Exploring valrubicin’s effect on *Propionibacterium acnes*-induced skin inflammation *in vitro* and *in vivo*

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Abstract

Acne is a common skin disease involving colonization with *Propionibacterium acnes* (*P. acnes*), hyperproliferation of the follicular epithelium and inflammatory events. Valrubicin is a second-generation anthracycline, non-toxic upon contact, and available in a topical formulation. Valrubicin’s predecessor doxorubicin possesses antibacterial effects and previously we demonstrated that valrubicin inhibits keratinocyte proliferation and skin inflammation suggesting beneficial topical treatment of acne with valrubicin. This study aims to investigate valrubicin’s possible use in acne treatment by testing valrubicin’s antibacterial effects against *P. acnes* and *P. acnes*-induced skin inflammation *in vitro* and *in vivo*. Valrubicin was demonstrated not to possess antibacterial effects against *P. acnes*. Additionally, valrubicin was demonstrated not to reduce mRNA and protein expression levels of the inflammatory markers interleukin (IL)-1β, IL-8, and tumor necrosis factor (TNF)-α *in vitro* in human keratinocytes co-cultured with *P. acnes*. Moreover, *in vivo*, valrubicin applied both topically and intra-dermally, was not able to reduce signs of inflammation in mouse ears intra-dermally injected with *P. acnes*. Taken together, this study does not support beneficial antibacterial and anti-inflammatory effects of topical valrubicin treatment of acne.

Introduction

Acne vulgaris is one of the most common skin diseases worldwide affecting more than 85% of all adolescents.1 The disease presents a significant clinical problem with severe social and psychological implications. The pathogenesis of acne is multifactorial and usually implicated follicular colonization with the gram-positive anaerobe *Propionibacterium acnes* (*P. acnes*) and inflammation upon manifestation.1,2 *P. acnes* has previously been shown to stimulate secretion of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-8 and tumor necrosis factor (TNF)-α.3 Current acne treatments present side effects such as skin irritation, bacterial resistance, teratogen effects, and chronically rendering acne as a disease with persistent need for new and improved treatment modalities.1

Valrubicin (N-trifluoroacetyladriamycin-14-valerate) is a second-generation anthracycline currently FDA-approved for intra-vesical treatment of bacillus Calmette-Guérin-refractory bladder cancer.5 Valrubicin is derived from doxorubicin, which exhibits both antibiotic and antineoplastic activities.5,6 Valrubicin excels by lacking contact and cardiac toxicity.6 Valrubicin’s improved safety profile and excellent tissue penetration7 has led to the development of a cream formulation facilitating topical application and possible treatment of hyperproliferative skin diseases as demonstrated by alleviation of psoriasis and prevention of skin tumor development in mouse models.7,8 An effect ascribed to valrubicin’s ability to inhibit keratinocyte proliferation and skin inflammation.7,9

In this study, we aim to investigate whether topical valrubicin treatment may play a future role in acne treatment, thus valrubicin’s antibacterial effect against *P. acnes* is tested together with valrubicin’s anti-inflammatory effect on *P. acnes*-induced inflammation *in vitro* and *in vivo*. Valrubicin did not demonstrate antibacterial effects nor did it reduce expression levels of IL-1β, IL-8, and TNF-α *in vitro*, or reduce signs of inflammation *in vivo*. In conclusion, this study does not support beneficial antibacterial and anti-inflammatory effects of topical valrubicin treatment of *P. acnes*-associated acne. The submitted manuscript includes primary normal human epidermal keratinocytes (NHKs), isolated from healthy donors undergoing skin reductive surgery. All donors have signed a donor consent letter and the project is approved by the regional ethical committee (Den videnskabs- absetiske Komité for Århus Amt) with journal number 20030125. The submitted manuscript also includes animal experimentation which is approved by the Danish Animal Experiments Inspectorate (Dyreforsøgstilsynet) with journal number 2011/561-1958.

