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Activation of clindamycin phosphate by human skin

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Aims: To investigate the relative antimicrobial activity of clindamycin phosphate (CP) and clindamycin (Cly) and to examine the effect of skin homogenates on the activity of CP.

Methods and Results: Minimum inhibitory concentrations (MIC) were determined against dermally relevant organisms and bactericidal activity was studied using time-kill methodology. The effect of skin homogenates on the antimicrobial activity of CP was studied by well-diffusion assay. The MIC of Cly was substantially lower than that of CP in all susceptible organisms. Clindamycin also showed greater bactericidal activity (rate of kill) than CP. Phosphatases in skin homogenates activated CP at pH 4–8 with a maximal activation at pH 4.

Conclusions: Phosphatases within the skin have been shown to convert CP to the more potent form Cly.

Significance and Impact of the Study: Conversion to Cly is a major determinant of antimicrobial activity in the skin layers following topical application of CP.

INTRODUCTION

Acne lesions affect the pilosebaceous units of the skin on the face, back and chest. The pathophysiological features of acne are overproduction of sebum, the abnormal desquamation of the sebaceous follicle epithelium and the proliferation of micro-organisms such as *Propionibacterium acnes* and *Staphylococcus epidermidis* (Ebling and Cunliffe 1996). It has been suggested that the bacterial lipases of *P. acnes* are involved in the aetiology of acne by breaking down sebaceous oils to liberate fatty acids which cause inflammation (Cortan *et al*. 1994). Thus the rationale behind antibiotic treatment of acne is to reduce microbial colonization of the pilosebaceous units, thereby reducing the lipase-catalysed formation of free fatty acids (Gollnick and Schramm 1998; Toyoda and Morohashi 1998). Clindamycin (Cly), erythromycin and tetracycline are the most widely prescribed topical antibiotics for mild–moderate acne (Eady *et al*. 1982; Eady *et al*. 1996; Gollnick and Schramm 1998; Toyoda and Morohashi 1998).

Clindamycin therapy offers several advantages over other antibiotics, including a lower incidence of resistance amongst the resident skin bacteria and greater efficacy in reducing the number of propionibacteria (Toyoda and Morohashi 1998; Fluhr *et al*. 1999; Kurokawa *et al*. 1999). Unfortunately, the systemic side-effects of Cly are numerous and include the potentially fatal condition pseudo membranous colitis (Dhawan and Thadepalli 1982; van Hoogdalem 1998). Topical administration allows the delivery of Cly directly to its site of action while theoretically minimizing systemic absorption and thus reducing side-effects. However, factors such as the form of Cly (phosphate ester or hydrochloride salt) and the composition of the delivery vehicle have been found to affect the extent of systemic exposure to Cly (van Hoogdalem 1998). For this reason the phosphate ester is preferred for topical administration because it is believed to result in less transdermal absorption. However, the biopharmaceutical and antimicrobial properties of clindamycin phosphate (CP) and Cly are important factors for formulation design.

While the clinical efficacy of topical CP (Toyoda and Morohashi 1998) and the susceptibility of *P. acnes* to Cly *in vitro* (Fluhr *et al*. 1999; Kurokawa *et al*. 1999) are well documented, the relative activities of CP and Cly against *P. acnes* and other micro-organisms of the skin microflora have not been reported. At present, topical formulations contain an excess of CP. In order to develop improved topical formulations of CP, the relative antimicrobial activities of CP and Cly, the time-kill profiles of both compounds and the mechanisms involved in the dermal bioconversion of CP to Cly must be considered. Preliminary

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studies have revealed limited conversion of CP to Cly by micro-organisms of the skin surface. The aims of this study were (i) to compare the antimicrobial activities of CP and Cly against dermally relevant micro-organisms and (ii) to determine the effect of skin homogenates on the conversion of CP to Cly.

MATERIALS AND METHODS

Materials

Orthophosphoric acid, potassium dihydrogen orthophosphate, disodium phosphate, hydrochloric acid, sodium hydroxide and 4-chloro-*m*-cresol (chlorocresol) were obtained from BDH Laboratory Supplies (Poole, UK). Alkaline phosphatase (type XVII from human placenta; P1391), acid phosphatase (from human semen; P1649) and phosphatase inhibitor cocktails I and II were supplied by Sigma Chemical Company (Poole, UK). Clindamycin hydrochloride and CP were purchased from Fluka (Poole, UK). Tryptic soya agar and tryptic soya broth (TSB) were prepared by Oxoid (Basingstoke, UK). The micro-organisms used in this study were *P. acnes* NCTC 3973, *Candida albicans* NCPF 3179, *Staph. aureus* NCIMB 9518, *Staph. epidermidis* NCTC 2548, *Pseudomonas aeruginosa* NCIMB 10421 and *Proteus vulgaris* NCTC 4175. Deionized water was used throughout the study using an Option 3 Water Purifier (Elga, High Wycombe, UK).

