ORIGINAL ARTICLE

Effects of coadministration of natural polyphenols with doxycycline or calcium modulators on acute *Chlamydia pneumoniae* infection *in vitro*

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Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases. Here we present the first attempt to design a combination treatment targeted against *Chlamydia pneumoniae* infection using coadministration of natural phenolics with calcium (Ca^{2+}) modulators, and also the concomitant administration of these compounds with doxycycline. An *in vitro* acute *C. pneumoniae* model in human lung epithelial cells was used and Loewe additivity model was applied to evaluate the effects. In general, the phenolic compounds, quercetin, luteolin, rhamnetin and octyl gallate did not improve the antichlamydial effect of doxycycline, and, in some cases, resulted in antagonistic effects. The combination of doxycycline and Ca^{2+} modulators (isradipine, verapamil and thapsigargin) was at most additive, and at subinhibitory concentrations of doxycycline, often even antagonistic. The Ca^{2+} modulators showed no inhibitory effects on *C. pneumoniae* growth alone, whereas the coadministration of Ca^{2+} modulators with phenolic compounds resulted in potentiation of the antichlamydial effect of phenolic compounds. Verapamil (100 μM) was synergistic with low quercetin and luteolin concentrations (0.39 and 1.56 μM), whereas 10 μM isradipine was synergistic with high quercetin, rhamnetin and octyl gallate concentrations (12.5 μM and 100 μM). Use of thapsigargin with the phenolic compounds resulted in the most intense synergism. Interaction indices 0.12 and 0.14 were achieved with 0.39 μM luteolin and 10 and 100 nM thapsigargin, respectively. To conclude, the observed results indicate that the Ca^{2+} modulators potentiate the antichlamydial effects of the phenolic compounds.

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INTRODUCTION

Chlamydia pneumoniae is an intracellular Gram-negative bacterium infecting readily the epithelial and smooth muscle cells, as well as monocytes and macrophages. The bacterium causes acute respiratory tract infections, and is estimated to be responsible for 5–10% of community-acquired pneumonia.¹ The persistent infection of *C. pneumoniae* has been associated with the development of several inflammatory diseases. For example, the association between *Chlamydia pneumoniae* and atherosclerosis has been indicated by several approaches including *in vivo* animal studies, seroepidemiological studies in humans, demonstration of the presence of the bacteria in atherosclerotic lesions and atherosclerotic changes in vascular tissue.^{2–5}

Antimicrobial therapy is heading toward multidrug treatments because microbial resistance toward commonly used antibiotics has increased.⁶ The multidrug treatment approach has already been used for long against difficult pathogens that typically develop resistance

to antibiotics such as *Mycobacterium tuberculosis* or respond poorly to single-drug treatment such as *Helicobacter pylori*. Moreover, in serious cases of *C. pneumoniae* infection, such as *Chlamydia*-induced arthritis, prolonged treatment with multiple antibiotics has been successfully used.⁷

Most antibiotics limit infections by inhibiting central microbial biosynthetic pathways, thus having relatively little effect on the metabolically inactive forms of pathogens.⁸ Occasionally, *in vivo* antibiotic treatment of acute *C. pneumoniae* infections with standard clinical doses has failed.⁹ These treatment failures may promote the development of persistent infection. In fact, some of the first-choice antibiotics, such as doxycycline and rifampicin, have been reported to promote the development of persistent infection at subinhibitory concentrations.¹⁰ It has been suggested that *C. pneumoniae* infection of macrophages is often in a persistent form, which explains the observed refractory behavior of *C. pneumoniae* infection to antibiotic treatment in cells of monocyte-macrophage line.^{11,12} Thus, new

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treatments are needed to fight the chlamydial infections and avoid the treatment failures.