Materials and Methods

*P. acnes bacteria*

Three different *P. acnes* strains: 1.4.L1, 12.1.L1 and 30.2.L1, were included in this study. 1.4.L1 and 12.1.L1 are sequence type (ST)18 and strongly associated with moderate to severe acne while 30.2.L1 is ST27 and associated with healthy skin. Strains were kindly provided by Professor Mogens Kilian, Institute of Biomedicine, Aarhus University, Denmark, and isolated from acne patients as previously described.10,11 *P. acnes* were inoculated on 5% blood agar or reinforced clostridium agar and incubated under anaerobic conditions at 37°C. Further growth was accomplished by inoculation on dialysis membranes (3500 Dalton, 3 Spectra/Por® no.: 132725, Spectrum Laboratories, Breda, the Netherlands) on 5% blood agar for four days or by re-suspension in brain heart infusion broth until reaching log phase growth. Bacteria were harvested, washed and re-suspended in isotonic saline water and used immediately.

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Antibacterial assay

TYEG agar supplemented with 2 μg/mL of furazolidone to inhibit growth of staphylococci, was used to test the antibacterial activity of valrubicin against *P. acnes*. Valrubicin in different concentrations and formulations were loaded onto sterile filter discs and placed on the surface of inoculated agar plates. Valrubicin 250 mg/mL in acetone (kindly provided by Valderm ApS, Lyngby, Denmark), 25 mg/mL in acetone/DMSO [1:10], 5 μg/mL in acetone/sterile water [1:10,000], 40 mg/mL (Valstar® in cremophor/ethanol [1:1], kindly provided by Valderm ApS), 10 mg/mL (Valstar® in cremophor/ethanol/sterile water [1:1:4], 10 mg/mL in cream (1% batch 7077/001, kindly provided by Valderm ApS), 10 mg/mL in cream gel (1% batch 7078/001, kindly provided by Valderm ApS) and the vehicles: acetone, acetone/DMSO [1:10], acetone/sterile water [1:10,000], Cremophor® L (CS135, Sigma-Aldrich, Broendby, Denmark)/ethanol [1:1], Cremophor® L (CS135, Sigma-Aldrich)/ethanol/sterile water [1:1:4], cream and gel vehicles (kindly provided by Valderm ApS) were tested. Discs with penicillin (5 μg/mL) were re-suspended in isotonic saline solution, and new medium was removed, cells washed in PBS (Invitrogen), and new medium was added. *P. acnes* strain 1.4.L1, freshly prepared, was cultivated in keratinocyte serum-free medium (DMEM, Invitrogen, Carlsbad, CA, USA) at standard conditions (37°C, 5% CO₂, and 90% humidity) as previously described. 24 hrs prior to co-culturing NHEKs with *P. acnes*, medium was removed, cells washed in PBS (Invitrogen), and new medium without antibiotics added. *P. acnes* were re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% FBS without antibiotics prior to co-culture. The *P. acnes* strain 1.4.L1, associated with acne, was used. In house, unpublished results, demonstrated that freshly prepared *P. acnes*, harvested in log phase growth, stimulated a greater inflammatory response compared with thawed *P. acnes*, thus freshly prepared *P. acnes* were used throughout the study.

The effect of valrubicin on *P. acnes*-induced expression levels of inflammatory cytokines in NHEKs was determined by assessing five different treatment groups. Group 1 was not co-cultured with *P. acnes*, and served as negative control. Groups 2, 3, and 4 were co-cultured with *P. acnes* (strain 1.4.L1, freshly prepared) 30 minutes post *P. acnes* challenge, group 3 and 4 were furthermore treated with valrubicin (5.0 μg/mL, diluted 1:5,000 in acetone) or vehicle (acetone diluted 1:5,000). Group 5 was treated with valrubicin (5.0 μg/mL diluted 1:5,000 in acetone) alone. Each treatment group was performed in triplicate from each of the four donors.