Antimicrobial activity of clindamycin phosphate and clindamycin

The *in vitro* susceptibility of the six organisms listed above to both CP and Cly was determined. Minimum inhibitory concentration (MIC) values were determined using twofold dilutions of CP or Cly in sterile water. The cultures were incubated in double-strength TSB at 37°C for 48 h for aerobic bacteria and 72 h for anaerobic bacteria. Anaerobic conditions were maintained using an anaerobic jar and Anaero*Gen*™ sachets (Oxoid). The MIC values were recorded as the lowest CP or Cly concentration at which visible growth was prevented. Replicate determinations ($n = 3$) were performed on three separate cultures of each organism.

The bactericidal activity of CP and Cly was investigated using *P. acnes* or *Staph. epidermidis* at densities of 10^{10} and 10^{11} organisms ml^{-1}, respectively. The test organism was incubated in TSB whilst shaking with CP or Cly ($0{\cdot}5 \times$ MIC, MIC, $2 \times$ MIC, $4 \times$ MIC or $8 \times$ MIC) or a sterile water control for up to 3 h at 37°C. Total viable counts were performed at 0, 1 and 3 h. Data were analysed by expressing growth as the change in $\log_{10}$ cfu ml^{-1} compared with the count at 0 h.

Effect of human skin homogenates on the antimicrobial activity of clindamycin phosphate

Full-thickness, human abdominal skin (from caucasian female patients aged 35–47 years) was obtained following abdominoplasty and either stored at 4°C and used within 3 h of surgical removal or stored at –20°C until use. Any subcutaneous fat was trimmed with a scalpel and the skin (2·5 g) cut into small pieces (2–3 mm^2) and homogenized (in 6×30 s bursts at 4°C) in 10 ml phosphate-buffered solution (pH 4 or 10) at 4°C using an Ultra-Turrax homogenizer (Staufen, Germany) until a uniform suspension was produced.

The activity of CP and the effect of incubating CP with phosphatases and skin homogenates were determined by a well-diffusion assay. Molten agar (approx. 45°C) was seeded with *Staph. epidermidis* (100 μl of an overnight culture per 20 ml agar), dispensed into sterile Petri dishes and allowed to solidify. Wells of 80 mm diameter were made and 75-μl aliquots of solution or skin suspension dispensed into them. Control solutions included: buffer solutions (pH 4–10) alone; skin homogenates (0·25 g ml^{-1}) alone; cocktail inhibitor I and cocktail inhibitor II (Ck II) alone and acid phosphatase (ACP) (0·4 units) or alkaline phosphatase (ALP) (0·4 units) in the presence and absence of inhibitors. Cocktail inhibitor I inhibits ALP activity and Ck II inhibits ALP and ACP activity. Test solutions included 0·075% (w/v) CP alone and with: (i) acid or alkaline phosphatase in the presence or absence of inhibitors; (ii) skin homogenates in the presence or absence of inhibitors and (iii) skin homogenates at pH 4–8. After incubating the plates for 24 h at 37°C, the antimicrobial activity was assessed by measurement of the circular zones of inhibition around the wells.

RESULTS

Antimicrobial activity of clindamycin phosphate and clindamycin

In this study, concentrations of Cly equivalent to those deliverable by topical products (< 1% w/v) were demonstrated to produce antimicrobial activity against *Staph. aureus*, *Staph. epidermidis* and *Pr. vulgaris*, selected to represent the skin surface microflora, and *P. acnes*. The activity of Cly was markedly greater than that of CP but the relative activity against *C. albicans* and *Ps. aeruginosa* could not be determined (Table 1).

The antimicrobial action of CP and Cly at low effective concentrations was studied using time-kill methodology. The profiles obtained showed that, although Cly was used at lower concentrations because of its greater potency, it was more bactericidal than CP (Fig. 1a, b). During the 1 h experiment CP did not achieve > 3 $\log_{10}$ reductions (cfu ml^{-1})

Table 1 The minimum inhibitory concentration (MIC) of clindamycin phosphate (CP) and clindamycin (Cly) against the test micro-organisms (n = 3, mean ± S.E.M.)*

Organism	MIC of CP (w/v %)	MIC of Cly (w/v %)	Relative activity CP/Cly
Propionibacterium acnes	0·1 ± 0·05	0·008 ± 0·002	12·5
Candida albicans	>1	>1	N/A
Staphylococcus aureus	0·02 ± 0·005	0·006 ± 0·001	3·3
Staphylococcus epidermidis	0·003 ± 0·0006	0·001 ± 0·0006	3·0
Pseudomonas aeruginosa	>1	>1	N/A
Proteus vulgaris	0·4 ± 0·1	0·009 ± 0·007	44·4

