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Citation	Fiamegos, Yiannis C., Panagiotis L. Kastritis, Vassiliki Exarchou, Haley Han, Alexandre M. J. J. Bonvin, Jacques Vervoort, Kim Lewis, Michael R. Hamblin, and George P. Tegos. 2011. Antimicrobial and Efflux Pump Inhibitory Activity of Caffeoylquinic Acids from <i>Artemisia absinthium</i> against Gram-Positive Pathogenic Bacteria. PLoS ONE 6(4): e18127.
Published Version	doi://10.1371/journal.pone.0018127
Accessed	February 19, 2015 8:45:55 AM EST
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:8160879
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Antimicrobial and Efflux Pump Inhibitory Activity of Caffeoylquinic Acids from *Artemisia absinthium* against Gram-Positive Pathogenic Bacteria

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Abstract

Background: Traditional antibiotics are increasingly suffering from the emergence of multidrug resistance amongst pathogenic bacteria leading to a range of novel approaches to control microbial infections being investigated as potential alternative treatments. One plausible antimicrobial alternative could be the combination of conventional antimicrobial agents/antibiotics with small molecules which block multidrug efflux systems known as efflux pump inhibitors. Bioassay-driven purification and structural determination of compounds from plant sources have yielded a number of pump inhibitors which acted against gram positive bacteria.

Methodology/Principal Findings: In this study we report the identification and characterization of 4',5'-O-dicaffeoylquinic acid (4',5'-ODCQA) from *Artemisia absinthium* as a pump inhibitor with a potential of targeting efflux systems in a wide panel of Gram-positive human pathogenic bacteria. Separation and identification of phenolic compounds (chlorogenic acid, 3',5'-ODCQA, 4',5'-ODCQA) was based on hyphenated chromatographic techniques such as liquid chromatography with post column solid-phase extraction coupled with nuclear magnetic resonance spectroscopy and mass spectroscopy. Microbial susceptibility testing and potentiation of well know pump substrates revealed at least two active compounds; chlorogenic acid with weak antimicrobial activity and 4',5'-ODCQA with pump inhibitory activity whereas 3',5'-ODCQA was ineffective. These intial findings were further validated with checkerboard, berberine accumulation efflux assays using efflux-related phenotypes and clinical isolates as well as molecular modeling methodology.

Conclusions/Significance: These techniques facilitated the direct analysis of the active components from plant extracts, as well as dramatically reduced the time needed to analyze the compounds, without the need for prior isolation. The calculated energetics of the docking poses supported the biological information for the inhibitory capabilities of 4',5'-ODCQA and furthermore contributed evidence that CQAs show a preferential binding to Major Facilitator Super family efflux systems, a key multidrug resistance determinant in gram-positive bacteria.

Citation: Fiamegos YC, Kastritis PL, Exarchou V, Han H, Bonvin AMJJ, et al. (2011) Antimicrobial and Efflux Pump Inhibitory Activity of Caffeoylquinic Acids from *Artemisia absinthium* against Gram-Positive Pathogenic Bacteria. PLoS ONE 6(4): e18127. doi:10.1371/journal.pone.0018127

Editor: Jörg Hoheisel, Deutsches Krebsforschungszentrum, Germany

Received: October 22, 2010; **Accepted:** February 25, 2011; **Published:** April 4, 2011

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Funding: Funding for Y. Fiamegos was provided by E.U. Large Scale Facilities program, contracts HPRI-CT-2001-00178, 'LC-NMR analysis of traditional herbal pharmaceuticals' (project no 2002-31) and 'On line LC-SPE-NMR system in analyzing the bioactive constituents of Artemisia extract', (Project no 2003-011) of the Wageningen NMR center. Funding was provided for G. Tegos by the National Institutes of Health (NIH, 5U54MH084690-02, Center Driven Initiative 1) and Massachusetts Technology Transfer Center (MTTC), by the National Institutes of Health for K. Lewis (NIH, R01GM59903) and M. R. Hamblin (NIH, R01AI050875). Funding for P. Kastritis was provided by the Netherlands Organization for Scientific Research through a VICI grant (700.56.442) to A. Bonvin. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Multi-drug resistant microbial infections caused by Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* represent an exponentially growing problem affecting communities worldwide. Efflux mechanisms have become broadly recognized as major components of resistance to many classes of

antibiotics [1,2]. Some efflux pumps selectively extrude specific antibiotics while others, referred to as multidrug efflux pumps (MEPs), expel a variety of structurally and functionally diverse compounds [3] in addition to a variety of other physiological roles [4]. A novel and promising approach to deal with multidrug resistance is to improve the clinical performance of various antibiotics by employing efflux pump inhibitors (EPIs) [5,6]. Plants

have been explored comprehensively as potential sources of antimicrobials [7,8]. It has been established that disabling MEPs in Gram-negative species using a combination of MEP mutants and synthetic EPIs leads to a striking increase in the activity of numerous plant compounds [9]. Several *Berberis* medicinal plants (*Berberis repens*, *B. aquifolia*, and *B. fremontii*) that produce the plant antimicrobial berberine also synthesized an inhibitor of the *Staphylococcus aureus* NorA MEP identified as 5'-methoxyhydrocarpin (5'-MHC) [10]. Identification of 5'-MHC intensified the search for natural EPIs of plant origin. Bioassay driven purification and structural determination of compounds from various plant sources yielded a number of EPIs acting against Gram-positive bacteria with activities similar to 5'-MHC [11,12,13,14,15,16]. Gram-negative bacteria and fungi comprise the main groups of plant pathogens, and it is important to determine whether the antimicrobial/EPI synergy is a general mechanism of plant defense.

Artemisia is a fairly large genus within the family of the *Asteraceae* (*Compositae*), with more than 200 individual species [17]. *Artemisia absinthium*, best known as the principal ingredient in the infamous Absinthe drink, has been used medicinally since the times of ancient Greece, and also in western European systems of traditional medicine [17]. It was recently reported that the essential oils occurring in flowers and aerial parts from *A. absinthium* have antimicrobial properties [17]. Moreover aqueous extracts of *A. absinthium* are rich in caffeoyl and dicaffeoylquinic acids, which are known to inhibit HIV-1 integrase from integrating the reversibly transcribed viral DNA into host cell DNA [18]. Furthermore, these components are hepatoprotective, anti-histaminic, hypocholesterolemic, anti-spasmodic, and potentially antimicrobial. They also demonstrate high antioxidant activity [19] due to their o-quinone moieties. Caffeoylquinic acids could inhibit oxidative damage to both low-density lipoprotein [20] and linolenic acid [21] by scavenging reactive oxygen species (ROS) related to cancer and cardiovascular diseases. It has been reported that chlorogenic acid (5-*O*-caffeoylquinic acid) had inhibitory effects on carcinogenesis in the large intestine, liver, and tongue, and exhibited protective effects on oxidative stress *in vivo* [22].

The effort to discover plant EPIs, a process ranging from identifying a hit to isolating a pure compound, presents a distinct bottleneck: the lack of comprehensive, chemically and biologically representative natural product extract libraries. Analysis of natural product extracts is time-consuming and laborious, consisting of the

isolation of different components of the extract and the successive identification of these compounds by spectrometric data acquisition. We have identified EPI activity against Gram-positive bacteria in *A. absinthium* extracts. We dramatically expedited the analysis of *A. absinthium* aquatic infusions using analytical techniques (LC-SPE-NMR and LC-MS), revealing caffeoylquinic acids as key components. Subsequent antimicrobial susceptibility testing of berberine uptake and a competitive efflux assay employing the pure compounds was conducted. An array of pathogens, including knock out and over expression MEP mutants, strongly supported the hypothesis that 4',5'-ODCQA behaves as a designated EPI for the major facilitator super family (MFS) MEPs of the Gram-positive bacteria: *S. aureus*, and *Enterococcus faecalis*.

