexane). Recrystallization ($CH_2Cl_2$ and hexane) afforded 115 mg of **4a** (14% overall yield from 3,4-dihydroxybenzaldehyde) as a white solid: mp 145 °C (lit,[19] 138–140 °C); $^1$H NMR matches literature; $^{13}$C NMR ($CDCl_3$): $\delta$ 35.48, 41.09, 113.89, 115.30, 117.17, 120.95, 126.2, 127.13, 128.35, 128.65, 139.39, 141.07, 145.56, 147.60, 168.14. CI-MS $m/z$ 284 ($MH^+$, 100). HRCI-MS: Calcd for $C_{17}H_{18}NO_3$; 284.1287. Found: 284.1288.

The following compounds were prepared following the same procedure:

#### 5.4.2. 3-(2-Fluoro-4,5-dihydroxyphenyl)-*N*-phenethylacrylamide (4b)

Recrystallization ($CH_2Cl_2$ and hexane) afforded 80 mg of **4b** (7% overall yield from 2-fluoro-4,5-dihydroxybenzaldehyde) as a white solid: mp 145 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ (ppm): 2.77 (t, $J$ = 6.9 Hz, 2H), 3.39 (q, $J$ = 6.6 Hz, 2H), 6.40 (d, $J$ = 15.9 Hz, 1H), 6.60 (d, $J$ = 12.3 Hz, 1H), 6.91 (d, $J$ = 7.8 Hz, 1H), 7.28 (m, 5H), 8.18 (s, 1H), 9.17 (s, 1H), 9.89 (s, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$: 35.44, 41.11, 102.81 ($J_{C-F}$ = 26 Hz), 113.07 ($J_{C-F}$ = 5 Hz), 113.29, 119.27 ($J_{C-F}$ = 6 Hz), 126.20, 128.35, 128.64, 133.36, 139.37, 142.09, 148.67 ($J_{C-F}$ = 11.55 Hz), 155.77 ($J_{C-F}$ = 243 Hz), 167.88; CI-MS $m/z$ 302 ($MH^+$, 100). HRCI-MS: Calcd for $C_{17}H_{17}NO_3F$; 302.1192. Found: 302.1194.

#### 5.4.3. 3-(3-Fluoro-4,5-dihydroxyphenyl)-*N*-phenethylacrylamide (4c)

Recrystallization ($CH_2Cl_2$ and hexane) afforded 135 mg of **4c** (15% overall yield from 3-fluoro-4,5-dihydroxybenzaldehyde) as a white solid: mp 154 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ (ppm): 2.76 (d, $J$ = 7.2 Hz, 2H), 3.39 (d, $J$ = 6.6 Hz, 2H), 6.36 (d, $J$ = 15.6 Hz, 1H), 6.81 (d, $J$ = 6 Hz, 1H), 6.86 (s, 1H), 7.23 (m, 5H), 8.09 (t, $J$ = 5.1 Hz, 1H), 9.46 (s, 1H), 9.71 (s, 1H); $^{13}$C NMR (DMSO-$d_6$) $\delta$: 35.43, 41.1, 106.41 ($J_{C-F}$ = 20 Hz), 110.59, 118.79, 126.10, 126.22, 128.36, 128.64, 135.23, 139.34, 140.06, 147.57 ($J_{C-F}$ = 6 Hz), 152.28 ($J_{C-F}$ = 238 Hz), 167.67; CI-MS $m/z$ 302 ($MH^+$, 100). HRCI-MS: Calcd for $C_{17}H_{17}NO_3F$: 302.1192. Found: 302.1188.

#### 5.4.4. 3-(3-Fluoro-4-hydroxy-5-methoxyphenyl)-*N*-phenethyl acrylamide (4d)

An additional column chromatography purification (1:1.5 EtOAc/hexane) to remove traces of the *Z*-isomer, afforded 100 mg of **4d** (22% overall yield from 3-fluoro-4-hydroxy-5-methoxybenzaldehyde) as a white foam: $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 2.91 (t, $J$ = 6.9 Hz, 2H), 3.68 (q, $J$ = 6.2 Hz, 2H), 5.68 (s, 1H), 5.82 (s, 1H), 6.20 (d, $J$ = 15.4 Hz, 1H), 6.79 (s, 1H), 6.65 (dd, $J$ = 1.6 Hz, $J$ = 10.8 Hz, 1H), 7.28 (m, 5H), 7.50 (d, $J$ = 15.4 Hz, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$: 31.92, 37.13, 52.74, 102.72, 104.76 ($J_{C-F}$ = 19 Hz), 115.81, 122.65 ($J_{C-F}$ = 9 Hz), 122.87, 125.00, 125.09, 131.72 ($J_{C-F}$ = 14 Hz), 135.13, 136.53, 144.69 ($J_{C-F}$ = 6 Hz), 147.02 ($J_{C-F}$ = 242 Hz), 162.22; CI-MS $m/z$ 316 ($MH^+$, 100). HRCI-MS: Calcd for $C_{18}H_{19}NO_3F$; 316.1349. Found: 316.1351.