*Three determinations of MIC were made in each experiment. Each experiment was repeated three times.
N/A, Not applicable.

in the number of bacteria at any concentrations used against either test organism, apart from the 8 × MIC concentration against *Staph. epidermidis*. Clindamycin achieved > 3 $\log_{10}$ reductions (cfu ml^{-1}) against *Staph. epidermidis* at concentrations of MIC and above and against *P. acnes* at 4 × MIC and above. The lower sensitivity of *P. acnes* might be due to its slower growth rate. The difference in growth rate can be seen in the smaller increase in the number of *P. acnes* compared with the increase in the number of *Staph. epidermidis* in the controls over the 1 h duration of the time-kill experiment (Fig. 1a, b).

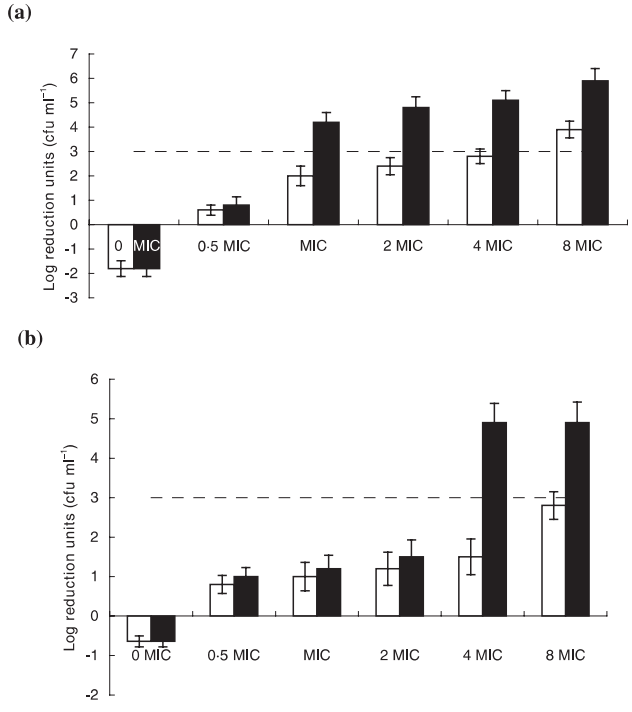

Fig. 1 Bactericidal activity ($\log_{10}$ reduction cfu ml^{-1}) of clindamycin (■) and clindamycin phosphate (□) at low effective concentrations (0 minimum inhibitory concentration (MIC), 0·5 MIC, 1 × MIC, 2 × MIC, 4 × MIC or 8 × MIC). Data obtained after 1 h incubation with (a) *Staphylococcus epidermidis* or (b) *Propionibacterium acnes* using time-kill methodology similar to that reported by Credito *et al.* (1999) who regarded >3 $\log_{10}$ reductions cfu ml^{-1} (shown by dotted horizontal line) as significant bactericidal activity. Control results (0 MIC) indicate bacterial growth over 1 h

Activation of clindamycin phosphate by phosphatases and skin homogenates

Mixtures of CP and phosphatase enzymes or skin homogenates were added to wells bored in *Staph. epidermidis*-impregnated agar plates and the antimicrobial activity was indicated by reproducible circular zones of inhibition around the wells. The suitability of the test system was determined using ACP or ALP to activate a concentration of CP (0·075% w/v) which produced no zones of inhibition of bacterial growth in the well diffusion assay. Cocktail inhibitor I, which inhibits only ALP activity, abolished 96% of the activation of CP by ALP but had no effect on ACP. Cocktail inhibitor II, which inhibits ACP and ALP activity, prevented 95 and 97% of the activation by both enzymes, respectively. No zones of inhibition were produced when, in control experiments, the cocktail inhibitors alone were added to the well diffusion assay.