Results and Discussion

Antimicrobial and EPI activity of extracts

The experimental approach to detect EPI activity was to test the combined action of a plant extract (the no alkaloid fraction) with berberine added at a sub inhibitory concentration. Extracts that inhibited cell growth in the presence of berberine and had no activity when added alone were likely to contain an EPI. Chloroform extracts of leaves from *A. absinthium* had no antimicrobial activity alone at up to 500 µg/ml (**Table 1**), but inhibited *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* growth completely in the presence of 30 µg/ml berberine (15 µg/ml berberine for *Bacillus* cells), a concentration one-eighth the MIC for this substance (data not shown).

The three main components of the extract consisting 40% of the total mass were identified via LC-UV-SPE-NMR [23] and LC-MS. The molecular ion in the ESI negative mode of the first peak was $m/z = 353$ and the MS^2 gave a characteristic chlorogenic acid fragmentation pattern [24]. The structure was confirmed by the respective 1H -NMR analysis. The second and third main components of the extract were co-eluted from the chromatographic column. By the spectral data obtained it was concluded that two dicaffeoylquinic acid isomers were present (3',5'-ODCQA and 4',5'-ODCQA) (**Figures S1, S2**).

Antimicrobial and EPI activity of CQAs

The *A. absinthium* extract was tested against a panel of human and plant pathogenic bacteria both for direct antimicrobial and EPI activity. The extract exhibited neither antimicrobial activity

Table 1. Antimicrobial susceptibility for *A. absinthium* and acquired CQA derivatives.

Strain	MIC (µg ml ⁻¹)					
	Extr	3',5'-ODCQA	4',5'-ODCQA	5'-OCQA	1',3'-ODCQA	1',5'-ODCQA
<i>S. aureus</i> 8325-4	256	>128	>128	128	>128	>128
<i>norA</i>	256	>128	>128	64	>128	>128
<i>NorA++</i>	256	>128	>128	>128	>128	>128
CA-MRSA	256	>128	>128	>128	>128	>128
<i>B. cereus</i>	128	>128	>128	64	>128	>128
<i>E. faecalis</i>	256	>128	>128	64	>128	>128
<i>norA</i>	256	256	>256	32	>256	>256
<i>E. coli</i>	>256	>256	>256	>256	>256	>256
<i>C. albicans</i>	>256	>256	>256	>256	>256	>256

All MIC determinations were performed in triplicate.

doi:10.1371/journal.pone.0018127.t001

nor EPI activity in *Escherichia coli* or *Candida albicans* (concentration range 150–500 µg/mL). It showed no apparent direct antimicrobial activity (MICs in the range of 128–256 µg/mL for all Gram-positive bacteria tested), but completely inhibited growth in concentrations lower than 50 µg/mL when berberine was added in sub inhibitory concentrations (30 µg/mL for *S. aureus* and *E. faecalis*, and 15 µg/mL for *B. cereus*).

We obtained 5'-O-caffeoylquinic (5'-OCQA) acid and peracetyl derivatives 1',3'- and 1',5'-ODCQA, 3',5'-ODCQA and 4',5'-ODCQA, and tested their direct antimicrobial and EPI activity (Table 1). 5'-OCQA was the only molecule with weak direct antimicrobial activity against the Gram-positive bacteria (*B. cereus* and *E. faecalis*, MIC 64 µg/ml). There was a 4-fold difference between MICs in *S. aureus* 8325-4 and the isogenic NorA mutant providing supporting evidence for 5'-OCQA as a potential substrate of MFS efflux systems. All other derivatives were virtually inactive with significantly higher MICs (>128 µg/ml) against all microorganisms in the panel tested.

Toxicity studies

Hemolysis experiments using sheep erythrocytes and DCQA derivatives demonstrated no hemolytic activity at 50 µM, a concentration higher than the MICs for Gram-positive and significantly higher than the concentration used for antimicrobial potentiation (Figure 1). There are a few brief reports discussing the toxic effect of 5'-OCQA on human oral squamous cell carcinoma (HSC-2) and salivary gland tumor (HSG) cell lines, as compared with that against human gingival fibroblast (HGF), and erythrocytes in a mM range [25]. The toxic effect is attributed to the potential of the compound to generate reactive oxygen species (ROS). A dose-response experiment using erythrocytes was also able to detect toxicity of 5'-OCQA at fairly low concentrations, (1-

5 µM) whereas 3',5'- and 4',5'-ODCQAs did not exhibit any cell-lytic properties at significantly higher concentrations (50 µM). This trend, together with existing studies [22,26], suggest that 5'-OCQA is toxic at high concentrations, and implies that the other derivatives appear to have a different interaction pattern with mammalian cells, suggesting that a therapeutic window may exist in both cases. 3',5'-ODCQA and 4',5'-ODCQA showed extremely low toxicity to the blood cells indicating that the antimicrobial activity and toxicity patterns are relatively similar related to the differences in substitution moieties.

Potentiation of antibiotics by 4',5'-ODCQA

We conducted a checkerboard assay using berberine and CQAs against *S. aureus* 8325-4 (Table 2). This method supports evidence for synergy, competition or antagonism for selected compounds as it is reflected by the FIC index (FICI). A FICI of ≤0.5 indicates synergistic interaction. FICI is calculated as the sum of the FICs of each agent. The FIC of each agent is calculated as the MIC of the agent in combination divided by the MIC of the agent. This indicated a synergistic effect for 4',5'-ODCQA, and competition for the other two derivatives. We employ a clinical MRSA strain (CA-300) and conducted extensive checkerboard assays using an array of known MFS substrates and 4',5'-ODCQA suggesting a promising range of activity both in different strains and clinically relevant compounds (Table 3). We further evaluated the ability of 4',5'-ODCQA to potentiate designated substrates of MFS and known antibiotics against a panel of Gram-positive phenotypes (Tables 4, 5). The activity of all of the MFS substrates increased significantly in the presence of the compound against the wild-type strains tested (8-fold for berberine, and 4-8-fold for EtBr and fluroquinolones). The addition of 4',5'-ODCQA in the more susceptible *E. faecalis* NorA knock out mutant (Table 5) increased the susceptibility of EtBr and norfloxacin (2–4 fold). This result indicated the possible additional participation of the QacA MEP system which has been implicated in the efflux of NorA substrates [12,27]. Besides this anticipated complication, the ability of 4',5'-ODCQA to potentiate antibiotics was significantly weaker in the case of the NorA knock out phenotypes, indicating a likely mode of action associated with this MFS efflux system. Moreover the *S. aureus* with NorA overexpression showed significant potentiation of all antimicrobials and gave susceptibilities that were comparable with the wild type counterpart in the absence of 4',5'-ODCQA (8-fold for berberine, between 4- 8-fold for EtBr and fluroquinolones). Results were similar when a *B. cereus* were used (data not shown), implying a wider range of activity for 4',5'-ODCQA.