Measurement of inflammatory cytokine mRNA and protein expression levels

To analyze mRNA expression levels, cells were harvested 2.5 hrs post *P. acnes* co-culture (2 hrs post *P. acnes* or vehicle treatment) by transferring cells to ice, washing in ice-cold PBS (Invitrogen) and adding RNA-lysis-buffer including β-mercaptoethanol (SV 96 Total RNA Isolation System, Promega, Madison, WI, USA). Lysed cell samples were stored at −80°C until processed for RNA purification. RNA was isolated according to the SV Total RNA Isolation System protocol (Promega) and cDNA and quantitative real-time reverse transcription-polymerase chain reaction (PCR) was performed as previously described. Expression of IL-1β, IL-8 and TNF-α was determined by TaqMan® Gene Expression Assays (Assay ID: IL-1β: Hs00174097_m1, IL-8: Hs00174103_m1, TNF-α: Hs01113624_g1, Applied Biosystems, San Francisco, CA, USA). RPLP0 was used as internal reference (Assay ID: RPLP0: Hs99999902_m1, Applied Biosystems). The expression of each gene was analyzed in triplicate. To analyze expression levels of secreted proteins, cell supernatants were harvested 2.5 hrs post *P. acnes* stimulation (12 hrs post *P. acnes* or vehicle treatment), snap-frozen in liquid nitrogen and stored at −80°C until processed in the enzyme-linked immunosorbent assay (ELISA) as previously described by use of DuoSet® ELISA development kits (IL-1β/L-1F2: DY201, CXC1/L-8: DY208, and TNF-α: DY210; R&D Systems, Abingdon, UK). The expression of each protein was analyzed in triplicate.

**P. acnes**-induced ear inflammation mouse model

C57BL/6J female mice (6-10 weeks old) (Taconic, Ry, Denmark) were housed under standard conditions with a 12 hrs light/dark cycle and controlled temperature. Animals were fed a standard rodent laboratory diet and when not otherwise stated given water ad libitum. During intra-dermal injection of *P. acnes*, measurement of ear thickness, and topical and intra-dermal injection treatment with valrubicin or vehicle, mice were anaesthetized in isoflurane (2.3.75%). At the end of the experiment, mice were sacrificed by cervical dislocation. Animal experiments were approved by The Danish Experimental Animal Inspectorate. Ear inflammation was induced in mouse ears by challenging ears with intra-dermal injections of 10 μL (6×10⁷) or 15 μL (9×10⁷) freshly prepared *P. acnes* (strain 1.4.L1) re-suspended in isotonnic saline water. No ear inflammation was observed in control mice intradermally injected with only isotonnic saline water after 24 hrs (data not shown). To test the effect of valrubicin as topical treatment a short term (1 day) and a long term (2 weeks) experiment was performed. Valrubicin (10 μg/mL dissolved in acetone) and vehicle (acetone, group 2, n=16) and ear thickness was measured before (0 hrs) and 24 hrs after challenge. In the long-term experiment, mouse ears were divided into five groups where groups 1 and 2, and groups 3 and 4 were challenged with 6×10⁷ or 9×10⁷ freshly prepared *P. acnes*, respectively. 30 minutes post challenge and daily thereafter throughout the study, ears were treated topically with valrubicin (group 1, n=7 and group 3, n=8) or vehicle (acetone, group 2, n=4 and group 4, n=8). As control, an additional group of mouse ears (group 5, n=8) was left untreated but treated daily with valrubicin throughout the study. To test the effect of intra-dermal valrubicin treatment, 2 weeks post challenge, mouse ears were treated daily for 3 days with intra-dermal injections of valrubicin (Valstar®, 10 μL, group 1, n=12) or vehicle (cremophor/ethanol [1:1], 10 μL, group 2, n=11). As control, an additional group of challenged mouse ears was left untreated (group 3, n=9).

**Ear thickness** was measured by a Mitutoyo digimatic indicator (Mitutoyo, Kawasaki, Japan) prior to and at indicated time points after ear challenge.

