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# Inhibitory effect of the natural product betulin and its derivatives against the intracellular bacterium *Chlamydia pneumoniae*

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ABSTRACT

*Chlamydia pneumoniae* is a universal pathogen that has been indicated to play a part in the development of asthma, atherosclerosis and lung cancer. The complete eradication of this intracellular bacterium is in practice impossible with the antibiotics that are currently in use and studies on new antichlamydial compounds is challenging because *Chlamydia* research lacks the tools required for the genetic modification of this bacterium. Betulin is a natural lupane-class triterpene derived from plants with a wide variety of biological activities. This compound group thus has wide medical potentials, and in fact has been shown to be active against intracellular pathogens. For this reason, betulin and its derivatives were selected to be assayed against *C. pneumoniae* in the present study.

Thirty-two betulin derivatives were assayed against *C. pneumoniae* using an acute infection model in vitro. Five promising compounds with potential lead compound characteristics were identified. Compound **24** (betulin dioxime) gave a minimal inhibitory concentration (MIC) of 1 μM against strain CWL-029 and showed activity in nanomolar concentrations, as 50% inhibition was achieved at 290 nM. The antichlamydial effect of **24** was confirmed with a clinical isolate CV-6, showing a MIC of 2.2 μM. Previous research on betulin and its derivatives has not identified such a remarkable inhibition of Gram-negative bacterial growth. Furthermore, we also demonstrated that this antichlamydial activity was not due to $PLA_2$ (EC 3.1.1.4) inhibition caused by the betulin derivatives.



## 1. Introduction

*Chlamydia pneumoniae* is a Gram-negative human respiratory pathogen that causes acute respiratory infections. It has been estimated that *C. pneumoniae* infection is the causative agent in 5–10% of community-acquired pneumonia, bronchitis and sinusitis cases [1]. This intracellular bacterium also causes a chronic infection that has been associated with atherosclerosis [2,3], asthma [4,5], lung cancer [6] and Alzheimer's disease [7,8]. In these diseases, *C. pneumoniae* seems to act as a triggering factor, although comprehensive data in this respect is yet to be gathered. Serological reports indicate that this bacterium is endemic worldwide and infects practically everyone at least once in their lifetime [9].

This intracellular bacterium has been shown to survive 30 days of antibiotic treatment in a cell culture mimicking chronic infection [10], and similar persistence has been shown in animal models [11,12].Chronic infection can also be reactivated to acute infection with cortisone acetate [13]. Mostly used antibiotics against *C. pneumoniae* are azithromycin (macrolide), doxycycline (tetracycline) and rifampicin (rifamycin). Rifampicin is highly effective against *Chlamydia* in vitro, but *Chlamydia* have the capability to develop a resistance to this compound [14], thus making it a non-ideal treatment and clinical use of rifampicin is often limited to multidrug treatments of tuberculosis and other difficult infections. Doxycycline is widely used against chlamydia but may induce persistence instead of eradication if the cellular concentration remains subinhibitory [15]. These antibiotics prevent the bacterial protein synthesis as they interfere with different parts of the RNA interpretation, and thus they have little effect on metabolically inactive elementary

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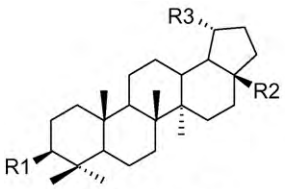
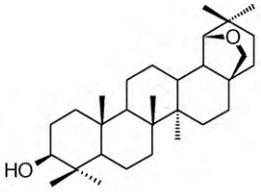
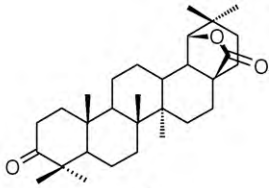
**Table 1**
Structures of betulin (**1**) and derivatives **2–27**, their inhibition of *C. pneumoniae* growth as determined by the TR-FIA method ($n = 6$) and their effect on host cell viability in two different concentrations ($n = 3$).



Basic structure



26



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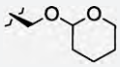
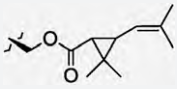
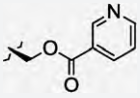
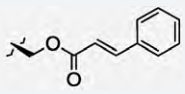
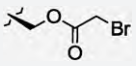
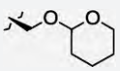
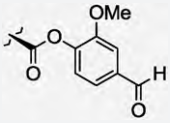
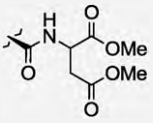
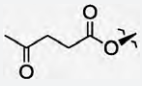
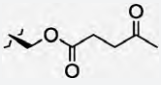
| Compound | R1 | R2 | R3 | Inhibition % at 1 μM (±SEM %) | Host cell viability at 1 μM (%) | Host cell viability at 8 μM (%) |
|---|---|---|---|---|---|---|
| **1** | OH | $CH_2OH$ |  | 53 (4) | 99 | 91 |
| **2** | OH | $CH_2OH$ |  | 44 (7) | 99 | 86 |
| **3** | OH | $CO_2H$ |  | 19 (4) | 85 | 65 |
| **4** | OH |  |  | 51 (5) | 102 | 89 |
| **5** | OH | CH=NOH |  | 100 (2) | 78 | 59 |
| **6** | OH |  |  | 6 (15) | 104 | 113 |
| **7** | OH |  |  | 0 (0) | 103 | 106 |
| **8** | OH |  |  | 0 (0) | 75 | 99 |
| **9** | OH |  |  | 48 (6) | 106 | 96 |
| **10** | OH | $CH_2OAc$ |  | 22 (12) | 106 | 88 |
| **11** | – | $CH_2OH$ |  | 39 (17) | 99 | 82 |
| **12** | – | $CH_2OAc$ |  | −6 (5) | 106 | 93 |
| **13** | OAc | $CH_2OAc$ |  | −10 (15) | 104 | 87 |
| **14** | OAc | $CH_2OH$ |  | −1 (15) | 106 | 77 |
| **15** | OAc |  |  | 3 (9) | 107 | 92 |