4',5'-ODCQA enhances accumulation of berberine in Gram-positive bacteria

Berberine is a useful model plant antimicrobial whose accumulation in microbial cells can be conveniently monitored by measuring the fluorescence of berberine bound to DNA. We used three different Gram-positive bacteria, *S. aureus*, *E. faecalis*,

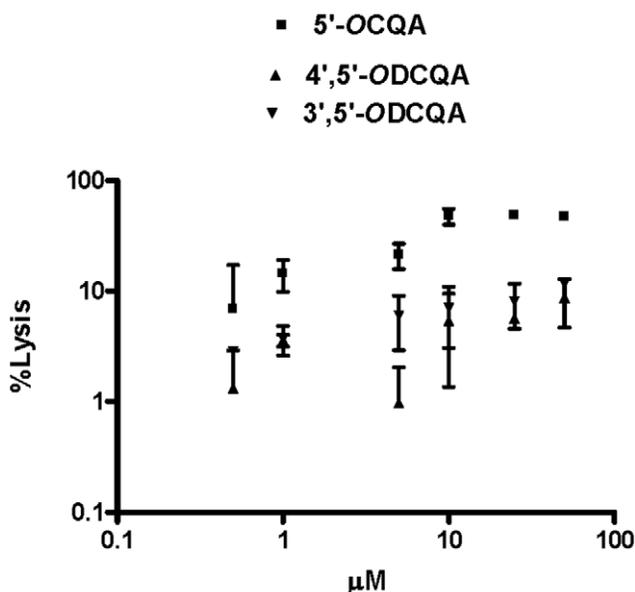


Figure 1. Hemolytic activity of caffeoylquinic acids towards sheep erythrocytes. The CQA induced significantly more (*) hemolysis, while DCQAs induced significantly virtually no hemolysis. The data were analyzed by two-way repeated-measurement ANOVA for the factors of different derivatives and derivative concentrations followed by post hoc testing by the Holm-Sidak method ($P < 0.05$). Values are means \pm SEM ($n = 3$). Note the log scales on both the x and y axes.

doi:10.1371/journal.pone.0018127.g001

Table 2. Checkerboard assay for interaction of DCQAs with BERB against *S. aureus*.

Compound	FIC (compound)	FIC (BERB)	FICI
5'-OCQA	0.50	0.50	1.00
3',5'-ODCQA	1.00	1.00	1.00
4',5'-ODCQA	0.125	0.0625	0.5

doi:10.1371/journal.pone.0018127.t002

Table 3. Checkerboard assay of 4',5'-ODCQA with antimicrobials against MRSA.

Antibiotic (Dye)	FIC (antibiotic or dye)	FIC (4',5'-ODCQA)	FICI
CIPRO	0.20	0.00078	0.0039
NOR	0.10	0.00039	0.0039
LEVO	0.40	0.00156	0.0039
BERB	0.0312	0.0625	0.50
EtBr	0.0625	0.0156	0.0625

^aEvaluated by the checkerboard method recommended by the CLSI and expressed as the FIC index (FICI). A FICI of ≤ 0.5 indicates synergistic interaction. FICI is calculated as the sum of the FICs of each agent. The FIC of each agent is calculated as the MIC of the agent in combination divided by the MIC of the agent alone.

CIPRO, ciprofloxacin **NOR**, norfloxacin **LEVO**, levofloxacin **BERB**, berberine, **EtBr**, Ethidium Bromide.

doi:10.1371/journal.pone.0018127.t003

and *B. cereus*, to evaluate with a functional assay the EPI potential of CQAs. We monitored an increase in berberine fluorescence, assessing the rate of penetration and the level of accumulation of berberine in *S. aureus* cells, in response to an established NorA inhibitor (INF₂₇₁) [28,29] and the selection of CQA derivatives (**Figure 2A**) compared to berberine alone. Rapid accumulation was also observed in other strains tested (*E. faecalis*, **Figure 2B** and *B. cereus* data not shown). In all cases the potentiation of uptake was maximal for 4',5'-ODCQA. The effect was equal or higher than that seen with INF₂₇₁. Accumulation of berberine was observed in the presence of 3',5'-ODCQA and 5'-OCQA, although the rate and level of uptake were lower compared with 4',5'-ODCQA, and closer to the baseline of berberine. This data agrees with the EPI activity and the checkerboard assay data.

To study efflux, we loaded cells with berberine or the combination of berberine with the compound at concentrations significantly higher than those used for antimicrobial susceptibility testing and then transferred them into fresh medium. **Figure 3A** (*S. aureus*) and **Figure 3B** (*E. faecalis*) show that berberine efflux was inhibited in the presence of 4',5'-ODCQA both in wild type strains as well as the *S. aureus* NorA overexpressing strain. The lack

Table 4. Potentiation of antimicrobials by 4',5'-ODCQA in *S. aureus*.

	<i>S. aureus</i> 8325-4		NorA -/- <i>S. aureus</i> (MIC µg/mL)		NorA+/+ <i>S. aureus</i>	
	alone	+4',5'-ODCQA	alone	+4',5'-ODCQA	alone	+4',5'-ODCQA
BERB	256	16	16	8	>512	64
EtBr	64	4	8	8	256	64
CIPRO	1	0.2	0.4	0.8	1	0.4
NOR	1	0.1	0.2	0.2	2	0.8
MOXI	1	0.2	0.4	0.4	1	0.4
LEVO	1	0.4	0.8	0.4	4	1

The strains *S. aureus* 8325-4 (wt) and the isogenic pair of knock out and overexpression NorA mutant were used. All MIC determinations were performed in triplicate. * 4',5'-ODCQA was added at a final concentration of 10 µg/mL.

MOXI, moxifloxacin.

doi:10.1371/journal.pone.0018127.t004

Table 5. Potentiation of antimicrobials by 4',5'-ODCQA in *E. faecalis*.

	OGRF1 (MIC µg/mL)		norA-/- <i>E. Faecalis</i> (MIC µg/mL)	
	alone	+4',5'-ODCQA	alone	+4',5'-ODCQA
BERB	512	64	32	16
EtBr	128	8	16	4
CIPRO	2	0.8	1	1
NOR	1	0.2	0.4	0.2
MOXI	1	0.1	0.2	0.4
LEVO	1	0.1	0.2	0.1

The strains OGRF1(wt) and the knock out NorA mutant were used. All MIC determinations were performed in triplicate. 4',5'-ODCQA was added at a final concentration of 10 µg/mL.

doi:10.1371/journal.pone.0018127.t005

of any effect in NorA knockout strains provides additional evidence that 4',5'-ODCQA is inhibiting NorA.

Biofilm inhibition with CQAs

In order to determine if members of the natural products of the CQAs family were able to inhibit biofilm formation, alone and in combination with a representative panel of antimicrobials, a standard biofilm assay was conducted. The data are shown for *S. aureus* in **Figure 4A** and for *E. faecalis* in **Figure 4B**. Only 5'-OCQA, when added alone, had a significant reduction in biofilm viability in both strains whereas both DCQAs did not. Challenging biofilms with CQA derivatives in combination with sub-inhibitory concentrations of berberine, ethidium bromide, and the fluoroquinolone moxifloxacin gave a statistically significant decrease in viability of biofilms in the presence of added 4',5'-ODCQA. The killing effect was increased 2 to 4 logs. In the presence of 50 µg/ml 4',5'-ODCQA, it was more pronounced in *E. faecalis* than *S. aureus* for all antimicrobials tested. CQA enhanced the killing effect of the compounds used in a less dramatic fashion when it was added in both biofilm species at the same concentration. By contrast 3',5'-ODCQA had no apparent effect in either microorganism tested, regardless of the antimicrobial employed. Quantitation of biofilm formation and subsequent inhibition with or without treatments was followed by reduction of OD₆₀₀ after Crystal Violet staining and Colony Forming Units (CFU) determinations. Results from the CV stained biofilms were compatible with CFU determinations but were omitted in the presentation for simplicity.

Molecular modeling studies

In order to gain structural insights into the interaction of the potential EPIs (5'-OCQA, 4',5'-ODCQA and 3',5'-ODCQA) with two MES a. an ABC transporter [30] and b. a MFS transporter [31] we used HADDOCK [32]. High quality models could be obtained for all six protein-ligand complexes. Docking results are consistent with our antimicrobial susceptibility tests and the high-throughput competitive efflux assays performed in this study: All CQAs can successfully enter the binding site of both efflux pumps (**Figure 5**) remaining in the binding pockets throughout the simulation runs. In addition, CQAs are predicted to have different interaction energies with each efflux system: Their preferential binding to the MFS transporter is governed by hydrophobic interactions uniformly found in all solutions (**Table 6**). Conversely, when interacting with the ABC transporter, electrostatic forces (ionic interactions and hydrogen bonds) are dominant.