**Statistics**

For statistical analysis the two-tailed Student’s t-test was employed. P<0.05 was considered significant.

**Results**

Valrubicin’s antibacterial effect against *P. acnes*

Valrubicin’s antibacterial effect against *P. acnes* was tested by employing different concentrations and formulations of valrubicin
Valrubicin's effect on

**P. acnes-induced inflammation in keratinocytes in vitro**

Valrubicin's effect on *P. acnes*-induced inflammation in keratinocytes in *vitro* was tested in NHEKs co-cultured with freshly prepared *P. acnes*. *P. acnes* only significantly induced an increase in the mRNA expression level of IL-1β (Figure 1a) but not IL-8 (Figure 1b) and TNF-α (Figure 1c). The observed increased mRNA expression level of IL-1β and the existing expression levels of IL-8 and TNF-α were not affected by valrubicin treatment (P=0.98 for IL-1β, P=0.52 for IL-8, and P=0.88 for TNF-α). *P. acnes* significantly increased protein expression levels of both IL-1β (Figure 1d), IL-8 (Figure 1e) and TNF-α (Figure 1f), however, valrubicin treatment was demonstrated not to reduce the *P. acnes*-induced expression levels of IL-1β (P=0.54), IL-8 (P=0.94), and TNF-α (P=0.76).

Valrubicin's effect on *P. acnes*-induced skin inflammation *in vivo*

Valrubicin's effect on *P. acnes*-induced skin inflammation *in vivo* was tested by treating mouse ears, challenged by injection of *P. acnes*. Short term (1 day) topical valrubicin treatment demonstrated no reduction in ear thickness (Figure 2a); ear thickness in both valrubicin and vehicle treated mouse ears was increased approximately 2-fold after 24 hrs. Long term (2 weeks) daily topical valrubicin treatment was tested in repeated experiments where mouse ears were injected with the same amount of *P. acnes* (Figure 2b) or 30% fewer *P. acnes* (Figure 2c). No reduction in ear thickness was seen in valrubicin treated mouse ears compared with vehicle treated ears, at any time point during the 2 weeks. Ear thickness, in both the valrubicin and vehicle treated mouse ears, increased approximately 3-fold (Figure 2b) and 2-fold (Figure 2c) after 24 hrs. Mouse ears treated with valrubicin alone, demonstrated no change in ear thickness during the 2 weeks. Repeated acetone-applications have previously been demonstrated, by our group, not to affect ear thickness. In a final experiment, to facilitate the ability of valrubicin to locate directly into the site of inflammation, valrubicin was injected intra-dermally into the mouse ears (Figure 2d). No effect of valrubicin as compared with vehicle, on ear thickness, was observed.

### Table 1. Valrubicin's antibacterial effect on *P. acnes*.

<table>
<thead>
<tr>
<th>Valrubicin concentration and formulation</th>
<th>1.4.L1</th>
<th>12.1.L1</th>
<th>30.2.L1</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mg/mL in acetone</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>25 mg/mL in acetone/DMSO (1:10)</td>
<td>*</td>
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<tr>
<td>5 μg/mL in acetone/sterile water (1:10,000)</td>
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</tr>
<tr>
<td>40 mg/mL (Valstar®) in cremophor/ethanol (1:1)</td>
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<td></td>
</tr>
<tr>
<td>10 mg/mL (Valstar®) in cremophor/ethanol/sterile water (1:1:4)</td>
<td>*</td>
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<tr>
<td>10 mg/mL in cream (1% batch)</td>
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</tr>
<tr>
<td>10 mg/mL in cream gel (1% batch)</td>
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</table>

* indicates no clearing of the bacterial layer. Vehicles alone demonstrated no antibacterial effect and penicillin, used as positive control, showed strong bacterial clearing in all three *P. acnes* strains.