**Table 1** (*Continued*)

| Compound | R1 | R2 | R3 | Inhibition % at 1 μM (±SEM %) | Host cell viability at 1 μM (%) | Host cell viability at 8 μM (%) |
|---|---|---|---|---|---|---|
| **16** | OAc | CN |  | 75 (5) | 98 | 93 |
| **17** | O= | $CH_2OAc$ |  | −13 (29) | 107 | 82 |
| **18** | O= | CHO |  | 60 (4) | 72 | 65 |
| **19** | O= | $CO_2H$ |  | 53 (5) | 56 | 32 |
| **20** | O= | $CO_2H$ |  | 100 (3) | 95 | 48 |
| **21** | O= | $CO_2Me$ |  | 74 (5) | 95 | 94 |
| **22** | O= |  |  | 0 (0) | 101 | 104 |
| **23** | O= |  |  | 52 (4) | 103 | 94 |
| **24** | =NOH | CH=NOH |  | 95 (2) | 86 | 58 |
| **25** |  |  |  | 6 (19) | 72 | 61 |
| **26** | OH | [a] | [a] | 48 (5) | 102 | 68 |
| **27** | O= | [a] | [a] | 6 (5) | 103 | 105 |

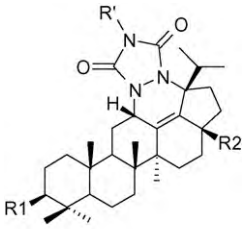
[a] See structures above.

bodies (EB) and only partial effect on the aberrant chlamydial bodies (AB) seen in chronic infections [16–18]. Due to the complex developmental cycle of *Chlamydiae*, including its restricted intracellular habitat, and the lack of host cell free cultivation methods, chlamydiocidal compounds are difficult to discover [19,20]. The intracellular location especially hinders the efficacy of many antichlamydial compounds and is the main reason why *Chlamydia* research still lacks the tools for genetic manipulation. This shortage of genetic tools seriously hinders studies related to chlamydial gene expression and *Chlamydia*-induced changes in host cell gene expression. In addition, *C. pneumoniae* has been cultivated from many tissues in the diseases presented above and the antichlamydial compound must thus penetrate all of these tissues, including blood monocytes, in amounts that successfully eradicate this pathogen in vivo, which is seldom the case [21]. In addition to general antibiotics, some in vitro chlamydiocidal compounds are known, for example a natural flavonoid, rhamnetin, was shown to inhibit chlamydial growth and recurrence completely at a concentration of 50 μM [22].

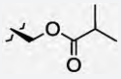
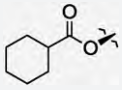
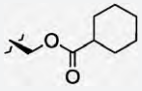
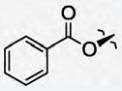
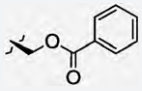
Betulin is a well-known natural lupane triterpene with various pharmacological activities [23]. It is a compound that is widely distributed in nature and can be easily extracted from birch bark, a side product of forest industry [24], and betulin-derived compounds have most widely been studied for their anticancer activity. It has been proposed that betulin and its natural derivative, betulinic acid, inhibit the $sPLA_2$ (secreted phospholipase A2, EC 3.1.1.4) enzyme [25], intracellular isoforms of which have recently been studied as essential components for chlamydia gaining lipids for its replication requirements [26]. The derivatives of betulin have also shown a wide range of anti-viral, anti-protozoal and anti-fungal activities, in addition to their activities against Gram-positive bacteria. However, to our knowledge these compounds exhibit no remarkable activity on Gram-negative bacteria [27–30]. Our previous results [31–33] have shown that betulin derivatives are active against intracellular *Leishmania* parasites and alphaviruses, and thus, they are attractive compounds for antichlamydial screening. The anti-HIV derivative of betulinic acid, Bevirimat (MPC-4326), has been successfully tested in phase II clinical trials, and phase III clinical trials are planned for 2009/2010 [34]. The toxicity, tissue distribution and metabolism of betulin as well as its derivatives have also been under investigation [35–37].

Continuing our research on nature-derived antichlamydial compounds [22,38], a group of betulin derivatives that have previously shown activity against intracellular pathogens [31–33]

**Table 2**
Structures of betulin derivatives **28–32**, their inhibition of *C. pneumoniae* growth as determined by TR-FIA method ($n = 6$) and their effect on host cell viability in two different concentrations ($n = 3$).




**28-32**

| Compound | R1 | R2 | R′ | Inhibition % at 1 μM (±SEM %) | Host cell viability at 1 μM (%) | Host cell viability at 8 μM (%) |
|---|---|---|---|---|---|---|
| **28** | OAc | $CH_2OAc$ | Ph | 1 (6) | 95 | 91 |
| **29** | | | $CH_3$ | 33 (14) | 103 | 85 |
| |  |  | | | | |
| **30** | | | $CH_3$ | 42 (7) | 101 | 92 |
| |  |  | | | | |
| **31** | | | Ph | 11 (5) | 102 | 103 |
| |  |  | | | | |
| **32** | OAc | $CH_2OAc$ | $CH_2CH_3$ | 55 (4) | 104 | 92 |

was selected to be assayed against *C. pneumoniae* in an acute infection model using time-resolved fluorescence (TRF) based screening assay and microscopic techniques. Furthermore, the correlation of the previously described $PLA_2$ inhibition with the antichlamydial activity of betulin derivatives was examined by enzyme inhibition assay on $sPLA_2$.

## 2. Experimental

### 2.1. Compounds

The chemical structures of the betulin-derived triterpenoids are presented in Tables 1 and 2. We have previously reported many of these compounds, the characterization data and the syntheses of these betulin derivatives [31–33], with the exception of two compounds, **26** and **27**, presented below.