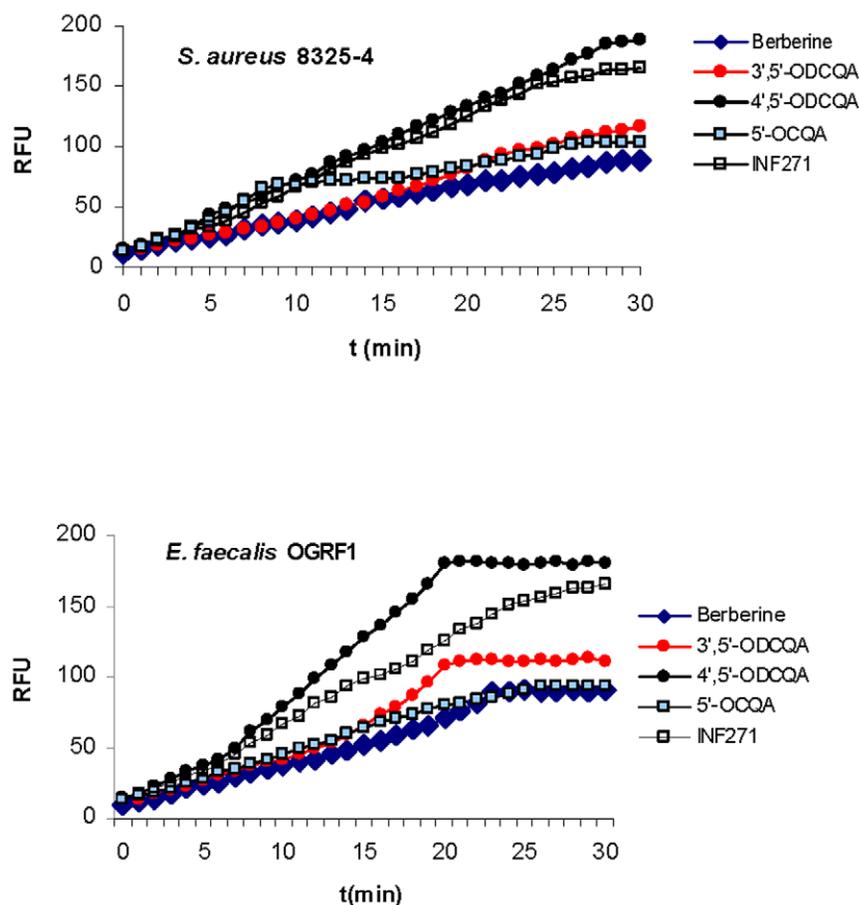


Figure 2. Berberine accumulation with caffeoylquinic acids in Gram-positive bacteria. The uptake of berberine with no addition or with the addition of 5'-OCQA, 3',5'-ODCQA, 4',5'-ODCQA, or INF₂₇₁ by cells of *S. aureus* (A), *E. faecalis* (B), was measured by determination of the increase in fluorescence following binding to DNA and is expressed as relative fluorescence units (RFU). Berberine was present at a concentration of 30 $\mu\text{g/ml}$, and 5'-OCQA, 3',5'-ODCQA, 4',5'-ODCQA, INF₂₇₁ were added at the same final concentrations used for the MIC determinations. doi:10.1371/journal.pone.0018127.g002

Interactions of the caffeoylquinic acids with the glycerol-3-phosphate transporter (PDB ID: 1PW4)

5'-OCQA exhibits strong binding to the glycerol-3-phosphate transporter (see **Table 6** and **Figure 5, A**). Besides the strong desolvation energy component (-40.1 ± 10.2 kcal/mol), it is also stabilized by increased electrostatics (-140.9 ± 18.3 kcal/mol). For example, two Arg residues (R45 and R269) have been observed to be crucial for the inhibition of the MFS transporter. In our results, their side chains form salt bridges with the oxygen atoms of the 5'-O-caffeoylquinic acid (distance < 5.5 Å) (**Figure 5A**). K80, located at the transporter's active site, forms a salt bridge with the $-\text{COOH}$ group of 5'-OCQA. Results are also very consistent as nearly all models fall into under one cluster, with an average positional root mean square displacement from the overall lowest-energy structure equal to 0.3 ± 0.1 Å.

For 3',5'-ODCQA, the docking results in two low-energy conformations (**Table 6**). Although their interaction energies are quite similar and their location resembles that of the 'classical inhibitor', striking differences in their hydrogen bond network are observed (For details, see Figure S3). 4',5'-ODCQA is found in the binding site in an orientation similar to that of 3',5'-ODCQA. However, the significantly larger buried surface area (BSA) and more favorable van der Waals energies are indicative of a tighter fit into the binding site of the MFS efflux system than the other

inhibitors; in particular 3',5'-ODCQA does not show such an extent in complementarity.

Interactions of caffeoylquinic acids with the Sav1688 ABC transporter (PDB ID: 2HYD)

The ABC transporter was modeled in its open conformation (**Figure 5B**) (**Figure S4** and **Table S1**), using as template the crystal structure of its close sequential and structural homologue, the P-glycoprotein in its open form (PDB ID: 3G61) [33]. All caffeoylquinic acids successfully entered the active site of the ABC transporter (**Figure 5B**). Besides the favorable Electrostatics (**Figure 5B**), the desolvation penalty is quite large (**Table 6**). Results are rather similar to the ones shown above for the MFS transporters with one major cluster with over 140 members. All other clusters were small in size (< 10 structures were included). Binding of this molecule to the ABC transporter is, however, not as energetically favored as to the MFS transporters (for comparison of the interactions, see **Table 6** and **Figure 5A** and **5B**). For the other two EPIs, more clusters were obtained. As was already the case with the docking to the MSF transporters, in all solutions, 4',5'-ODCQA occupies a much larger area in the binding site compared to 3',5'-ODCQA; van der Waals interactions of these EPIs also follow the same principle (**Table 6**).

All the above mentioned results can rationalize the strong observed binding of 4',5'-ODCQA and 5'-OCQA and the lower