Calcium (Ca^{2+}) channel blockers have been shown to inhibit the growth of some extracellular and intracellular pathogens and to have combinatory effects with certain antimicrobials.¹³ High doses of verapamil have been shown to inhibit the growth of *Chlamydia*,¹⁴ and most interestingly, Ca^{2+} channel blockers have been shown to improve antibiotic susceptibility of persistent *C. pneumoniae* infection, presumably by reactivating the persistent infection to more easily treatable acute infection.^{15,16}

We have previously shown that phenolic compounds, common in our daily food, are potent and valid inhibitors of *C. pneumoniae*, *in vitro* and *in vivo*, even with doses achievable from the nutritional sources.^{17,18} Phenolic compounds have been associated with a wide range of biological activities; among others, they have been shown to affect Ca^{2+} channel regulation in mammalian cells at physiological concentrations. As reviewed by Scholz *et al.*,¹⁹ the type and strength of the effect depends highly on the compound concentration and on the cell and channel type. In our earlier work with rat pituitary GH_4C_1 cells expressing L-type Ca^{2+} channels, luteolin and octyl gallate inhibited the uptake of Ca^{2+} from extracellular media, whereas quercetin increased the uptake, and rhamnetin showed no effect on the Ca^{2+} ion uptake at a concentration of $20 \mu\text{g ml}^{-1}$ ($63\text{--}71 \mu\text{M}$).²⁰ These phenolic compounds sharing good antichlamydial activity, but showing differential Ca^{2+} -modulating activities, were chosen for the current study.

In this study we investigated whether coadministration of doxycycline, Ca^{2+} modulators and phenolic compounds in appropriate dose could produce a synergistic antimicrobial effect on acute chlamydial infection *in vitro*. Two clinically used Ca^{2+} channel blockers were used, isradipine being highly selective inhibitor of L-type Ca^{2+} channels and verapamil, which is relatively non-selective Ca^{2+} channel inhibitor. In addition, thapsigargin, a selective inhibitor of the sarco/endoplasmic reticulum Ca^{2+} -ATPase pump was included in the study.

RESULTS

Dose-dependent inhibition of *C. pneumoniae* growth by single compounds

First, the dose-dependence of the antichlamydial effect of Ca^{2+} channel blockers, isradipine and verapamil, sarco/endoplasmic reticulum Ca^{2+} -ATPase pump, inhibitor thapsigargin, doxycycline, the three flavonoids quercetin, luteolin and rhamnetin, and an alkyl gallate octyl gallate, were studied in a model for acute *C. pneumoniae* infection in human lung epithelial (HL) cells.

The treatment of the chlamydial cultures with Ca^{2+} channel blockers (up to $100 \mu\text{M}$ concentration) had minor or moderate effects

on the number of chlamydial inclusions. Isradipine was the more efficient from the two tested Ca^{2+} channel blockers in reducing chlamydial growth, as treatment with $10 \mu\text{M}$ isradipine resulted in 56% inhibition of chlamydial growth, and with $100 \mu\text{M}$ isradipine, 87% inhibition of number of chlamydial inclusions was achieved, yet this higher concentration also decreased host cell viability to 54%. (Table 1) Verapamil, on the other hand, gave 28% inhibition of chlamydial growth at a concentration of $100 \mu\text{M}$, whereas treatment with $10 \mu\text{M}$ concentration markedly increased the number of chlamydial inclusions. Lower concentrations of verapamil resulted in no significant change in the number of chlamydial inclusions. Thapsigargin showed 53% inhibition at $1 \mu\text{M}$ concentration, but also decreased HL cell viability at this concentration. Lower thapsigargin concentrations showed no antichlamydial effects.

The treatment of chlamydial cultures with doxycycline, quercetin, luteolin, rhamnetin and octyl gallate resulted in dose-dependent inhibition of chlamydial growth as measured by the number of chlamydial inclusions. Sigmoidal dose-response curves for the antichlamydial effects of doxycycline, the three flavonoids and octyl gallate were obtained (data not shown). The IC_{50} values determined by the curves were 49 nM ($\log \text{IC}_{50} -7.3 \pm 0.26$) for doxycycline, $46 \mu\text{M}$ (-4.3 ± 0.17) for quercetin, $16 \mu\text{M}$ (-4.8 ± 0.26) for luteolin, $50 \mu\text{M}$ (-4.3 ± 0.25) for rhamnetin and $4.2 \mu\text{M}$ (-5.4 ± 0.18) for octyl gallate.

