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Stefania Zauli, Annarosa Virgili, Vincenzo Bettoli

Dipartimento di Scienze Mediche, Sezione di Dermatologia, Università di Ferrara, Italy.

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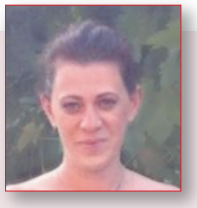

Stefania Zauli

SUMMARY

The role of P. acnes in the pathogenesis of acne is well established. The evidence suggests a gradual increase of strains of P. acnes resistant to the most commonly topical antibiotics used in acne treatment over the last 20 years. Obviously the presence of P. acnes resistant to antibiotic reduces the efficacy of the acne treatment increasing the psychological discomfort and reducing the patient's compliance.

This paper includes in vivo study and the results of in vitro report (Evocutis Report EVO-0436 Sept 2013) of adjuvant topical for acneic skin based on micronized silver, zinc acetate and lauric acid (trade name Diakon Krem; manufacturer: Valetudo-Divisione Biogena, Italy). The aim of in vitro ***test is to verify the activity of Diakon Krem against five selected P. acnes strains (sensitive, resistant C*, resistant E*, resistant C+E* and resistant C+E+T*), using an agar dilution minimum inhibitory concentration (MIC) assay.***

The MIC value observed on the different tested strains (sensitive, resistant C, resistant E, resistant C+E, resistant C+E+T) is the same, 0,156% v/v, it means that 100 ml of solution contain dissolved 0,156 ml of Diakon Krem.

All P. acnes isolates irrespective of antibiotic genotype/phenotype or geographical origin were equally susceptible to Diakon Krem, hence no MIC change has been observed.

After that, the efficacy and the tolerability of the adjuvant topical (Diakon Krem) have been assessed in the treatment of mild facial acne vulgaris in a prospective observational clinical study.

The clinical study including 25 patients affected by mild facial acne vulgaris, has shown a mean reduction of 70% of the medium Leeds score and a mean reduction of 33% of the medium psychological distress.

Twenty patients (80%) referred a positive Patient Global Assessment (PGA): 8 patients referred a "great improvement", 4 a "moderate improvement" and 8 a "mild improvement".

None of the patients complained any side effects and the topical was well tolerated by all the subjects.

In conclusion, Diakon Krem is not influenced by P. acnes antibiotic-resistant in vitro, ***and seems efficacy to reduce acne severity and well tolerated*** in vivo.

**C = Clindamycin, E = Erythromycin, T = Tetracycline.*

Key words: Acne vulgaris; P. acnes; Antibiotic resistance; MIC; Micronised silver; Zinc acetate; Lauric acid.

Introduction

Acne has a prevalence of over 90% among adolescents and persists into adulthood in approximately 12%-14% of cases with psychological and social implications [1].

Although not a fatal disease, it can produce both physical and emotional scarring, as well as psychological stress. Hence, treatment of the disease is important [2].

Acne formation begins with the microcomedone, a clinically invisible lesion. Microcomedones then develop into visible acne lesions: comedones, papules, pustules, and nodules. The degree of inflammation is variable [3].

Lesions occur primarily on the face, neck, upper back and chest. When assessing the severity of the acne, one needs to consider the distribution (back, chest, upper arms), type and number of lesions (comedones, papules, pustules, nodules) and the presence or absence of scarring [4].

Acne is an androgen-dependent disorder of pilosebaceous follicles (or pilosebaceous unit). There are four primary pathogenic factors, which interact to

produce acne lesions: sebum production by the sebaceous gland, alteration in the keratinization process at the level of the infrainfudibulum, *Propionibacterium acnes* (*P. acnes*) follicular colonization, and release of inflammatory mediators [5].
Follicular colonisation by *P. acnes* plays a critical role in the development of inflammatory acne. Chemotactic factors induced by *P. acnes* attract neutrophils, monocytes, and lymphocytes to the pilosebaceous unit. Furthermore, *P. acnes* induces initiation of sebum production in facial follicles, and stimulates the production of proinflammatory cytokines such as TNF-α, IL-1β, IL-8, and IL-12 mediated by toll-like receptor 2.
In addition, *P. acnes* releases lipases, proteases, and hyaluronidases which contribute to tissue injury. In response to *P. acnes*, keratinocytes can produce massive amounts of reactive oxygen species (ROS) by NAD(P)H oxidase through activation of the scavenger receptor CD36 to eliminate the bacteria and generate inflammation [6].
Topical antibiotics alone can be useful in the treatment of acne. Clindamycin and erythromycin inhibit protein synthesis by irreversibly binding to the ribosomal 50S subunit of *P. acnes*. Topical antibiotics act against this organism, in addition to having anti-inflammatory properties, making these medications useful in the treatment of acne [7].
Prior to the mid 1970s, resistance to *P. acnes* was not identified [2]. By 1979, *Crawford* and colleagues had detected the first indication of resistance to topical erythromycin and clindamycin, which was followed by the emergence of tetracycline-resistant *P. acnes* in the early eighties. Since then, the incidence of antibiotic resistance in acne has continued to rise across the globe, from 20% in 1978 to 72.5% in 1995, with combined resistance to erythromycin and clindamycin more prevalent than resistance to tetracycline. Evidence suggests that it is the use of topical erythromycin and clindamycin, the most commonly used topical antibiotics in acne, that has contributed to the gradual increase in resistance over the last 20 years [8].
These findings indicate the need to develop strategies to minimize the use of antibiotics in acne therapy.
The aim of *in vitro* study was to test *in vitro* activity of adjuvant topical for acneic skin with antibacterial ingredients, micronised silver and zinc acetate, strengthened by the presence of and lauric acid, against five selected *P. acnes* strains (sensitive, resistant C*, resistant E*, resistant C+E* and resistant C+E+T*), using an agar dilution minimum inhibitory concentration (MIC) assay.