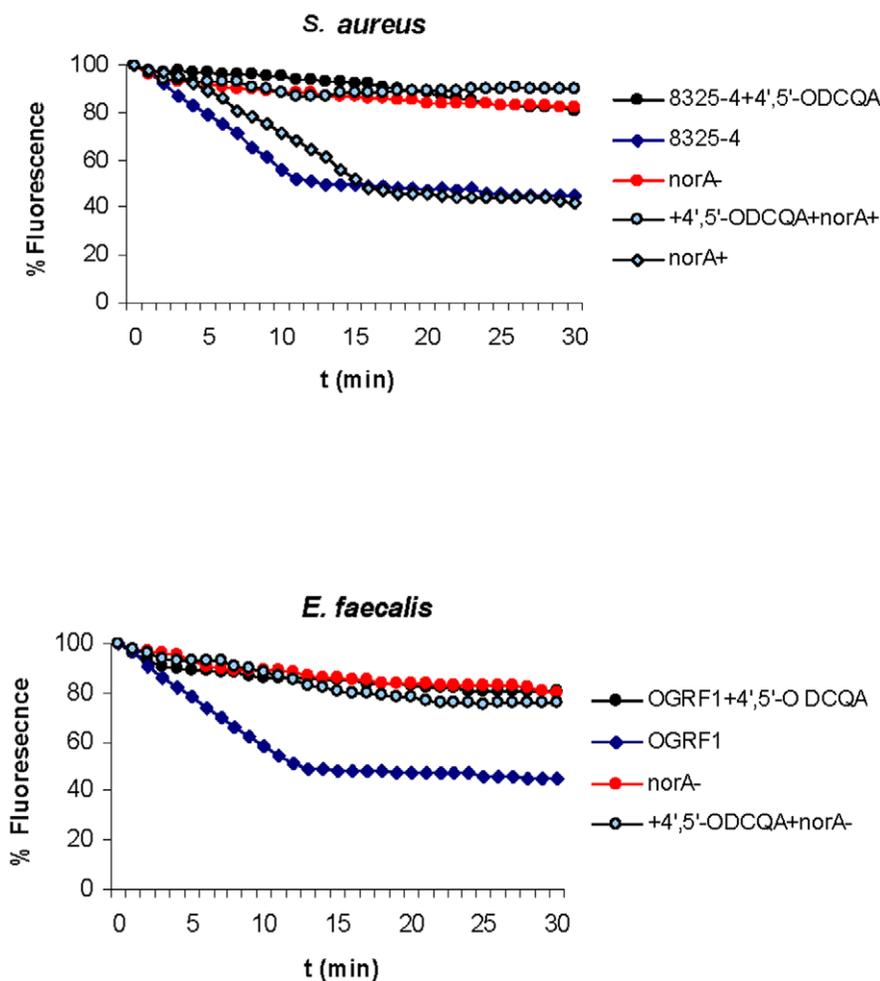


Figure 3. Efflux potentiation by 4',5'-O-dicaffeoylquinic acid in *S. aureus* (A), *E. faecalis* (B). Efflux was measured using berberine by determination of the decrease in fluorescence following binding to DNA and is expressed as % fluorescence. Cells were loaded with berberine at a concentration of 100 $\mu\text{g/ml}$, and then resuspended in buffer in the absence or presence of 4',5'-ODCQA, at the same final concentrations used for the MIC determinations.

doi:10.1371/journal.pone.0018127.g003

efflux pump inhibition of 3',5'-ODCQA. They suggest that, for the MFS efflux systems, all molecules could potentially inhibit the active site; however, 5'-OCQA exhibits in both cases a substantially smaller interaction region showing less complementarity. The docking of all CQAs in the binding sites of the ABC transporter, reveals an abundance of charged interactions. 3',5'-ODCQA shows a decreased complementarity within the active site compared to that of 4',5'-ODCQA. Observed interactions with residues within the active sites are *similar* to the ones described in the literature for other EPIs [34,35].

We, therefore, formulate a hypothesis consistent with the derived energetics, that CQAs show a preferential binding to MFS efflux systems, rather than to ABC transporters. The reported energies provide insights into the interaction of the EPIs with efflux systems and contribute to a working hypothesis regarding the molecular mechanisms behind their observed inhibitory activities. They should, however, be interpreted with caution and only in terms of estimates of potential of binding and not as binding affinities [36]. Studies including Surface Plasmon Resonance or Isothermal Titration Calorimetry to determine K_d 's or ΔG 's will offer further insights in this primary hypothesis.

Synopsis

An array of natural product screening and synthesis campaigns led to the identification of a variety EPI chemotypes targeting NorA. The list includes catechin gallates [37], the resin glycosides and tetrasaccharide agents of *Ipomoea murucoides* [38], polyacylated oligosaccharides from the medicinal Mexican morning glory species [39], N-caffeoylphenalkylamide derivatives [40], citral derived amides [41] kaempferol glycoside from *Herissantia tiubae* (Malvaceae) [42] and a set of plant-based alkaloids against methicillin-resistant *S. aureus* (MRSA) [43]. A series of plant phenolic compounds have been functioning as ethidium bromide EPIs in *Mycobacterium smegmatis* [44]. A series of synthetic efforts have been concentrated in paroxetine and femoxetine [45], piperine and fluoroquinolone as the basis for designing structural analogues [46,47], phenothiazines [48] with emphasis on thioridazine and chlorpromazine [49]. All these explorations are focusing in the bioassay driven purification and structural determination of natural EPIs employing an EtBr efflux assay. In retrospective this venture offers a rapid methodological alternative to the direct isolation of active compounds as well as attempts to enrich the EPI discovery process with an extensive and rationalized secondary validation of lead chemotypes. The