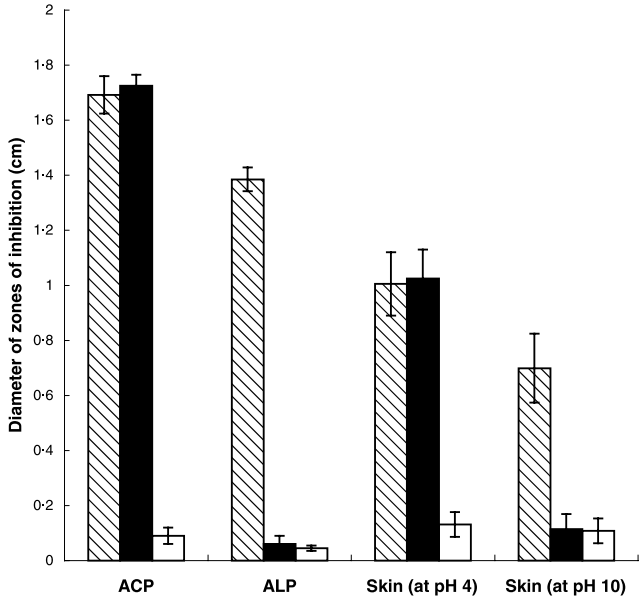

Fig. 2 Zones of inhibition obtained by well-diffusion assay after incubation of clindamycin phosphate (CP; 0·075% w/v) with acid phosphatase (ACP), alkaline phosphatase (ALP) or human skin homogenates at pH 4 or 10 (n = 6, mean ± S.D.). ▨, CP alone; ■, CP with cocktail inhibitor I, ALP inhibition; □, CP with cocktail inhibitor II, ALP + ACP inhibition

When skin homogenates were used in place of the phosphatase enzymes, similar results were obtained indicating the ability of both acid and alkaline phosphatases in the skin to activate CP (Fig. 2). The experiments were conducted at pH 4, which is selective for ACP, and pH 10, which is selective for ALP, and the role of each enzyme was again confirmed by the use of selective inhibitors. In further experiments, CP was spiked into skin homogenates at pHs which reflect the physiological pH gradient from the acid mantle surface of the skin to the neutral dermis. The zones of inhibition were maximal at pH 4 (0·93 cm), when activation would be exclusively due to ACP activity, but also substantial at pH 6 (0·78 cm) and pH 8 (0·72 cm), where activation would reflect a combination of submaximal ACP and ALP activities.

DISCUSSION

The results obtained (Table 1) were in accordance with the recognized antimicrobial profile of Cly, which is effective against many Gram-positive and Gram-negative anaerobic bacteria (Verhoef and Levison 1999). Similarly, the lack of activity of Cly against *C. albicans*, a fungal organism, and *Ps. aeruginosa*, a Gram-negative aerobe, broadly corresponded with the recognized spectrum of activity and potency of Cly (Dhawan and Thadepalli 1982; Verhoef and Levison 1999). Although often regarded as an inactive prodrug of Cly (Verhoef and Levison 1999), CP had antimicrobial activity against the same organisms as Cly, albeit at between three and 44 times higher concentrations (Table 1).

Clindamycin is regarded primarily as having a bacteriostatic action, although it is recognized to have concentration-dependant bactericidal activity against certain staphylococci and anaerobic bacteria (Verhoef and Levison 1999). Bacterial protein synthesis inhibitors, such as Cly, also exert a pronounced postantibiotic effect against susceptible species, probably as a result of persistence at their ribosomal binding site.

The enhancement of antimicrobial activity in the well-diffusion system was used as an indication of the conversion of CP to Cly. Direct measurement of CP and Cly levels in the skin by methods such as high-performance liquid chromatography has proved impractical because of low drug concentrations and the variability of extraction efficiency. Well-diffusion assays are reliable, inexpensive to use and are routinely employed to test the potency of antibiotics against a wide range of organisms.

In this study, subinhibitory concentrations of CP in the presence of skin homogenates and phosphatase inhibitors still possessed some activity against *Staph. epidermidis*. It is possible that skin contains enzymes other than phosphatases that may convert CP to Cly or another active metabolite such as 1-dethiomethyl-1-hydroxy clindamycin (Riebe and Oesterling 1972). In addition, some preliminary studies have indicated the inhibition of phosphatase activity in the skin to be incomplete and it is possible that residual phosphatase activity may account for the activation of CP in the presence of the inhibitors. Furthermore, the viability of the skin was crucial to achieve maximum activation of CP. Previous studies by our group have shown that skin from a single individual that had been stored frozen for 1 week exhibited only 78% of the ability to activate CP compared with when freshly homogenized (Amr *et al*. 2000). The lower level of activation observed with stored frozen skin probably reflects a loss of phosphatase enzyme activity during freezing, storage and thawing.

Commercially available topical preparations of CP are presented in different delivery vehicles, including gels and alcoholic lotions, which will affect the penetration and distribution of CP in the skin. The simultaneous conversion of CP to the more potent Cly adds to the complexity of targeting antimicrobial activity to the site of action. However, if the localization of antimicrobial activity in the skin could be assessed following the application of modified formulations, it would be possible to optimize the retention of effective antimicrobial concentrations in the skin and minimize systemic exposure to Cly, thus reducing any possible side-effects. The well-diffusion assay developed for this study could be used to screen the effectiveness of topical formulations of antimicrobial prodrugs. Moreover, topical application of such formulations to skin contained in Franz cells (Waranuch *et al*. 1999), followed by separation of the skin layers after diffusion has been allowed to occur, might allow the distribution of antimicrobial activities to be further examined.

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