The inhibition of *C. pneumoniae* by combinations with doxycycline

Doxycycline (70 , 35 and 18 nM) was combined with the three flavonoids and octyl gallate. When combined with quercetin, most samples within the combination treatments gave higher inclusion numbers than untreated controls, resulting in negative inhibition percentages; however, the inhibition values of both doxycycline and quercetin alone were consistent with the obtained dose-response curves. As a result of the negative inhibition values obtained for most of the combinations, the interaction indices (*I*) calculated for the different doxycycline-quercetin combination showed mostly strong-to-moderate antagonism (*I*-values >2 , in most combinations) (Table 2). However, an *I*-value of 0.9 was achieved with 70 nM doxycycline + $6.25 \mu\text{M}$ quercetin combination, indicating an additive effect.

Similar data were obtained when doxycycline was combined with the two other flavonoids, luteolin and rhamnetin. Most of the samples treated with the combinations in different doses resulted in negative inhibition values and thus calculated *I*-values indicate strong antagonizing effects between the agents. Moreover, doxycycline-octyl gallate combinations showed some negative inhibition; antagonizing or indifferent rather than additive or synergistic effects for all the concentration combinations (Table 2).

Table 1 Effect of the studied compounds on HL cell viability (ATP level), measured with a commercial kit CellTiter Glo (Promega, Madison, WI, USA) after 70 h exposure

Viability % (s.e.m. %)							
Concentration (μM)	Quercetin	Luteolin	Rhamnetin	Octyl gallate	Thapsigargin	Verapamil	Isradipine
100	89 (4.6)	47 (3.8)	81 (1.0)	21 (39.7)	—	81 (0.4)	54 (0.8)
10	109 (2.7)	89 (1.2)	89 (10.2)	97 (1.8)	—	81 (0.3)	81 (1.0)
1	105 (1.0)	102 (1.0)	99 (1.0)	109 (0.5)	61 (0.8)	82 (0.2)	79 (1.5)
0.1	101 (0.5)	102 (1.2)	101 (1.0)	111 (0.6)	68 (3.5)	88 (0.5)	98 (3.1)
0.01	101 (1.0)	102 (1.0)	104 (2.1)	114 (4.1)	90 (1.0)	—	—
0.001	—	—	—	—	87 (2.7)	—	—

Data show host cell viability percentages (mean $\pm$ s.e.m. of three replicates).

Table 2 Results from the experiments with combinations of doxycycline and natural phenols on the *C. pneumoniae* infection in HL cells. Interaction indices, inhibition percentage of *C. pneumoniae* growth in parenthesis and host cell viability percentages in square brackets, are presented

	μM	Doxycycline		
		0.07	0.035	0.018
Quercetin	100	1.20 (94) [82]	2.25 (68) [81]	1.55 (73) [78]
	50	2.30 (51) [99]	2.97 (38) [97]	>10 (0) [99]
	25	>10 (14) [107]	>10 (–11) [104]	>10 (–29) [106]
	6.25	0.91 (45) [105]	>10 (–11) [102]	>10 (–47) [101]
	1.25	>10 (16) [107]	>10 (–21) [104]	>10 (–37) [106]
Luteolin	25	0.95 (90) [81]	2.09 (68) [83]	2.95 (51) [82]
	12.5	>10 (–23) [102]	>10 (–80) [102]	>10 (–1) [102]
	6.25	>10 (–1) [109]	>10 (–111) [106]	>10 (–70) [106]
	1.25	1.57 (45) [105]	>10 (–55) [104]	>10 (–45) [104]
	0.39	1.45 (41) [100]	>10 (–78) [100]	>10 (–36) [101]
Rhamnetin	100	1.00 (96) [94]	0.85 (87) [94]	0.63 (96) [97]
	50	2.17 (63) [92]	2.54 (36) [94]	4.89 (18) [97]
	25	2.29 (50) [91]	>10 (–15) [91]	1.65 (31) [94]
	6.25	2.31 (26) [97]	>10 (–21) [102]	>10 (–55) [100]
	1.25	1.67 (52) [102]	2.14 (12) [102]	>10 (–47) [109]
Octyl gallate	50	–	–	–
	25	–	–	–
	12.5	>10 (5) [90]	>10 (–21) [91]	>10 (14) [89]
	3.125	2.03 (52) [103]	2.04 (29) [105]	4.93 (7) [102]
	0.78	1.56 (48) [107]	>10 (–18) [113]	>10 (–15) [108]
		Interaction index		Effect
		0–0.75	Synergy ^a	
		0.76–1.25	Additive	
		1.26–2.00	Indifferent	
		>2.00	Antagonism	