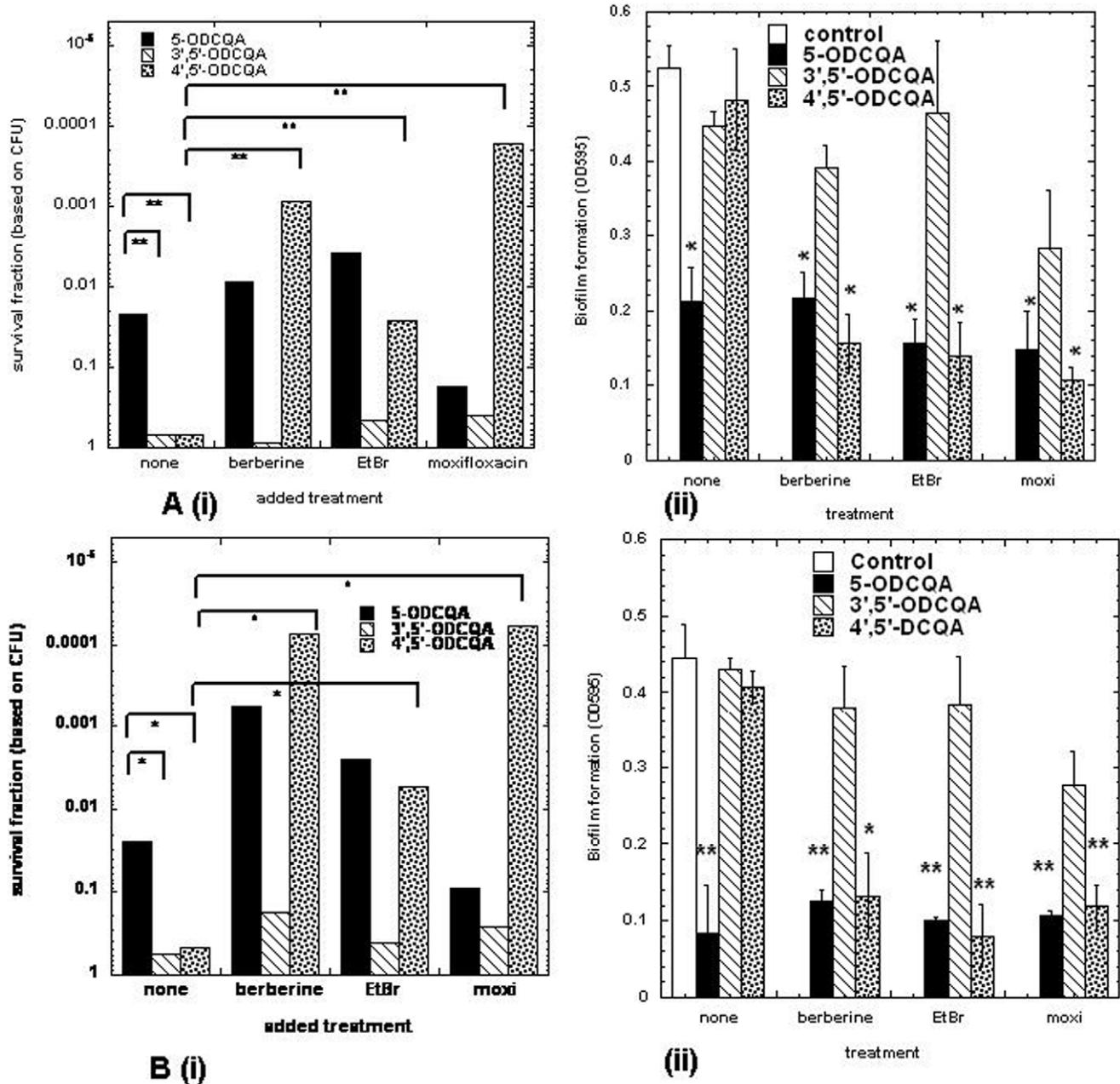


Figure 4. Potentiation of biofilm inactivation by CQAs in combination with model antimicrobials. (A) Biofilms of *S. aureus* 8325-4 were significantly more sensitive to berberine, ethidium bromide and representative fluoroquinolones in the presence of the EPI 4',5'-ODCQA. Antimicrobials in sub-lethal/inhibitory concentrations (100 $\mu\text{g/ml}$ for berberine, 10 $\mu\text{g/ml}$ for EtBr and 2 $\mu\text{g/ml}$ for moxifloxacin respectively) were added to 48 hr biofilms that had been grown in media with and without CQAs. After 2 h of exposure to antimicrobials the biofilm density was assessed by determining the number of CFUs per microtiter well (i) and the absorbance of crystal violet (CV)-stained biofilms at 600 nm (ii). In the presence of CQAs (50 $\mu\text{g/ml}$), (B) Biofilms of *E. faecalis* OGRF1 strain showed large increases in EtBr susceptibility in the presence of 4',5'-ODCQA. 48 hr biofilms were exposed to sub-inhibitory concentrations of antimicrobials (50 $\mu\text{g/ml}$ for berberine, 8 $\mu\text{g/ml}$ for EtBr and 1 $\mu\text{g/ml}$ for moxifloxacin respectively) for 2 h after growth in media with and without CQAs. The size of the viable biofilm was found by calculating the number of CFUs per microtiter well (i) and the absorbance of crystal violet (CV)-stained biofilms at 600 nm (ii).for three independent experiments and statistical significance determined by a paired two-tailed t test). doi:10.1371/journal.pone.0018127.g004

antimicrobial and toxicity profile of caffeoylquinic acids combined with prior art and information regarding their impact in antioxidant defenses in mammalian systems suggests a potential similar role in bacteria. The direct antimicrobial activity of CQA implies an array of possibilities including effect in the cell envelope and requires a robust target based validation and investigation.

Materials and Methods

Microbial strains and culture conditions

The following bacterial strains were used in this study: *S. aureus* 8325-4 (wild-type); K1758 (8325-4 ΔnorA); QT1 8325-4 *norR::cat* (NorA is overexpressed [50], *S. aureus* Community Associated

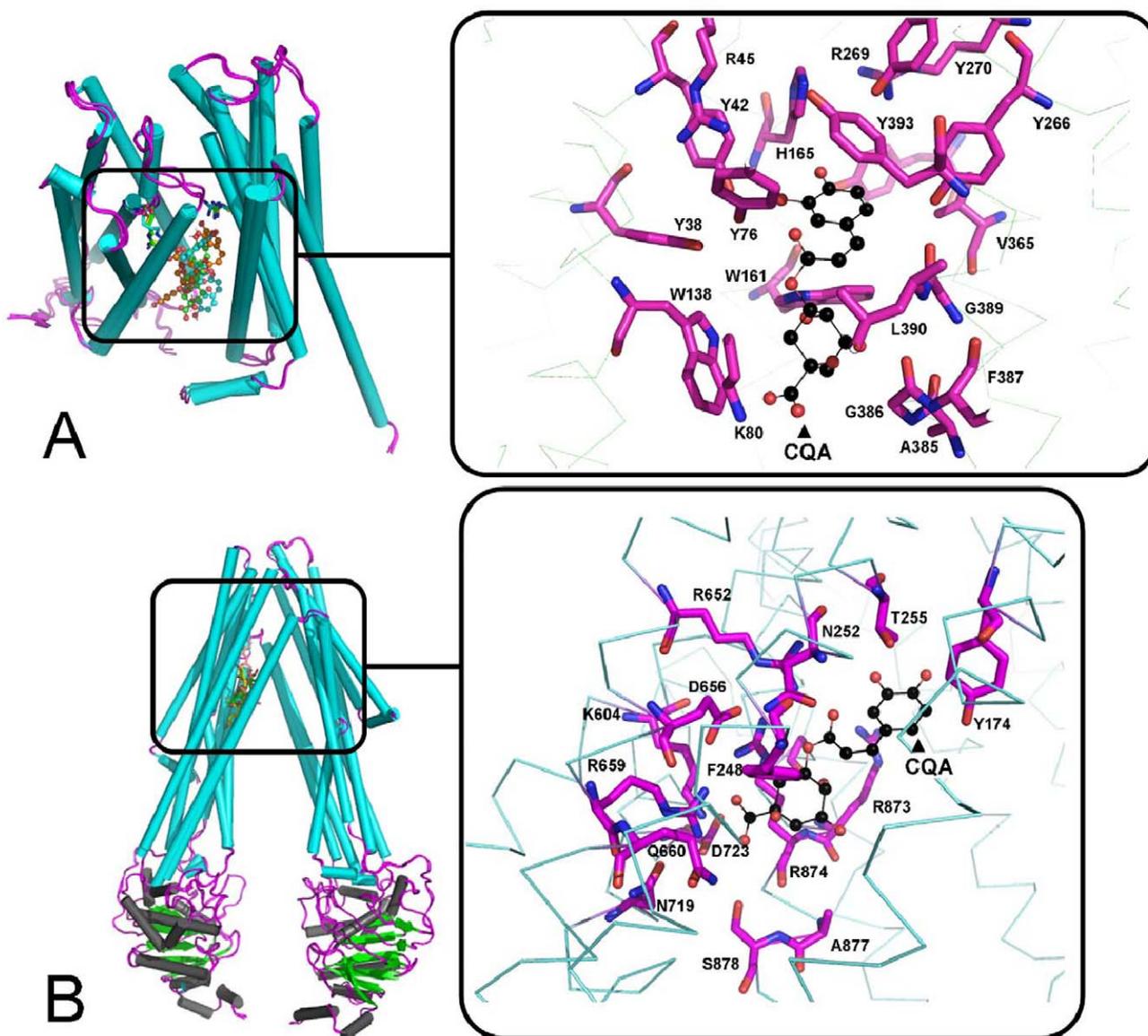


Figure 5. Docking results and interactions within the binding pockets of MES for of all CQAs. All CQAs can successfully enter the binding sites of both efflux systems (A, B). See also Table 3 for the calculated energetics.
doi:10.1371/journal.pone.0018127.g005

(CA)-MRSA USA300 (Human isolate/Clinical Microbiology, MGH), *E. faecalis* OGRF1 (Fus^r Rif^r; *p*-Cl-Phe^r), *E. faecalis* GW4481 (OG1RFΔ*emeA*, [51], *Escherichia coli* K12 (ATCC), prototrophic wild type reference strain *Candida albicans* DAY185 [52]. Bacterial cells were cultured in Mueller–Hinton Broth (MHB) and yeast cells in YPD (unless is otherwise stated). Cell growth was assessed with a spectrophotometer (Shimadzu, Mini 1240) at 600 nm (OD₆₀₀).

Chemicals

All solvents used in chromatography were of HPLC grade from Lab-Scan Analytical Sciences Ltd. (Dublin Ireland); Formic acid from Merck (Haarlem, The Netherlands) and H₂O was produced in-house (Milli-Q Water Purification System, Millipore). Acetonitrile-d₃ (99.8%) was obtained from Cambridge Isotope Laboratories (Apeldoorn, The Netherlands). Antibiotics (erythromycin, tetracycline) and antimicrobials (berberine, ethidium bromide,

rhodamine 6G, chlorogenic acid) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fluroquinolones (norfloxacin, ciprofloxacin, levofloxacin and moxifloxacin) were from the Massachusetts General Hospital Pharmacy (<http://www.massgeneral.org/services/pharmacy.aspx>) INF₂₇₁ was provided by Chembridge Inc. San Diego, CA 1',3' & 1',5'-ODCQA as well as peracetylderivatives of both DCQAs, were kindly provided by Jiri Slanina (Department of Biochemistry Faculty of Medicine Masaryk University in Brno [53] and 3',5'-ODCQA, 4',5'-ODCQA by Cfm Oskar Tropitzsch e.K. (Markredwitz, Germany).