Allobetulin **26** was synthesized according to the literature by refluxing a mixture of betulin (**1**) (UPM Kymmene, Lappeenranta, Finland) (2.0 g, 4.5 mmol), *p*-toluenesulfonic acid monohydrate (Sigma–Aldrich, Lyon, France) (4.0 g, 21 mmol) and water (MilliQ) (0.75 ml) in $CHCl_3$ (Fluka, Buchs, Switzerland) (70 ml) for 3 h [39]. The resulting mixture was cooled and washed with saturated aqueous $NaHCO_3$ (Sigma–Aldrich, Schnelldorf, Germany) solution ($3 \times 65$ ml), and water (100 ml) and then dried over anhydrous $Na_2SO_4$ (Merck, Darmstadt, Germany). Removal of the solvent *in vacuo* gave compound **26** (1.86 g, 93%) as an off-white solid. M.p. 263–264 °C. $R_f$ 0.8 (1:3 EtOAc-*n*-hexane); $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 0.76 (s, 3 H), 0.79 (s, 3 H), 0.84 (s, 3 H), 0.91 (s, 3 H), 0.92 (s, 3 H), 0.97 (s, 6 H) 3.19 (dd, $J = 5.4$, 11.1 Hz, 1 H), 3.43 (d, $J = 7.8$ Hz, 1 H), 3.52 (s, 1 H), 3.79 (dd, $J = 1.2$, 7.5 Hz, 1 H). $^{13}C$ NMR (75 MHz, $CDCl_3$): $\delta$ 13.5, 15.4, 15.7, 16.4, 18.2, 20.9, 24.5, 26.2, 26.4, 26.4, 27.4, 27.9, 28.8, 32.7, 33.9, 34.1, 36.2, 36.7, 37.2, 38.8, 38.9, 40.5, 40.6, 41.4, 46.8, 51.0, 55.4, 71.2, 78.8, 87.8. FTIR ($\nu$, $cm^{-1}$): 768, 1041, 1386, 1451, 2868, 2937, 3431. MS (direct, EI+): $m/z$ 424; Anal. ($C_{30}H_{50}O_2$) C, H: calculated, 81.39, 11.38; found, 81.29, 11.64.

28-Oxyallobetulone **27** was similarly synthesized by refluxing a mixture of betulonic acid 19 (1.0 g, 2.2 mmol), *p*-toluenesulfonic acid monohydrate (2.0 g, 10.5 mmol) and water (0.30 ml) in $CHCl_3$ (50 ml) for 3 h. The resulting mixture was cooled and washed with saturated aqueous $NaHCO_3$ solution ($3 \times 50$ ml), and water (50 ml) and then dried over anhydrous $Na_2SO_4$. Removal of the solvent *in vacuo* gave the crude product, which was purified by column chromatography on silica gel (1% MeOH in $CH_2Cl_2$) (Merck, Darmstadt, Germany) to yield compound **27** (191 mg, 19%) as a white solid. Characterization data were identical to those reported in the literature [39,40]. M.p. 296–298 °C. $R_f$ 0.7 (1%, MeOH:99% $CH_2Cl_2$); $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 0.88 (s, 3 H), 0.94 (s, 3 H), 0.94 (s, 3 H), 0.95 (s, 3 H), 1.02 (s, 6 H), 1.07 (s, 3 H) 2.45 (m, 2 H), 3.94 (s, 1 H). $^{13}C$ NMR (75 MHz, $CDCl_3$): $\delta$ 13.5, 15.3, 16.3, 19.5, 20.9, 21.4, 23.9, 25.5, 26.5, 26.6, 27.8, 28.7, 31.9, 32.3, 33.0, 33.5, 34.0, 36.9, 39.8, 40.5, 46.6, 47.3, 50.5, 55.0, 76.4, 85.9, 179.8, 217.9. FTIR ($\nu$, $cm^{-1}$): 968, 1386, 1449, 1461, 1707, 1763, 2867, 2945. MS (direct, EI+): $m/z$ 454; Anal. ($C_{30}H_{46}O_3$) C, H: calculated, 79.25, 10.20; found, 78.98, 10.37.

### 2.2. Cell lines

HL cells [41] were grown in RN-medium consisting of 500 ml of RPMI 1640 medium (BioWhittaker, Lonza, Basel, Switzerland), supplemented with 7% fetal bovine serum (FBS) (BioWhittaker, Lonza, Basel, Switzerland), 2 mM L-glutamine (BioWhittaker, Lonza, Basel, Switzerland) and 20 μg gentamicin (Fluka, Buchs, Switzerland) per ml.

*Chlamydia pneumoniae* strain CWL-029 (kindly provided by M. Leinonen, National Institute for Health and Welfare, Finland) was grown and purified as described in Alvesalo et al. [22].

### 2.3. Primary screen

The time-resolved fluorometric immunoassay (TR-FIA) method was performed according to Tammela et al. [42] with small modifications. Briefly: The HL cell-line (human lung) was used, and $6 \times 10^4$ cells were distributed in RN-medium in each well of a 96-well plate (Wallac Isoplate 1450-516, PerkinElmer Finland, Turku, Finland). After 24 h, cells were infected with 0.2 MOI (multiplicity of infection) of strain CWL-029 in the presence of 0.5% cycloheximide (Sigma–Aldrich, St. Louis, USA) and centrifuged 550 × *g* for one hour (Heraeus Multifuge 3 s, Thermo Fischer Scientific, Vantaa, Finland) and kept at 37 °C, 5% $CO_2$ and 95% humidity for a second hour. After this, the infective suspension was removed, and the betulin derivatives and control compound rifampicin (83907, Sigma–Aldrich, St. Louis, USA) were added to the wells in six replicates for each compound. Then, the plates were incubated at 37 °C, 5% $CO_2$ and 95% humidity for 70 h. Incubation was terminated by fixing the cells with methanol (Mallinckrodt Baker, Phillipsburg, USA).

The following day, the plates were labeled with assay buffer (PerkinElmer Finland, Turku, Finland) that was supplemented with europium-conjugated chlamydial antibody (100 ng/ml) (Perkin Elmer Life and Analytical Sciences – Wallac, Turku, Finland). Plates were incubated for 30 min at 37 °C and washed six times with wash solution (PerkinElmer Finland, Turku, Finland) using a platewasher (BW 50, Biohit, Helsinki, Finland). Enhancement solution was added to the wells, and the plates were shaken at low intensity for 5 min on a plateshaker (Delfia Plateshaker, PerkinElmer Finland, Turku, Finland). Signals were measured using a multilabel plate reader (Victor, PerkinElmer Finland, Turku, Finland).

### 2.4. Autofluorescence and label binding

All of the compounds were tested for autofluorescence following the TR-FIA procedure described above, but no *C. pneumoniae* or antibodies were added. The same procedure was repeated without the addition of the *C. pneumoniae*, but while still adding the antibodies to investigate whether the compounds reacted with the antibodies themselves.