^aCombinations in which compound concentrations exceeded those required for maximal inhibition resulted in apparently high interaction index values. These numeric values reflect the reaching of the upper plateau of the dose–response curves rather than antagonizing effects by the combinations.
—host cell viability below 67%.

The effect of combined treatment of infected cultures with doxycycline and the Ca^{2+} modulators was investigated, as this issue has earlier been addressed in persistent infection of *C. pneumoniae* in macrophages.¹⁵ In our model of acute *C. pneumoniae* infection in HL cells, the inhibitory effect of doxycycline (from 0.018 to 0.28 μM) was not markedly increased in the concomitant treatment of isradipine (100, 10, 1 μM), verapamil (100, 10, 1 μM) or thapsigargin (1 μM , 100 or 10 nM). Table 3 presents the inhibition percentages resulting from each of the combinations, together with the *I*-values calculated for the combinations. As indicated by the *I*-values, many of the combined inhibitory concentrations of doxycycline and each of the Ca^{2+} modulators show additive ($0.75 < I \leq 1.25$), however not synergistic ($I \leq 0.75$), effects on inhibition of chlamydial growth. On the other hand, the combinations with subinhibitory concentrations of doxycycline and Ca^{2+} modulators were often indifferent ($1.25 < I \leq 2$) or antagonistic (*I*-values > 2).

The inhibition of *C. pneumoniae* by natural phenol and Ca^{2+} modulator combinations

The effect of combining the phenolic compounds with Ca^{2+} modulators were studied in similar experiments by titrating the effect of each phenolic compounds with 100, 10 and 1 μM isradipine or verapamil, or with 1 μM , 100 nM and 10 nM thapsigargin. Majority of the combinations with 100 μM isradipine lowered the host cell viability below 2/3 of control and thus those results are not presented.

When quercetin and rhamnetin were assayed with 10 μM isradipine, *I*-values of approximately 0.5 were achieved in the presence of 50 μM of 100 μM rhamnetin and quercetin. Thus, 10 μM isradipine functioned synergistically with the high polyphenol concentrations. Furthermore, 1 μM isradipine, which showed no antichlamydial effect when administered alone, was able to potentiate the antichlamydial effect of 100 and 50 μM of quercetin and rhamnetin. In the case of isradipine–luteolin combinations, increased numbers of chlamydial inclusions compared with untreated controls, resulting in negative inhibition values, were seen for most concentration combinations (Table 4). Thus, the *I*-values for these combinations gave values higher than 2 and were classified as antagonistic effects. Antagonism was also seen in most of the isradipine–octyl gallate combinations.

Changing the Ca^{2+} channel blocker from isradipine to verapamil resulted in somewhat similar results, however, with some exceptions in the case of quercetin. When the highest verapamil concentration (100 μM) was combined with 0.39 and 1.56 μM luteolin, *I*-values of 0.52 and 0.58 were achieved, indicating that these agents have a moderate synergistic effect in the antichlamydial assay. Verapamil (100 μM) was moderately synergistic also with 1.56 μM quercetin (*I*-value 0.63). Lower verapamil concentrations (10 and 1 μM) resulted in negative inhibition values (up to threefold number of chlamydial inclusion compared with untreated controls) independent of luteolin, quercetin (except for 100 μM) or rhamnetin concentration present in the assay media. Moreover, in the case of verapamil and octyl gallate combinations, the counts were up to fourfold compared with untreated controls, and thus heavy or moderate antagonism was indicated for all concentration combinations.