![Figure 1](image-url)

*Figure 1. Valrubicin's effect on *P. acnes*-induced mRNA and protein expression levels of inflammatory cytokines by NHEKs. NHEKs from four healthy donors, were cultured in the presence of freshly prepared *P. acnes*. The effect of valrubicin on *P. acnes*-induced mRNA expression levels of IL-1β (a), IL-8 (b), and TNF-α (c) was evaluated after 2 hrs of stimulation. mRNA expression levels were normalized to the expression levels of the housekeeping gene, ribosomal protein large P0 (RPLP0) and to the untreated control (no *P. acnes* bacteria added). The effect of valrubicin on *P. acnes*-induced protein expression levels of IL-1β (d), IL-8 (e), and TNF-α (f) was evaluated after 12 hrs of stimulation. Protein expression levels were determined by ELISA and normalized to the untreated control (no *P. acnes* bacteria added). Values are presented as mean ± standard deviation (NHEKs, n=4 donors, cultured in triplicate).*
Discussion

Several treatment modalities exist for acne, however, some may be associated with adverse side effects and therapeutic failure.1 Thus, research is focused on discovering new and improved treatments to expand the current treatment palette.

The role of *P. acnes* in the pathogenesis of acne has been discussed, as *P. acnes* is also a commensal of healthy skin.2 Recent findings, by multi-locus sequence typing (MLST), however, have shown that *P. acnes* can be divided into different strains associated with either moderate to severe acne, healthy skin or opportunistic infections.10,12 Nevertheless, the majority of acne treatments, currently available, are associated with anti-inflammatory effects and not necessarily antibacterial effects. Interestingly, prolonged antibiotic treatment, associated with high degree of *P. acnes* resistance, often alleviates acne lesions; a fact explained by anti-inflammatory effects of the antibiotics.13,14

Valrubicin did not demonstrate anti-inflammatory effects on primary keratinocytes co-cultured with *P. acnes*, as measured by mRNA and protein expression levels of IL-1β, IL-8, and TNF-α. These findings were surprising as we previously demonstrated that 2 hrs of valrubicin treatment significantly reduced mRNA expression levels of IL-1β and IL-6 in 12-O-tetradecanoylphorbol 13-acetate (TPA) treated mouse ears,9 and because 2 hrs of valrubicin treatment demonstrated to significantly reduce mRNA expression levels of IL-1β, IL-8 and TNF-α in stimulated keratinocytes and immune cells (unpublished results). Moreover, we previously demonstrated that 8 hrs and 24 hrs of valrubicin treatment significantly reduced protein expression levels of IL-1β and IL-6 in TPA treated mouse ears.9 Also, we previously reported that the employed concentration of valrubicin (5.0 μg/mL) was able to inhibit proliferation of NHEKs in cell culture.7 Why valrubicin reduces TPA-induced inflammation and not *P. acnes*-induced inflammation is unclear.

IL-1β, IL-8 and TNF-α are expressed in human acne lesions,17,18 and *P. acnes* has previously been demonstrated to stimulate secretion of these pro-inflammatory cytokines in keratinocytes.20,21 The fact that IL-1β is up-regulated in acne skin has proposed beneficial effects of IL-1β-targeted therapy which was recently shown to be the case, in patients suffering from auto-inflammatory syndromes including acne-lesions.19,20

Topical valrubicin treatment did not reduce *P. acnes*-induced ear inflammation following short and long term treatments. In a similar study, by our group, Hauge et al. induced both acute and chronic inflammation in the same mouse strain by stimulating mouse ears topically with TPA.4 In this study, topical valrubicin treatment significantly reduced TPA-induced ear inflammation in both the acute and the chronic model. In both studies, topical valrubicin was applied in equal amounts and concentrations. Again, why valrubicin reduces TPA-induced inflammation and not *P. acnes*-induced inflammation is unclear.

Previous studies have successfully demonstrated anti-inflammatory effects in the employed *P. acnes* injected ear-inflammation model.17,29 Similar numbers of *P. acnes* were injected, however different *P. acnes* strains were employed. These studies demonstrated a similar 2-4 fold increase in ear thickness upon *P. acnes* injection which then decreased upon...