### 2.5. Dose–response experiments

The concentration effect of the most active compounds was studied with traditional immunofluorescence labeling (IF). Overall, $4 \times 10^5$ HL-cells/ml were distributed onto a 24-well plate (Cellstar, Greiner Bio-One, Frickenhausen, Germany) with Ø 13 mm # 1.5 coverslips (Menzel-Gläser, Braunschweig, Germany) in 1 ml of RN-medium and left to attach for 24 h. The medium was changed to 200 μl of RN-medium, supplemented with 0.5% of cycloheximide and 0.2 MOI of *C. pneumoniae,* and plates were centrifuged 550 × *g* for one hour and kept a second hour at 37 °C, 5% $CO_2$ and 95% humidity. After this, the infective suspension was removed and betulin derivatives and the control compound rifampicin were added to the wells, three replicates of each. The wells were incubated at 37 °C, 5% $CO_2$ and 95% humidity for 70 h, and incubation was terminated by fixing the cells with methanol. The coverslips were removed and labeled with "Pathfinder® Chlamydia Culture Confirmation System" (Bio-Rad, Hercules, USA), according to manufacturer's instructions. The excess label was removed by dipping the coverslips twice into PBS and once into deionized water (MilliQ). The coverslips were mounted onto glass slides and inspected by fluorescence microscopy with a 200× magnification. The amount of the formed inclusions was counted from four eye fields of each coverslip, totaling 12 observations per compound.

### 2.6. Activity verification against a clinical isolate CV-6

The minimal inhibitory concentration (MIC) and the minimal chlamydiocidal concentration (MCC) of compound **24** were determined in a standardized assay, as previously described by Gieffers et al. [43]. In our procedure, we made one modification where centrifugation was changed from the original procedure to 1700 × *g*.

### 2.7. Evaluation of physicochemical properties

The evaluation of physicochemical properties was carried out based on the simple rules suggested by Lipinski et al. [44] and Norinder and Haeberlein [45].

### 2.8. Effect on host cell viability

All of the plates in this study were inspected under a microscope for abnormalities. The effect on host cell viability was determined by the commercial "CellTiter-Glo® Luminescent Cell Viability Assay" (Promega, Madison, USA) on the 96-well plates described earlier. The processing of plates and compounds was similar to the TR-FIA experiment (Section 2.3), but no infection was induced. Measuring of the adenosine triphosphate (ATP) content of cells was done 70 h after adding the compounds to the plate. To take this measurement, 200 μl of old growth medium that contained the compounds was removed and 100 μl of fresh medium was added. Then, 100 μl of CellTiter-Glo® Reagent was added, according to the manufacturer's instructions. Luminescence was determined with a Varioskan Flash multimode reader (Thermo Fischer Scientific, Vantaa, Finland).

### 2.9. Effect on guanosine biosynthesis

The infection protocol was followed as described earlier in Section 2.3, but after the infection, the compounds were added in RN-medium supplemented with 0.5% of cycloheximide and 25 μg/ml of guanosine (Sigma–Aldrich, St. Louis, USA).

### 2.10. Effect on phospholipase $A_2$ enzyme

The effect of betulin and its derivatives on $PLA_2$ enzymes was assayed according to the Sigma–Aldrich protocol for EC 3.1.1.4. Betulin derivatives were dissolved in ethanol (96%) at 0.5% (w/v) concentration, as described in Bernard et al. 2001 [25]. From this solution, 400 μl were then added to the reaction mixture just prior to the addition of the $PLA_2$ enzyme. The 10.6 ml of reaction mixture at the endpoint contained 2% of lecithin (Sigma–Aldrich, St. Louis, USA), 141 mM sodium chloride (Merck, Darmstadt, Germany), 4.7 mM calcium chloride (Merck, Darmstadt, Germany), 0.5 units of class IB phospholipase $A_2$ (P-8913, Sigma–Aldrich, St. Louis, USA) and approximately 400 μM of the compound of interest, depending on the molecular weight of compound. Sodium hydroxide (NaOH), purchased from Sigma–Aldrich (St. Louis, USA), was used to restore the solution to pH 8.

## 3. Results

### 3.1. Primary screen

A total of 32 betulin derivatives were screened using the TR-FIA method to examine their ability to inhibit the growth of *C.*

*pneumoniae*. The average assay quality parameter values from these screens were $Z'$ 0.34 [46], a signal to background ratio of 23.1 and a signal to noise ratio of 5.5. The compounds were also analyzed for autofluorescence and label binding properties to exclude false negative inhibition results from the TR-FIA. None of the compounds showed autofluorescence, nor did they change the binding of the europium-labeled antibody by themselves (data not shown).

Three derivatives showed a high (>80% growth inhibition) antichlamydial activity at a concentration of 1 μM, and 15 compounds were moderately active (20–80% growth inhibition) (Tables 1 and 2). The remaining derivatives were classified as non-active (<20% growth inhibition). Betulin (**1**) showed a 53% inhibition of *C. pneumoniae* growth at a concentration of 1 μM and was well tolerated by the host cells in the viability assay (Table 1). The other natural compound, betulinic acid (**3**), inhibited bacterium by 19% but had a much stronger effect on host cell viability. The esterification of position R2 at the C28 carbon (general structure shown in Table 1) in betulin was generally unsuccessful in terms of aiding the antichlamydial activity, with the exception of compound **9**, which resulted in a 47% inhibition. Conversion of betulin into betulin-28-oxime (**5**) produced a complete inhibition of the pathogen, and a similar effect was achieved by the 3,28-dioxime compound (**24**).

In addition to modifications at position R2, which proved significant for the modulation of the antichlamydial activity, we also studied the role of the R1 (C3 carbon) position (Table 1). In general, acetylation of R1 lowered the activity of the compound compared to betulin, with the exception of 3-*O*-acetylbetulinyl-28-nitrile (**16**), which inhibited chlamydial growth by 75%. In contrast to most R1-acetylated derivatives, the conversion of R1 into a ketone carbonyl improved or retained the antichlamydial activity of betulin. Betulonic acid derivatives **21** and **23** inhibited *C. pneumoniae* by 75% and 52%, and, interestingly, had no indications of host cell toxicity, unlike betulonic acid (**19**) itself.