The antichlamydial effect of the three flavonoids was potentiated by thapsigargin. Thapsigargin (10 and 100 nM), having no antichlamydial effect alone, resulted in *I*-values of 0.45 and 0.82, with 6.25 μM quercetin and *I*-values of 0.53 and 0.63 with 50 μM quercetin, respectively. Furthermore, 10 and 100 nM thapsigargin were also able to strongly potentiate the antichlamydial effect of 0.39 μM luteolin, providing *I*-values of 0.12 and 0.14, respectively. Furthermore, 100 nM thapsigargin moderately potentiated the inhibition of *C. pneumoniae* growth when combined with 25, 50 or 100 μM rhamnetin, showing *I*-values of 0.3–0.7. The beneficial effects on inhibition of *C. pneumoniae* were less prone in the combinations of thapsigargin with octyl gallate.

DISCUSSION

Plant polyphenolic compounds, such as flavonoids, are ubiquitous in most edible plants especially tea, onion, apple, berries and grapes. They are important metabolites of plants in defense against radiation and microorganisms.²¹ So far, most of the antibacterial agents from nature have been derived from microbial resources, but plant metabolites deserve consideration in this respect for at least two reasons. First, plants have exceptional ability to produce cytotoxic agents and second there is an ecological rationale that antimicrobial natural products should be present or plants following microbial attack to protect the producer from pathogenic microbes in its environment.²² We have previously shown that dietary phenolic compounds are effective against the intracellular bacterium *C. pneumoniae*.^{17,18} As it

Table 3 Results from the experiments with combinations of doxycycline and calcium modulators on the *C. pneumoniae* infection in HL cells

	μM	Isradipine			Verapamil			Thapsigargin		
		100	10	1	100	10	1	1	0.1	0.01
Doxycycline	0.28	2.00 (94) [86]	1.10 (93) [96]	1.01 (95) [103]	1.33 (98) [93]	1.03 (95) [97]	1.00 (85) [101]	1.33 (85) [90]	1.03 (69) [96]	1.00 (78) [106]
	0.14	2.00 (98) [87]	1.11 (88) [95]	1.01 (87) [100]	1.33 (91) [93]	1.03 (82) [96]	1.00 (88) [101]	2.33 (71) [92]	2.03 (78) [93]	1.00 (84) [107]
	0.07	1.50 (96) [86]	6.80 (16) [93]	1.53 (41) [100]	2.2 (39) [95]	1.9 (59) [98]	>10 (–13) [101]	5.33 (31) [93]	2.33 (29) [95]	2.03 (30) [105]
	0.035	1.25 (98) [84]	>10 (–37) [93]	>10 (–1) [96]	>10 (–7) [97]	>10 (–21) [103]	1.99 (12) [102]	>10 (11) [90]	2.94 (2) [99]	>10 (–15) [105]
	0.018	1.13 (98) [84]	>10 (–10) [91]	>10 (–38) [100]	1.76 (21) [94]	>10 (–1) [97]	>10 (–36) [101]	3.69 (26) [92]	2.00 (8) [95]	>10 (–1) [107]

	Interaction index	Effect
	0–0.75	Synergy
	0.76–1.25	Additive
	1.26–2.00	Indifferent
	>2.00	Antagonism ^a

Interaction indices, inhibition percentage of *C. pneumoniae* growth in parenthesis, and host cell viability percentages in square brackets, are presented.

^aCombinations in which compound concentrations exceeded those required for maximal inhibition resulted in apparently high interaction index values.

These numeric values reflect the reaching of the upper plateau of the dose–response curves rather than antagonizing effects by the combinations.