Plant Material & Aqueous infusions

Artemisia absinthium, growing wild in Epirus region (Northwestern Greece) was collected in the period from spring to summer. 2 g of dried aerial parts of *A. absinthium*, ground to pass a 0.4 mm sieve, were added to 50 ml of boiling double distilled water (DDW) and stirred for 30 min. The plant residue was then filtered, and the

Table 6. Energetic contributions and cluster sizes for each docking run.

	Efflux Pump PDB ID ^a	Cluster size ^b	RMSd ^d (Å)	Haddock Score (kcal/mol)	Van der Waals (kcal/mol)	Electrostatics (kcal/mol)	Desolvation (kcal/mol)	BSA (Å ²)
5'-OCQA	1PW4	198	0.3±0.2	-90.3±7.2	-22.0±0.7	-140.9±18.3	-40.1±10.2	671±38
	2HYD*	146	0.3±0.2	-1.3±4.6	-9.7±3.4	-161.5±24.2	40.6±9.3	498±83
3',5'-ODCQA	1PW4	116	0.3±0.2	-108.2±3.6	-27.7±2.8	-151.5±10.1	-50.2±3.5	825±32
		82	0.5±0.0	-98.1±4.4	-27.1±1.8	-146.7±20.7	-41.7±7.1	823±64
	2HYD*	7	0.3±0.2	0.1±8.0	-22.9±3.5	-182.0±18.6	58.5±2.5	716±57
		94 ^c	0.5±0.0	2.8±7.1	-17.9±5.7	-176.8±25.0	55.9±5.4	623±72
4',5'-ODCQA	1PW4	88	0.3±0.2	-108.1±11.0	-38.2±3.0	-137.1±12.2	-42.4±11.5	931±48
		109	0.5±0.1	-103.9±2.9	-32.8±3.4	-169.7±32.1	-37.2±6.3	952±22
	2HYD*	82	0.3±0.2	-8.7±3.9	-20.8±1.5	-185.7±32.5	49.1±5.7	766±70

See text for details and materials and methods for column header definitions.

^a1PW4 corresponds to the PDB ID glycerol-3-phosphate transporter from *Escherichia coli* and 2HYD* to the modeled open conformation of Sav1688 ABC transporter.

^bSize of top ranking clusters. Clustering was performed with a 7.5 Å cutoff based on pairwise interface ligand RMSDs, the RMSD of the ligand after fitting on the binding site of the receptor.

^cThis cluster scored second on the top; the first cluster included only 7 structures and the energetics were very similar (see Table).

^dRMSd is the average Root mean square displacement from the top four ranking model of a cluster from its top ranking member, calculated on the backbone atoms of the protein and all heavy atoms of the ligand.

doi:10.1371/journal.pone.0018127.t006

filtrate was lyophilized. The 40 mg of dried extract produced were analyzed for the determination of its main active components. Plant materials and aqueous infusions preparations as well as instrumentation used for the analysis of the constituents are reported in **Text S1**.

Susceptibility testing

Cells (10⁵/ml) were inoculated into MHB (RPMI for *C. albicans*) and dispensed at 0.2 ml/well in 96-well microtiter plates. Growth inhibition was determined by serial 2-fold dilution of test extract or compounds in 96-well microtiter plates to determine Minimal Inhibitory Concentrations (MICs). In order to identify compounds with EPI activity, extracts and compounds were serially 2-fold diluted in combination with 30 µg/ml berberine for gram-positive bacteria, 10 µg/ml erythromycin for *E. coli*, and 10 µg/ml rhodamine 6G (R6G) for yeast cells. An EPI was defined as a compound that completely prevented cell growth in the presence of sub-inhibitory antibiotic during an 18-hr incubation at 37°C or 24–48 hr at 30°C (*B. cereus*, *C. albicans*). All tests were done in triplicate by following the Clinical and Laboratory Standards Institute (CLSI, former NCCLS, National Center for Clinical Laboratory Standards) recommendations [54,55]. Growth was assayed with a microtiter plate reader (Spectramax PLUS384, Molecular Devices) by absorption at 600 nm. The checkerboard tests for DCQAs and antimicrobials were performed as previously described [56]. For each combination experiment (composed of a row or column in the matrix), the Fractional Inhibitory Concentration (FIC) of each agent was calculated: FIC = 1 additive effect; FIC < 1, synergy; FIC > 1 antagonism.

Hemolysis assay

The ability of CQAs to hemolyze sheep erythrocytes is based on the protocol of Ciornei *et al.* [57], with the following modifications [58]: sheep erythrocytes (Rockland Immunochemicals) were treated with CQAs (0.5–50 µg/ml) in PBS with DMSO at 2% and the supernatants for 2 h, using Triton X-100 and DMSO as controls, and were monitored on a microtiter plate reader (Spectramax PLUS384, Molecular Devices) at OD₅₄₀.

Berberine accumulation assays

Determination of berberine uptake was performed as described previously [9,59]. Cells were cultured with aeration at 37°C to an optical density at 600 nm (OD₆₀₀) of 1.8, pelleted, and washed twice with 20 mM HEPES-NaOH (pH 7.0) buffer. The cells were then resuspended to an OD₆₀₀ of 0.3 in 1 ml of HEPES buffer containing 10 µM glucose followed by incubation at 37 or 30°C for 1 h. The cells were centrifuged, washed, and resuspended at an OD₆₀₀ of 0.15 in HEPES buffer. Assays were performed in 96-well flat-bottom black plates (Costar) in a final volume of 200 µl. Berberine was added at 30 µg/ml, and fluorescence was measured with a Spectramax Gemini spectrofluorometer (Molecular Devices) at a 355-nm excitation wavelength and a 517-nm emission wavelength.

Berberine competitive efflux assay

Those were performed as described [59]. *S. aureus* and *E. faecalis* cells were cultured with aeration and mild agitation until they reached the late log phase (OD₆₀₀ between 0.9 and 1), pelleted by centrifugation (2 min, 12,000 rpm), and then washed and resuspended in 25 mM PBS (pH 7.4) containing 0.05 g L⁻¹ MgSO₄, 7 g L⁻¹ K₂HPO₄, 0.5 g L⁻¹ sodium citrate·3H₂O, 1 g L⁻¹ (NH₄)₂SO₄, 0.01 mg L⁻¹ folic acid, 0.05 mg L⁻¹ pyridoxine hydrochloride, 0.025 mg L⁻¹ riboflavin, 0.01 mg L⁻¹ biotin, 0.025 mg L⁻¹ thiamine, 0.025 mg L⁻¹ nicotinic acid, 0.025 mg L⁻¹ calcium pantothenate, 0.5 µg L⁻¹ vitamin B12, 0.025 mg L⁻¹ *p*-aminobenzoic acid, 0.025 mg L⁻¹ thiotic acid, and 4.5 mg L⁻¹ monopotassium phosphate. Cells were then resuspended to an OD₆₀₀ of 0.8 in buffer with 10 mM glucose. Cells were then loaded with either 30 µg/ml berberine and 10 µg/ml 4',5'-ODCQA and incubated at 37°C with aeration for 20 min. Cells were then centrifuged in a 4°C cold room, washed in ice-cold PBS, and added at an OD₆₀₀ of 0.3 to a chilled 96-well flat-bottom white microtiter plate (Costar) containing ice-cold 25 mM PBS and 10 mM glucose in a final volume of 200 µL. Fluorescence was measured with a Spectramax PLUS384 GeminiXS spectrofluorometer (Molecular Devices) at a 355-nm excitation wavelength and a 517-nm emission wavelength.