In addition to our studies on the R1 and R2 positions, the role of R3 position (the 20,29-double bond) on antichlamydial activity was studied in betulin (**1**) and betulonic acid (**19**). This activity was compared to compounds **2** and **20**, which had a hydrogenated double bond 20,29 (Table 1). Hydrogenation of the betulin double bond to 20,29-dihydrobetulin (**2**) resulted in unchanged activity, but reduction of betulonic acid to 20,29-dihydrobetulonic acid (**20**) led to 100% inhibition at a concentration of 1 μM.

As these results indicated that the antichlamydial activity tolerates modification in lupane skeleton, allobetulin (**26**) and 28-oxyallobetulone (**27**) were synthesized to increase structural variation in the assayed compounds (Table 1). Allobetulin was equal to betulin in antichlamydial activity (48%), but compound **27** was inactive.

In addition, compounds recently published by us [31] having a 1,2,4-triazole moiety fused to the hydrocarbon skeleton of betulin were also assayed against *C. pneumoniae* (Table 2). All heterocyclic derivatives that showed moderate activity shared a small (methyl or ethyl) substituent at position R′ (29, 30 and 32), whereas those having phenyl substituents at the R′ position (28 and 31) had no inhibitory effects.

The five most active compounds were compounds **5**, **16**, **20**, **21** and **24**, which showed inhibition percentages of 100%, 75%, 100%, 74% and 95%, respectively. These five compounds were selected for dose–response experiments described in the following section.

### 3.2. Dose–response experiments

Based on activities from the primary screening and structural analyses, we chose five compounds for further examination by traditional microscope-based IF assays. Only compound **24** was able to completely eradicate *C. pneumoniae* strain CWL-029 from the HL-cells, having a MIC of 1 μM and an 80% inhibition at a concentration of 500 nM, as determined by the IF method (Fig. 1). The other selected compounds showed detectable *C. pneumoniae* inclusions even in the highest concentrations used, although the sizes of the inclusions were clearly reduced compared to the infected controls.

To evaluate the differences between the TR-FIA and IF methods, the dose response was determined for compound **24** using TR-FIA (Fig. 2). The correlation of these two methods was good and the differences in the dose response curves were well explained by the methodological differences and the inclusion size distribution observed in IF microscopy. The antichlamydial effect of compound **24** continued to be in the nanomolar range, as 50% inhibition was achieved with a concentration of 290 nM as measured by IF and 125 nM as measured by TR-FIA, approaching the MIC of one of the most potent antichlamydial compounds known, rifampicin. Rifampicin was used as a control in all of the experiments and gave a MIC of 12 nM (0.010 μg/ml), as determined by TR-FIA, and an average inhibition of 97% in the IF experiments at the same concentration.

### 3.3. Activity verification against a clinical isolate

Compound **24** was assayed against the clinical isolate CV-6 and showed a MIC of 2.2 μM (1 μg/ml) and MCC of 8.8 μM (4 μg/ml), and thus can be classified as chlamydiocidal.

### 3.4. Evaluation of the physicochemical properties

All of the most active compounds were evaluated for their potential drug-like properties according to the Lipinski rule of five (Table 3) [44]. Also, the CNS penetration potential was evaluated according to Norinder and Haeberlein 2002 [45], which requires a nitrogen/oxygen atom total less than five and a *log P*−(N + O) that is positive (Table 3).

### 3.5. Effect on host cell viability

All of the wells in the plates used in this study were visually inspected under a microscope. The only case where the cell monolayer was found damaged was when the highest concentration (8 μM) of compounds **19** or **20** was used. In all other cases, the cell monolayer was visually comparable to infected and non-infected controls.

The effect of all compounds on host cell viability was measured after 70 h exposure to the compounds at 1 and 8 μM concentrations. Overall, 22 compounds did not have any remarkable effect on the host cell viability (threshold of >20% viability loss set prior

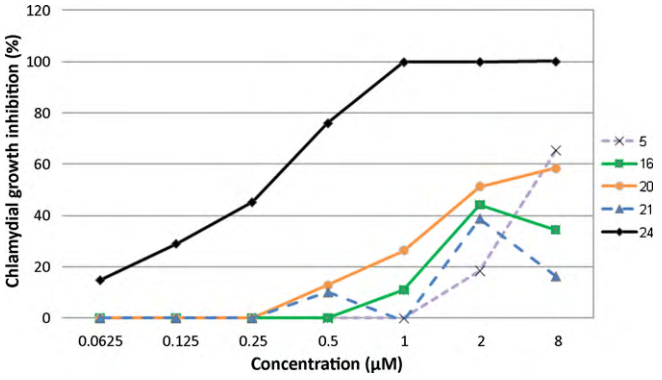



**Fig. 1.** The antichlamydial activity of the five selected compounds at concentrations ranging from 0.0625 to 8 μM, as measured by the IF method. Compound **24** induced a complete eradication of *C. pneumoniae* at concentrations of 1–8 μM. Data represent the mean value from 12 observations.

**Table 3**
The five most efficient antichlamydial compounds had potential drug-like properties according to the Lipinski rule of five with the exception of log *P*. As the compounds follow guidelines shown below, they are expected to cross the blood–brain barrier.

| Compound | H-bond donor <5 | H-bond acceptor <10 | [a]Freely rotatable bonds <5 | Molecular weight <500 | [a]log *P* <5 (25°C) | (N+O) <5 | log *P*–(N+O) >0 |
|---|---|---|---|---|---|---|---|
| **5** | 2 | 3 | 4 | 455.7 | 8.5 ± 0.5 | 3 | 5.5 |
| **16** | 0 | 3 | 3 | 479.7 | 9.7 ± 0.4 | 2 | 7.7 |
| **20** | 1 | 3 | 2 | 456.7 | 8.6 ± 0.4 | 3 | 5.6 |
| **21** | 0 | 3 | 3 | 468.7 | 8.8 ± 0.5 | 3 | 5.8 |
| **24** | 2 | 4 | 4 | 468.7 | 8.9 ± 0.5 | 4 | 4.9 |

[a] Assessed by Advanced Chemistry Development (ACD/Labs) Software V9.04 (Toronto, Canada).