Table 4 Results from the experiments with combinations of calcium modulators and natural phenols on the *C. pneumoniae* infection in HL cells

	μM	Isradipin		Verapamil			Thapsigargin		
		10	1	100	10	1	1	0.1	0.01
Quercetin	100	0.60 (92) [83]	0.51 (94) [89]	–	0.70 (90) [101]	2.01 (58) [102]	1.00 (91) [88]	1.05 (80) [93]	1.01 (84) [92]
	50	0.63 (81) [89]	0.75 (32) [101]	–	>10 (10) [104]	>10 (–13) [106]	0.67 (88) [98]	0.53 (80) [99]	0.63 (74) [94]
	25	7.00 (23) [90]	5.20 (13) [100]	–	>10 (1) [103]	>10 (–115) [104]	3.33 (36) [82]	>10 (–8) [84]	>10 (–1) [92]
	6.25	>10 (–85) [89]	>10 (–109) [95]	2.68 (17) [79]	>10 (–126) [97]	>10 (–91) [101]	1.21 (52) [77]	0.82 (27) [82]	0.45 (33) [93]
	1.25	>10 (–58) [87]	>10 (–83) [99]	0.62 (46) [91]	>10 (–126) [96]	>10 (–185) [98]	>10 (17) [75]	1.52 (14) [79]	>10 (–9) [96]
Luteolin	25	4.79 (38) [69]	>10 (3) [72]	–	5.10 (30) [70]	3.13 (40) [69]	–	–	–
	12.5	>10 (–44) [80]	>10 (–205) [89]	–	>10 (–43) [83]	>10 (–76) [84]	–	3.33 (30) [68]	3.15 (35) [85]
	6.25	>10 (–70) [89]	>10 (–242) [96]	–	>10 (–56) [92]	>10 (–91) [96]	3.56 (30) [77]	>10 (–28) [82]	>10 (–101) [92]
	1.25	>10 (–152) [94]	>10 (–40) [97]	0.58 (86) [87]	>10 (–54) [96]	>10 (–13) [96]	1.16 (57) [73]	>10 (–23) [85]	>10 (–53) [94]
	0.39	>10 (–163) [94]	>10 (–106) [95]	0.52 (71) [88]	>10 (–60) [99]	>10 (–10) [96]	1.04 (58) [70]	0.14 (58) [79]	0.12 (32) [90]
Rhamnetin	100	0.60 (85) [80]	0.51 (86) [84]	–	1.07 (76) [77]	1.01 (71) [83]	1.00 (100) [92]	0.70 (98) [95]	0.67 (89) [84]
	50	0.63 (73) [86]	0.51 (74) [85]	–	1.35 (59) [86]	4.10 (19) [89]	1.00 (81) [88]	0.60 (54) [91]	4.22 (20) [83]
	25	>10 (–17) [79]	1.25 (46) [83]	–	1.35 (33) [81]	2.18 (20) [83]	0.75 (76) [86]	0.30 (78) [91]	>10 (–57) [79]
	6.25	>10 (–40) [85]	>10 (–127) [88]	–	>10 (–123) [82]	>10 (–86) [86]	5.52 (19) [81]	>10 (–4) [84]	>10 (–50) [77]
	1.25	>10 (–250) [93]	>10 (–91) [94]	>10 (–124) [67]	>10 (–138) [91]	>10 (–57) [96]	>10 (–54) [76]	>10 (–126) [82]	>10 (–117) [88]
Octyl gallate	12.5	0.56 (85) [83]	>10 (4) [87]	3.27 (46) [87]	>10 (–20) [84]	>10 (–47) [86]	1.75 (78) [80]	1.30 (75) [88]	>10 (5) [95]
	3.125	>10 (0) [95]	>10 (–107) [100]	>10 (1) [90]	1.88 (21) [98]	3.25 (12) [103]	1.63 (51) [81]	>10 (–17) [90]	>10 (–71) [96]
	0.78	>10 (–155) [99]	>10 (–141) [100]	>10 (9) [90]	>10 (–289) [98]	>10 (–266) [102]	1.16 (47) [82]	0.89 (19) [86]	>10 (–54) [95]

	Interaction index	Effect
	0–0.75	Synergy
	0.76–1.25	Additive
	1.26–2.00	Indifferent
	>2.00	Antagonism ^a

Interaction indices, inhibition percentage of *C. pneumoniae* growth in parenthesis, and host cell viability percentages in square brackets, are presented.