Biofilm formation & Quantitation

Biofilm formation is assayed by the ability of cells to adhere on polystyrene surface of 24-well microtiter plates [60]. The indicated medium is inoculated from a 1:100 dilution from an overnight BHI culture. Plates are incubated at 37°C for 48 hrs. Compounds are added at the appropriate combinations and concentrations followed by additional overnight incubation at 37°C. Two identical microtiter plates were used for each independent experiment. For the first plate crystal violet (CV) solution was added to each well, the plates were incubated at room temperature, rinsed thoroughly with water and dissolved. CV-stained biofilms were solubilized in ethanol, and samples were transferred to a new polystyrene microtiter dish, and the absorbance was determined with a plate reader at 600 nm. For the second plate cells are dissolved followed by CFU determinations on BHI agar plates.

Statistics

For the toxicity experiments studies values are means of three separate experiments and bars are SEM. For biofilm inactivation studies, between means were tested for significance by a paired two-tailed *t* test. The significance level was set at $p < 0.05$.

Structure-activity relationships and comparative protein-ligand docking studies

Two efflux pump crystal structures [34,35] were selected to perform comparative docking of caffeoylquinic acids to their binding sites: the glycerol-3-phosphate transporter from *Escherichia coli* [35] (PDB ID: 1PW4) was the representative structure from the MFS, and Sav1866 from *Staphylococcus aureus* [34,35] (PDB ID: 2HYD), is an ABC transporter of Gram-positive bacteria. 2HYD was crystallized in the closed conformation. Since 2HYD shares very high structural similarity with P-glycoprotein [34,61], the open conformation was generated by flexible structural alignment with FATCAT (<http://fatcat.burnham.org/>) against the corresponding P-glycoprotein crystal structure [61] (PDB ID: 3G61). This approach concluded in a good quality model (see **Figure S3 and Table S1**).

For the docking trials, HADDOCK version 2.1 [62] was used. HADDOCK is a highly successful modeling approach that makes use of biochemical and/or biophysical interaction data such as chemical shift perturbation data, mutagenesis data, or bioinformatic predictions and thus, incorporates structural knowledge of the target to drive the docking procedure. The docking was performed with default parameters using the web server version of HADDOCK [63]. All calculations were performed with CNS1.2 [64]. Non-bonded interactions were calculated with the OPLS force field [65] using a cutoff of 8.5 Å. The electrostatic potential (E_{elec}) was calculated by using a shift function, while a switching function (between 6.5 and 8.5 Å) was used to define the Van der Waals potential (E_{vdw}). The HADDOCK score is used to rank the generated poses. It is a weighted sum of intermolecular electrostatic (E_{Elec}), van der Waals (E_{vdw}), desolvation (ΔG_{solv}) and ambiguous interaction restraint (AIR) energies with weight factors of 0.2, 1.0, 1.0 and 0.1, respectively.

The ambiguous interaction restraints to drive the docking were defined as follows. The ligand was treated as an active residue for all stages of the docking, while the residues within the protein binding site were only defined as active for the rigid-body docking stage and considered as passive the subsequent semi-flexible refinement stage. This strategy allows to effectively pulling the ligand within the binding site during rigid-body docking while allowing a more thorough exploration of the binding pocket

during the refinement stage. Residues considered active for 1PW4 were 38, 42, 45, 46, 134, 165, 166, 266, 269, 270, 299, 362 and 393, whereas for 2HYD*, the active residues were 26, 252, 255, 288, 291, 295, 604, 608, 611, 612, 614, 648, 722, 837, 866, 869, 870, 873, 874 and 896. Passive residues were defined automatically via the web-server, as those surrounding the active ones.

Supporting Information

Figure S1 400 MHz 1H-NMR spectrum of 1, in CD₃CN. The spin system corresponding to 5'-OCQA is revealed. The H-2/ and H-6/signals of the quinic moiety are overlapped by the CD₃CN-H₂O residual signals and, therefore, they are eliminated. (TIFF)

Figure S2 400 MHz 1H-NMR spectrum of 2A and 2B, in CD₃CN. The spin system of 3',5'-ODCQA (2A) is mainly indicated while that one of 4',5'-ODCQA (2B) is suggested in italics. The signals of the aromatic protons of 2B are greatly overlapped by those of the 2A isomer and, thus, are not indicated in the spectrum. (TIFF)

Figure S3 View of protein-ligand hydrogen bonds in the top two clusters of the modelled complex of 3',5'-ODCQA with 1PW4: (A) top ranking cluster, (B) second, most populated cluster. The different orientations of the 3',5'-ODCQA is reflected in the differences in the hydrogen bonding pattern. For example, 3',5'-ODCQA, show a different pattern of hydrogen bonds of the carbonyl groups, interacting in one case with the side chains of Y38, Y42 and W261 whereas in the second cluster the observed hydrogen bonds are different and fewer in number. This figure was generated with PyMol (www.pymol.org). (TIFF)

Figure S4 Ramachandran plot of the model of Sav1688 in the open conformation. The plot is based on an analysis of 118 structures of resolution of at least 2.0 Å and an R-factor no greater than 20%. A good quality model would be expected to have over 90% in the most favoured regions. Plot statistics: Residues in most favoured regions [A,B,L], 1001 (95.5%). Residues in additional allowed regions, [A,B,L,P], 43 (4.1%). Residues in generously allowed regions, [~A, ~B, ~L, ~P], 2 (0.2%). Residues in disallowed regions, 2 (0.2%). Number of non-glycine and non-proline residues, 1048 (in total: 100.0%). Number of end-residues (excl. Gly and Pro), 4. Number of glycine residues (shown as triangles), 74. Number of proline residues, 30. Total number of residues: 1156. (TIFF)

Table S1 PSVS (http://psvs-1_4-dev.nesg.org/) validation statistics of the modelled open form (2HYD*) and, for comparison, of the crystal structure 2HYD. (TIFF)

Text S1 Plant materials and aqueous infusions preparations as well as instrumentation used for the analysis of the constituents of *Artemisia absinthium* extracts. (DOC)

Acknowledgments

The authors wish to thank Dr. Christos. Nanos for collecting and botanical characterization of the plant material, Dr. Glen Kaatz (Division of Infectious Diseases, Wayne State University School of Medicine) for providing the NorA knock out strain, Dr. David Hooper (Division of Infectious Diseases, Massachusetts General Hospital) for the overexpression *S. aureus* NorA strain QT1, George Weinstock (Human Genome

Sequencing Center, Baylor College of Medicine) for the *E. faecalis* norA knock out mutant GW4481, Aaron Mitchell (Department of Microbiology, Columbia University) for the *Candida albicans* DAY185 reference strain, Jiri Slanina (Department of Biochemistry Faculty of Medicine Masaryk University in Brno) and M. Reinecke (Department of Chemistry, University of Arizona) for 1',3'- & 1',5'-ODCQA as well as peracetylderivatives of both caffeoylquinic acids, Dr. Frank R. Stermitz (Colorado State University) for his expert consultation and Dominique Perez at State University of New Mexico Center for Molecular Discovery for proofreading.

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Conceived and designed the experiments: YCF KL GPT. Performed the experiments: YCF PLK VE HH. Analyzed the data: YCF PLK AMJJB MRH GPT. Contributed reagents/materials/analysis tools: JV AMJJB KL MRH. Wrote the paper: YCF MRH GPT.

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