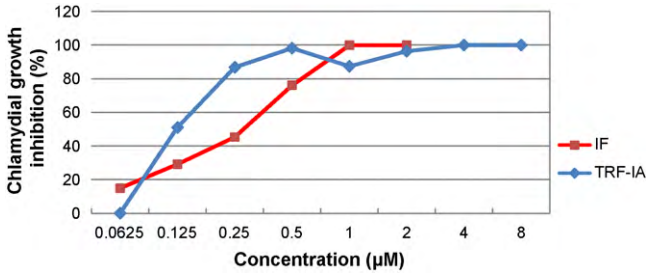



**Fig. 2.** The differences between IF and TR-FIA methods in assaying the antichlamydial effect of betulin derivatives were evaluated using compound **24**. The IF method was used to measure the number of inclusions, and TR-FIA was used to measure the overall chlamydial mass. Data represent the mean from twelve observations for IF and six for TR-FIA.

to the experiment) at either of the examined concentrations (Tables 1 and 2). At a concentration of 1 μM, only five compounds had an effect on the intracellular ATP concentrations of host cells and thus on the host cell viability. As the compounds were well tolerated by host cells, the host cell death was not an explanatory factor for the antichlamydial activity of these compounds.

For the five most active compounds, the dose-response effect on host cell viability was determined. Only compound **20** induced a loss of viability more than 20% when assayed at a 1 μM concentration (Fig. 3), which was the concentration used in the primary screen, whereas compounds **16** and **21** showed no effects on host cell viability at any concentrations and compounds **5** and **24** had no significant effect on host cell viability at concentrations ≤1 μM.

### 3.6. Effect on guanosine biosynthesis

The potential effect of betulin derivates on guanosine depletion was assayed by adding excess amounts of guanosine to growth medium concomitantly with the selected five betulin derivatives. The addition of 25 μg/ml of guanosine did not reverse the inhibitory effect of the five most active compounds (data not shown).

### 3.7. Effect on phospholipase $A_2$ enzyme activity

The effect of the five most active betulin derivates and five moderately or non-active compounds on the $sPLA_2$ class IB enzyme activity was measured in vitro. Compounds (**1**, **2**, **3**, **13** and **31**) were selected to study structure–activity relationships for the possible inhibition of the $PLA_2$ enzyme. Doubling the amount of ethanol did not affect the amount of NaOH consumed.

The concentration of betulin derivatives in the assay was 0.5% (w/v) (approximately 400 μM depending on the molecular weight). The inhibition of the $PLA_2$ enzyme was calculated as shown in the formula below and results are presented in Fig. 4.

$$\%\ \text{Inhibition of PLA}_2 = \frac{(A-C)-(B-C)}{A-C} \times 100$$

In this equation, *A* is the [NaOH] added to the $PLA_2$ control without inhibitor, *B* is the [NaOH] added to sample with the inhibiting compound and *C* is the [NaOH] added to the blank.

Our results showed that betulin (**1**) inhibited $PLA_2$ by 62.5% and that betulinic acid (**3**) inhibited $PLA_2$ by 25%. The oxime moiety at the R2 position gave the highest anti-$PLA_2$ effect, and these two

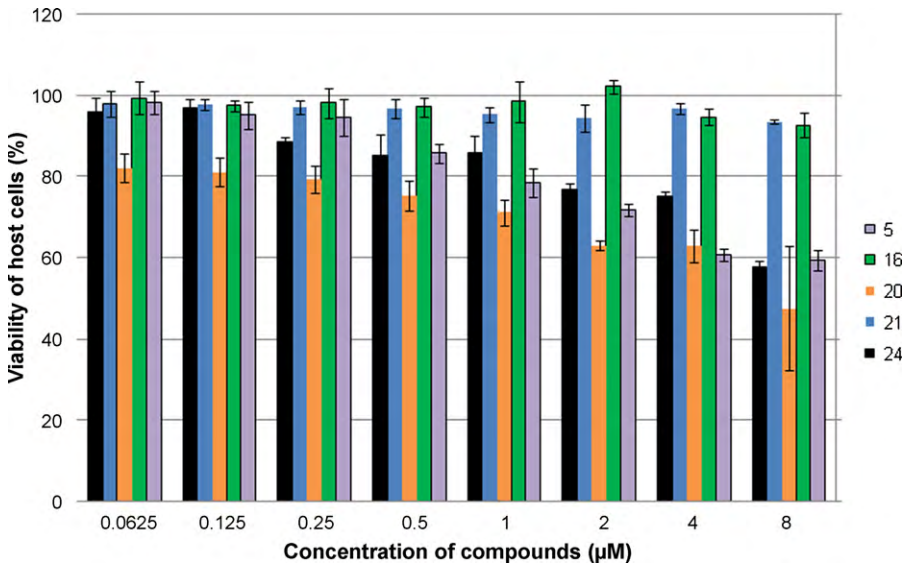



**Fig. 3.** Viability of HL cells after 70 h exposure to the five selected betulin derivatives at the concentrations used in this study. The viability has been adjusted using DMSO as a control (100%). Data represent the mean and ±SD of three experimental repetitions.

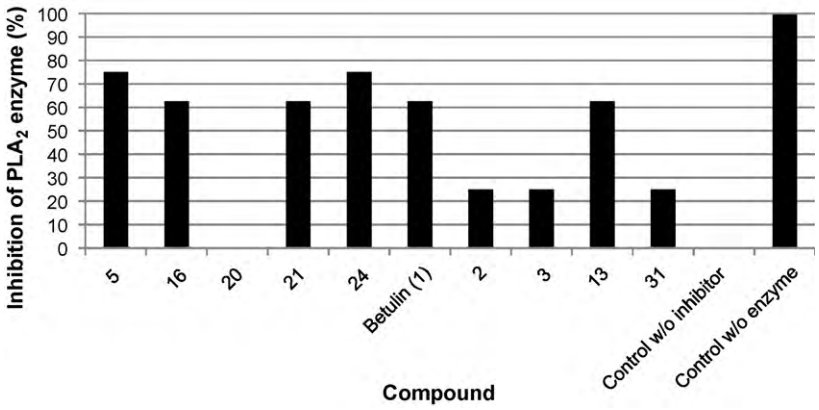



**Fig. 4.** The activity of betulin and its derivatives against the PLA2 enzyme was determined in vitro. The most active betulin derivatives were also good inhibitors of PLA2, with the exception of compound **20**, which did not show any effect on the enzyme. Inhibition was measured as the amount of NaOH required to re-establish pH 8 after the addition of compounds and enzyme compared to control experiments without the inhibitor and enzyme.

compounds (**5** and **24**) were also the most potent antichlamydial compounds. The hydrogenated double bond (R3) of the original betulin and betulonic acid in 20,29-dihydrobetulin (**2**) and 20,29-dihydrobetulonic acid (**20**), respectively, clearly diminished the anti-$PLA_2$ activity, as did the more dramatic modification of the 20,29 position in compound **31**. Compound **13**, which was completely inactive against *C. pneumoniae*, inhibited $PLA_2$ by 62.5%.