^aCombinations in which compound concentrations exceeded those required for maximal inhibition resulted in apparently high interaction index values. These numeric values reflect the reaching of the upper plateau of the dose–response curves rather than antagonizing effects by the combinations.

—=host cell viability below 67%.

has been proposed that natural compounds in combination with antibiotics could be a new strategy for developing therapies for infections caused by bacterial species and that natural plant products can potentiate the activity of antibiotics in combination,^{23,24}

we wanted to investigate whether coadministration of phenolic compounds with the antichlamydial compound doxycycline results in synergistic inhibition of acute *C. pneumoniae* infection. The combinations of natural phenols and doxycycline did not improve

antichlamydial activity compared with the activity of compounds alone, except for 100 μM rhamnetin, where additive and synergistic effects were received. However, lower concentrations of rhamnetin slightly diminished the antichlamydial effect of doxycycline. Quercetin, luteolin and octyl gallate clearly antagonized the activity of doxycycline in most of the concentration combinations. These *in vitro* results of the observed antagonism between doxycycline and natural phenols might give further support to the current user recommendations to avoid simultaneous intake of doxycycline with food.

The Ca^{2+} channel inhibitors have been shown to be of value in antimicrobial therapy by interacting with antimicrobial compounds and helping to prevent resistance.^{14,25,26} Azenabor *et al.*¹⁶ demonstrated that an L-type Ca^{2+} channel blocker, nifedipine, may enhance the antichlamydial effect of doxycycline in persistent infection cell model. Nifedipine treatment of macrophages infected with *C. pneumoniae* downregulated chlamydial hsp60 mRNA expression and upregulated major outer membrane protein expression, thus shifting the infection toward a form, which is more susceptible to antibiotic treatment. The authors concluded that the combination therapy using antibiotics and Ca^{2+} channel blockers should be evaluated for the treatment of chlamydial diseases. In our study, the combination of doxycycline and Ca^{2+} modulators against acute infection of *C. pneumoniae* in HL cells was at most additive and at subinhibitory concentrations of doxycycline often antagonistic.

C. pneumoniae has been shown to affect the cellular Ca^{2+} homeostasis in different ways. In *C. pneumoniae*-infected macrophages L-type Ca^{2+} channels are activated and intracellular Ca^{2+} concentrations are increased.²⁷ The entry of chlamydiae has been shown to cause redistribution of cellular Ca^{2+} stores in HeLa cells.^{28,29} Local high Ca^{2+} concentrations and Ca^{2+} -binding protein accumulate in the proximity of chlamydial aggregates and they are pivotal for the proper formation of chlamydial inclusions. In a recent microarray study by Alvesalo *et al.*,³⁰ expression levels of genes associated with regulation of Ca^{2+} homeostasis were significantly changed by *C. pneumoniae* infection in HL cells. The results presented in the current work indicate that Ca^{2+} modulators alone have only a minor effect on acute *C. pneumoniae* infection in HL cells, or in the case of verapamil, may even increase the number of chlamydial inclusions.

When studying phenolic compounds and Ca^{2+} modulators, more antagonistic combinations were found than synergic or additive combinations. However, 10 μM isradipine was synergistic with high quercetin and rhamnetin concentrations (100 μM and 50 μM), whereas in the presence of low quercetin/rhamnetin concentrations, the combinations increased the inclusion counts. Verapamil (100 μM) was synergistic with low quercetin and luteolin concentrations, but unfortunately, compromised cell viability limits the use of higher polyphenol concentrations. Although thapsigargin had no antichlamydial effect on its own, it was able to potentiate the growth inhibitory effect of quercetin, luteolin and rhamnetin on *C. pneumoniae*.