#### 5.4.5. 3-(3-Fluoro-4-hydroxyphenyl)-*N*-phenethyl-acrylamide (4e)

This process afforded 90 mg of **4e** (8% overall yield from 3-fluoro-4-hydroxybenzaldehyde) as a yellow foam with a 3:1 (*E*)-/(*Z*)-isomer ratio by $^1$H NMR: $^1$H NMR ($CDCl_3$) $\delta$ (ppm) major isomer: 2.91 (t, $J$ = 6.9 Hz, 2H), 3.68 (q, $J$ = 6.1 Hz, 2H), 5.66 (s, 1H), 6.18 (d, $J$ = 15.6 Hz, 1H), 7.00 (t, $J$ = 8.5 Hz, 1H), 7.12 (d, $J$ = 8.5 Hz, 1H), 7.18 (d, $J$ = 1.8 Hz, 1H), 7.28 (m, 5H), 7.52 (d, $J$ = 15.6 Hz, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$: 35.80, 41.30, 114.85 ($J_{C-F}$ = 19 Hz), 118.31, 118.66, 125.50, 126.95 ($J_{C-F}$ = 20 Hz), 127.18, 128.92, 128.99, 138.89, 140.85, 146.99 ($J_{C-F}$ = 14 Hz), 151.86 ($J_{C-F}$ = 241 Hz), 167.13; CI-MS $m/z$ 286 ($MH^+$, 100). HRCI-MS: Calcd for $C_{17}H_{17}NO_2F$; 286.1243. Found: 286.1242.

#### 5.4.6. 3-(2-Fluoro-4,5-dimethoxyphenyl)-*N*-phenethyl-acrylamide (4f)

Recrystallization from EtOAc and hexane gave 48 mg of **4f** (63% yield from 2-fluoro-4,5-dimethoxybenzaldehyde) as white crystals: mp 149 °C; $^1$H NMR ($CDCl_3$) $\delta$ (ppm): 2.92 (t, $J$ = 6.9 Hz, 2H), 3.68 (q, $J$ = 6.5, 2H), 3.88 (d, $J$ = 8.7, 6H), 5.90 (br s, 1H), 6.38 (d, $J$ = 15.6 Hz, 1H), 6.64 (d, $J$ = 12.0, 1H), 6.90 (d, $J$ = 7.20 Hz, 1H), 7.29 (m, 5H), 7.66 (d, $J$ = 15.6 Hz, 1H); $^{13}$C NMR ($CDCl_3$): $\delta$ 35.93, 41.09, 56.53 ($J_{C-F}$ = 9.96 Hz), 100.45 ($J_{C-F}$ = 28 Hz), 110.54 ($J_{C-F}$ = 4 Hz), 114.01 ($J_{C-F}$ = 13 Hz), 121.15 ($J_{C-F}$ = 7 Hz), 126.77, 128.99 ($J_{C-F}$ = 11 Hz), 134.03, 139.15, 145.67, 151.41 ($J_{C-F}$ = 10 Hz), 156.54 ($J_{C-F}$ = 248 Hz), 166.40; CI-MS $m/z$ 330 ($MH^+$, 100). HRCI-MS: Calcd for $C_{19}H_{21}NO_3F$; 330.1505. Found: 330.1506; elemental Anal. Calcd for $C_{19}H_{20}NO_3F$: C, 69.29; H, 6.12; N, 4.25. Found: C, 69.03; H, 6.12; N, 4.21.

### 5.5. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Lifeline Technologies (Walkersville, MD) and cultivated on 75 cm$^2$ 1% gelatin coated culture flasks using MCDB 131 cell culture media (Invitrogen, Carlsbad CA) supplemented with 2% fetal bovine serum, ascorbic acid, heparin, VEGF, hydrocortisone bFGF and heparin (Lifeline Technologies). The cells were grown to confluency at 37 °C in humidified atmosphere with 5% $CO_2$. HUVEC were then treated with Trypsin/EDTA and subcultivated onto gelatin coated 96 well multi-plates and used when confluent. Population doubling levels 2 through 5 were used in the described experiments.

### 5.6. Cytotoxicity assay

Stock CAPE and CAPE amide derivative solutions were dissolved in DMSO then diluted in MCDB 131 tissue culture media for use in the assays. Confluent HUVEC were treated with CAPE and the amide derivatives for 24 h at 37 °C at concentrations ranging from 10 to 60 μM. Following the 24 h incubation, the media was replaced with 10% CellTiter-Blue® Blue solution (Promega, Madison WI). HUVEC were incubated for 2 h at 37 °C then analyzed for fluorescence. The readings were taken at 545 nm excitation and 590 nm emission wavelengths on a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale CA). Cell viability was calculated from these fluorescence readings.

### 5.7. Cytoprotection assay

Confluent HUVEC were treated with CAPE and the amide derivatives for 5 h at 37 °C. After the 5 h incubation, the compounds were removed from the wells, and the cells were washed twice with MCDB 131 buffer. Stock hydrogen peroxide solution (50%, Sigma–Aldrich) was diluted in MCDB 131 buffer, and incubated in the cells following the buffer wash. HUVEC were incubated in the hydrogen peroxide for 1 h at 37 °C. The hydrogen peroxide was then removed. The cells were washed once with MCDB 131 media, and were then incubated in complete MCDB 131 media for 18 h at 37 °C. Following the 18 h period, the cells were treated with 10% CellTiter-Blue® solution and analyzed for viability. In the dose–response cytoprotection assay, percent cytoprotection for each compound was calculated by subtracting the average fluorescent reading of the negative control (HUVEC treated only with DMSO and hydrogen peroxide) from the fluorescent values of each well. This was then divided by the average fluorescence of the positive control (HUVEC treated only with DMSO) to obtain percent cytoprotection.

### 5.8. Statistical analysis

Data are reported as means ± standard deviation as a percentage of the control. Differences between the groups were first analyzed by ANOVA, and then evaluated by the Tukey-Kramer post hoc analysis. O'Brien's and Bartlett's tests showed that variances were equal among groups. $P$ <0.05 was considered significant. All statistical analysis was performed using the JMP program (SAS).

## Acknowledgments

This project was supported by the US Army Institute of Surgical Research, the Robert Welch Foundation (F-1298 and H-F-0032) and the TI-3D.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.080.

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