## 4. Discussion

Antichlamydial lead discovery is challenging, as the biology of *Chlamydia* is only partly understood. Genetic modifications have not been successful mostly because of the intracellular location of this bacterium, and this hinders the efforts to design new antichlamydial agents. Despite this problem, many aspects of the biology of *Chlamydia* infections are known from previous research and available genomic data. *Chlamydia* are known to be dependent on host cell metabolites, such as amino acids, purines and lipids, due to its intracellular location, similar to most obligate intracellular pathogens [20,47–50]. This intracellular habitat also requires other mechanisms for surviving, such as controlling host cell apoptosis and the evasion of host immuno defenses. *C. pneumoniae* affects the cytoskeletal structures of host cells [51] and is known to possess a Type III Secretion System (TTSS) [52], which mediates the secretion of effector proteins from the inclusion to the cytosol. All of the factors above provide potential targets for antichlamydial drug development, but due to the complexity of parallel *Chlamydia* survival mechanisms and the lack of tools for genetic modification [53], it is difficult to design antichlamydial compound and it is even more difficult to evaluate the mechanisms that are related to the inhibition of chlamydial growth.

In the current study, a set of 32 betulin derivates with known biological activities against intracellular pathogens were assayed at concentration of 1 μM for activity against *C. pneumoniae* using the TR-FIA method. The structure-activity relationships in this group were determined. A high number, 18 out of 33, of the compounds showed activity from moderate to high, 20–100% inhibition. The assay quality parameters showed that the TR-FIA assay was robust and could be used as a primary screen to differentiate the active compounds from non-active compounds in this complex cell assay. Since the mechanisms of entry are likely to be highly dependent on the host cells and the chlamydial strain used [53], we decided to put our efforts towards inhibiting chlamydial growth in established acute infection and to prove the capability of the selected betulin derivatives to inhibit *C. pneumoniae* growth inside host cells.

Betulin-derived compounds have been the subject of several bioactivity studies, including various intracellular pathogens like *Plasmodium* and *Leishmania* species and DNA and RNA viruses [31–34,54]. As betulin derivatives are also known to affect mammalian cell processes, such as neoplastic growth and immunological responses, one could hypothesize a general mechanism of action underlying many of these observed activities. One such explanation, typical to antiviral and immunosuppressive effects, is the depletion of cellular guanosine pools. Most of the derivatives evaluated in this study were also tested in our recent study on antiviral activity [33]. These derivatives showed an inhibition of viral replication that was independent of guanosine supply, as was also the case with the inhibition of *C. pneumoniae* found in the present study. This would indicate that the antimicrobial activity of betulin derivatives on intracellular microorganisms does not rely on this feature. Also, differences in the structure–activity relationships detectable for alphaviruses and *C. pneumoniae* indicated that a more pathogen-specific mode of action could exist. In the recent study targeting alphaviruses, most active derivatives shared free or acetylated hydroxyl groups at the R1 position and were esterified at the R2 position, whereas the R1-dihydro or R1-oxime derivatives were inactive [33]. In the current study on *C. pneumoniae,* almost the opposite was true: R1-oximes were among the most potent inhibitors and R1-dihydro derivatives also inhibited the pathogen, whereas most derivatives with R2 ester substituents lacked activity. Furthermore, the most potent anti-alphaviral derivative, 3,28-di-*O*-acetylbetulin (**13**), completely lacked activity against *C. pneumoniae,* and compound **24** was the most potent antichlamydial derivative and the only compound able to completely eradicate *C. pneumoniae* from cell cultures in both methods used.

The observed antichlamydial activity of allobetulin (**26**) and 20,29-dihydrobetulin (**2**) indicated that some related terpenoids might be successful as inhibitors of chlamydial growth, thus offering new potential chemical entities for antichlamydial drug research.

To scale the efficacy of betulin derivatives, the MIC of rifampicin and doxycycline, two inhibitors of RNA synthesis, were evaluated with TR-FIA and gave MIC-values of 12 nM (0.010 μg/ml) and 70 nM (0.031 μg/ml) respectively. Rifampicin was used at 12 nM concentrations as a reference antibiotic in IF experiments and yielded an average inhibition of 97%. Comparing these results to inhibition by betulin derivatives showed that betulin derivatives have the required efficacy for an antichlamydial lead compound.

Based on our TR-FIA results, the five most active and interesting compounds, **5**, **16**, **20**, **21** and **24**, were chosen for further analysis and their dose-response effect against *C. pneumoniae* was determined by IF. Compound **24** had a MIC of 1 μM against *C. pneumoniae* isolate CWL-029 and was the only compound that eradicated *C. pneumoniae* completely from the cell culture at the assayed concentrations. As the inhibitory effect differed between the two methods used in this study, TR-FIA and IF, the dependency of the methods was evaluated. The comparison of the *C. pneumoniae* growth inhibition of **24** by these two methods revealed a clear dependency between the results and the measured unit. The small size of the inclusions seen in IF experiments with subinhibitory concentrations of compounds correlated with the difference of the results gained from these two methods. The primary screening method, TR-FIA, measured the total chlamydial mass as the intensity of fluorescence and thus gave a good estimate of the total amount of *C. pneumoniae* developed. On the other hand, the IF method was based on fluorescence microscopy and gave the number of inclusions formed as the result, but omitted the inclusion size in the determination of inhibition.