To summarize our results, monotherapy with Ca^{2+} modulators was not inhibitory on chlamydial growth. Verapamil (10 and 1 μM) increased the number of *C. pneumoniae* inclusions in the acute infection model, and this effect was generally not reversed by inhibitory concentrations of the phenolic compounds. However, verapamil did not abolish the antichlamydial effect of doxycycline. High concentrations of quercetin and rhamnetin combined with isradipine and thapsigargin showed clear positive effects on the inhibition of *C. pneumoniae*, whereas other combinations of Ca^{2+} modulators and phenolic compounds only occasionally resulted as improved inhibition.

To conclude, high degree of dose dependence was associated with the observed effects in all cases. The coadministration of Ca^{2+} modulators with phenolic compounds resulted in synergistic inhibition of *C. pneumoniae* growth, whereas combination of these with doxycycline resulted in additive effects at best. Coadministration of phenolic compounds with doxycycline provided no marked beneficial effects. The present study suggest on the potential usefulness of phenolic compounds and Ca^{2+} modulators in combination for combating infections caused by this pathogen.

MATERIALS AND METHODS

Cell lines

HL cell line was used as host cells for the *C. pneumoniae* infection. The cells were grown as described in Salin *et al.*³¹ Briefly, the culture medium consisted of RPMI 1640 supplemented with 7.5% fetal bovine serum and 2 mM L-glutamine, all purchased from BioWhittaker, Lonza (Basel, Switzerland) and with 20 μg gentamycin (Fluka, Buchs, Switzerland) per ml. Cells were cultured to confluence in culture flasks (Greiner, Bio-One, Frickenhausen, Germany) in standard cell culture conditions (37°C, 5% CO_2 and 95% humidity). The *Chlamydia* strain used was a sequenced ATCC strain CWL-029 (VR-1310) and was grown and purified as described in Alvesalo *et al.*¹⁷

Compounds

The natural compounds, luteolin and rhamnetin were purchased from Extrasynthese (Genay, France), quercetin from Carl Roth GmbH (Karlsruhe, Germany) and octyl gallate from Fluka. Doxycycline was from ICN Biomedicals (Aurora, OH, USA) and verapamil and isradipine from Sigma-Aldrich (St Louis, MO, USA). Thapsigargin was a generous gift from Professor Kid Törnquist at Abo Akademi University, Turku, Finland. All stock solutions were made in DMSO (Mallinckrodt Baker, Phillipsburg, NJ, USA) at a concentration of 100 mM, except for doxycycline (0.56 mM) and thapsigargin (1 mM).

Antichlamydial assays

The antichlamydial assays were performed by an immunofluorescence method on a 24-well plate, quantifying the number of chlamydial inclusions in infected HL cells using a *Chlamydia*-specific anti-LPS antibody, as described earlier in Salin *et al.*³¹ and Keurulainen *et al.*³²

The antichlamydial effect of compound combinations was assayed by using a checkerboard method, with a single replicate.³³ Combinations were chosen, based on the activity results from dose-response experiments for single agents, and on each of the combination plates, the compounds were also present alone.

Cell viability assays

The effect of different concentrations of single compounds ($n=3$) and the studied combinations in different concentrations ($n=1$ for each concentration combination) on the host cell viability was determined using the luminescent ATP level determination method as described in Salin *et al.*³¹ All chemical treatments were well tolerated by the host cells (67% viability or higher of DMSO-treated control), unless otherwise stated in the text or tables.

Data analysis

All antichlamydial results represent mean and s.e.m. values from the number of replicates indicated for each experiment. The sigmoidal dose response curves and the IC_{50} values were determined by fitting the data from experiments with concentration series into nonlinear regression models using the GraphPad Prism software version 5.0. (GraphPad Software, Inc., La Jolla, CA, USA) For the analysis of concomitant administration of the chemical agents, Loewe model for additivity was applied by calculating the *I*-values for each combined dose. The equation for calculating *I* was as follows:

$$I = D_1/D_{X1} + D_2/D_{X2}$$

where D_1 and D_2 represent the concentrations of compounds in the combination, and D_{X1} and D_{X2} represent the concentrations of the drugs alone giving similar response.

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