The MIC (μg/ml) value of an antibiotic for a certain pathogen and the pharmacological properties of the antibiotic give indications for its clinical use. The differences in susceptibilities obtained by different laboratories depend partly on the strains of the microorganisms that are used. To support the antichlamydial activity found by TR-FIA and IF methods, the MIC and the MCC of compound **24** were determined in a standardized assay against a clinical isolate. As we wanted to use a strain known to induce acute as well as chronic infection, we chose the *C. pneumoniae* coronary artery isolate CV-6, recovered from a chronically infected atheromatous lesion of a 68-year-old male during routine restenosis surgery [55]. Compound **24** proved to be effective and bactericidal against the *C. pneumoniae* isolate CV-6, showing a MIC of 2.2 μM (1 μg/ml) and a MCC of 8.8 μM (4 μg/ml), which further confirmed the activity seen in previous experiments with this third assay against a clinical isolate.

Safety is another concern when pondering the suitability of a compound for drug development. The host cell viability was measured as the amount of ATP within the cells, as this is a highly sensitive method to detect cytotoxic effects [56]. The effects of all assayed-betulin derivates on the viability of host cells were determined to evaluate the potentiality of compounds as drug leads. The compounds showed clear structure dependent effects on host cells viability, and the carboxyl group at the R2 position (3, 19 and 20) caused the strongest cytotoxic effect. The effect on host cell viability was further assayed for the selected five compounds in dose-response experiments. Two of the compounds were excellently tolerated by host cells, even with the highest concentration used in the assays. When used in 1 μM concentration, only compound **20** was considered to affect host cell viability. Overall, compounds were well and consistently tolerated by HL-cells, thus implicating a good safety profile for these lead compounds. As the compounds were well tolerated by host cells, the host cell death was not an explanatory factor for the antichlamydial activity of these compounds.

Activity is only one parameter of drug development. Equally interesting is the evaluation of the tissue distribution and other ADME characteristics. Betulinic acid has been shown to penetrate to the tissues important for *C. pneumoniae* [36]. After 8 h of 500 mg/kg i.p. administration of betulinic acid to CD-1 mice, 148 μg/g was found in the lung, 72 μg/g in the heart and 183 μg/g in the brain tissues. In vitro results showed that compound **24** had a MIC range of 1–2.2 μM (0.5–1 μg/ml) for the *C. pneumoniae* strains tested, which indicates that it might be distributed in the tissues at chlamydiocidal amounts. Evaluation of the Lipinski parameters suggested that the bioavailability of the selected betulin derivatives is good. These compounds possessed less than 5 hydrogen donors and less than 10 hydrogen acceptors, and also had fewer than 5 rotatable bonds. High log *P* values might cause solubility problems, which can be solved by small modifications of the molecule that do not affect the activity, or by the prodrug approach. On the other hand, a positive log *P* value is beneficial for entering the brain, as the blood–brain barrier often limits entry of hydrophilic compounds [45]. Also, the molecular weights (455.7–479.7 g/mol) of these betulin derivatives agreed with the approximation of a good drug molecule. This is of further importance as the *C. trachomatis* inclusion membrane has been shown to prevent passive diffusion of molecules with weights above 520 Da [19]. If a molecule targets any functions inside the inclusion, it must be able to diffuse through the inclusion membrane or be taken actively in by one of the many transporters in this membrane, as reviewed by Saka and Valdivia 2010 [20]. Altogether, this kind of physicochemical profile is very promising for nature-derived compounds, as nature-derived drugs do not generally fit these limits.

The lack of known genes that code for chlamydial $PLA_2$ enzymes suggests that host-derived phospholipids and host $PLA_2$ enzymes are essential for this pathogen and thus a suitable drug target. HL-cells are known to express multiple $PLA_2$ genes [57] and host phospholipids are known to be modified by removal of the *sn*-2 positioned fatty acid by host $cPLA_2$ (cytosolic phospholipase A2) before they are imported into the inclusion [26,49]. *Chlamydia* seems to stimulate the production of host $cPLA_2$ and blockage of this pathway results in inhibition of the chlamydial growth [26], and it has also been suggested that *Chlamydia*-infected cells may increase $cPLA_2$ levels as a response of the innate immune system [20]. The $PLA_2$ enzyme family is also linked to many aspects of inflammation in atherosclerotic and neurological disorders, as well as to *C. pneumoniae*-induced chronic inflammation [58–61]. However, this work showed that class IB $sPLA_2$ inhibition did not correlate with the antichlamydial activity of these betulin derivatives, nor was the anti-$sPLA_2$ activity responsible for the decrease of host cell viability. Some of the antichlamydial derivatives did inhibit $sPLA_2$-IB, but inhibitory effects on the enzyme activity were also seen with some of the derivatives lacking antichlamydial activity. The anti-$sPLA_2$ effect caused by the betulin derivatives exerted a strong structural dependency on the nature of the substituents and the moieties at the 20,29 positions of the lupane skeleton (R3 position). As these structural differences result in changes also in physicochemical parameters and topological characteristics of the compounds, underlying factors like different permeability profiles and affinity to efflux systems may contribute to the lack of correlation between the effects on in vivo microbial growth and in vitro enzyme activity.

Phospholipase $A_2$ enzymes represent a large superfamily of structurally unrelated proteins, and both nonselective and selective inhibitors of these enzymes have been identified. The effects of betulin derivatives on different $PLA_2$s deserves further elaboration, as no potent cell permeable $sPLA_2$ inhibitors are yet known [62]. Information of the fitting of the betulin derivatives to different $PLA_2$s was provided by preliminary molecular docking studies with the Tripos Sybyl 8.0 program and with five crystal structures of the $PLA_2$ enzymes (1MKV, 3HSW, 3H1X, 2FNX and 1TGM), belonging to $PLA_2$ groups IB, IB, IIa, VIII and II, respectively. These preliminary results supported differences in $PLA_2$ binding between the betulin derivatives (data not shown).

The effectiveness of betulin derivatives against *C. pneumoniae* in vitro, the clear structure-activity relationships and the pharmacological profile suggested that betulin derivatives make good candidates for further drug development. Even with new potent antichlamydials, a multi-drug approach, such as targeting to simultaneously block multiple metabolic pathways, could be a

suitable approach to treat *C. pneumoniae* infection and might eradicate the pathogen more effectively than a single drug treatment, as has been the case with *Helicobacter pylori* and with many other pathogens. Our results demonstrate that betulin derivatives, and especially compound **24**, are highly potential candidates for the treatment of *C. pneumoniae* infection in vitro, with suitable lead compound properties, and thus require further studies to clarify their influences on chronic *C. pneumoniae* infections.

## Acknowledgements

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