*C = Clindamycin
*E = Erythromycin
*T = Tetracycline

The testing of anti-acne products for antimicrobial activity against *P. acnes* is a recognised method for determining potential efficacy, but is usually performed against a single strain. In this case highly defined panel over 30 strains of *P. acnes* was collected, including strains resistant to antibiotics commonly used in treating acne, of different antibiotic genotype/phenotype or geographical origin, to indicate whether tested material would be able to help reduce the prevalence of antibiotic resistance. Among this rich panel, we choose five selected *P. acnes* strains, representing the most common and worst type of antibiotic resistance that may occur (sensitive, resistant C, resistant E, resistant C+E, resistant C+E+T).
After that, the efficacy and the tolerability of the adjuvant topical (*Diakon Krem*) have been assessed in the treatment of mild facial acne vulgaris in a prospective observational clinical study.

Materials and Methods

The test *in vitro* was carried out at the *Evocutis Plc*, a leading dermatology company, with over 25 years' experience in skin microbiology and dermatology research (*R Bojar, DJ Fitgerald*, *in vitro* investigations into the antimicrobial activity against *Propionibacterium acnes*, *Evocutis Plc, EVO-0436 Sept 2013*).
The testing was carried out at ambient temperature. Environmental monitoring of the *Evocutis Class II Laboratory* is undertaken on a weekly basis to ensure low level background contamination is within pre-defined limits.
A stock solution of *Diakon Krem* was prepared to a concentration of 50% v/v with sterile dH_2O (Table 1A).
A series of doubling dilutions were then prepared to provide stock solutions for testing a final con-

Table 1A Preparation of test items.	
Final % (v/v)	Stock solution % (v/v)
2.5	Neat (100)
1.25	50
0.625	25
0.313	12.5
0.156	6.25
0.078	3.125
0.04	1.56
0.02	0.78
0.01	0.39
0.005	0.195
0.0025	0.1
0.001	0.05
0.0005	0.025
0.00025	0.0125
0 (Control)	dH_2O only

centration range of 1.25 – 0.00025 (0) % v/v. Sterile dH_2O alone acted as the positive (0) control. Aliquots (19.5 mL) of *Wilkins-Chalgren* (Oxoid, CM0619) were first melted at 90°C then cooled to 50-55°C. To prepare the plates, 0.5 mL of *Diakon Krem* (neat) or diluted *Diakon Krem* stock solutions were added to the cooled molten agar, mixed thoroughly and then poured into sterile triple-vented 90 mm petri-dishes. Three replicates were prepared at each test concentration.

Plates were allowed to cool to RT & then surface dried for 30 minutes in a laminar flow cabinet prior to storage (overnight) at 4°C.

The activity of the *Diakon Krem* was evaluated against an agreed panel comprising five *P. acnes* isolates (sensitive, resistant C, resistant E, resistant C+E and resistant C+E+T) (Table 1C). The *P. acnes* isolates were grown on *Wilkins-Chalgren* agar at 37°C under anaerobic conditions for 96 h and then prepared to an OD 600 nm of 0.2 (± 0.005) in *Wilkins-Chalgren* broth to yield ~1-2 × 10^8 cfu/mL.

The plates containing *Diakon Krem* (inc. control) were inoculated from low to high concentration using an AQS A400 multipoint inoculator to deliver spots of ~1-2 µL to give a final inoculum of ~1-2 ×105 cfu per spot. Plates were incubated at 37°C under anaerobic conditions for 72 h.

For quality control purposes each inoculum was checked for purity and antibiotic resistance profile to clindamycin (2 µg), erythromycin (5 µg), and tetracycline (10 µg) (Table 1B).

Following incubation, MIC was determined by noting the concentration where a marked reduction

Table 1B *P. acnes* panel quality control.							
Isolate	Phenotype	Mutations	OD	Purity	Zone of inhibition (mm)		
					Clin (2)	Ery (5)	Tet (10)
EVO-2	Sensitive	None	0.205	Pass	43.32	46.05	43.93
EVO-58	Resistant - C	Unknown	0.203	Pass	20.90	47.26	46.95
EVO-59	Resistant - E	2057	0.202	Pass	38.47	24.84	43.93
EVO-33	Resistant - C/E	erm (X)	0.201	Pass	0.00	0.00	39.08
EVO-44	Resistant - C/E/T	1058/2059	0.205	Pass	0.00	0.00	0.00

C = Clindamycin
E = Erythromycin
T = Tetracycline

Resistant		
Antibiotic	Breakpoints (mm)	
	Resistant	Sensitive
Clin (2)	< 25	≥ 25
Ery (5)	< 25	≥ 25
Tet (10)	< 32	≥ 32

Table 1C *P. acnes* panel isolation details.			
Isolate	Source	Year of isolation	Site of isolation
EVO-2	United States	Unknown	Wound
EVO-58	Sweden	2000	Skin
EVO-59	England	Pre 2000	Skin
EVO-33	Spain	1999	Skin
EVO-44	United States	Pre 2000	Skin

in the appearance of growth on the test plate as compared to the control plate. Examples of a marked change in growth include a change from a uniform spot of confluent growth to a film of growth or multiple tiny colonies, or one to several normal sized colonies – this follows guidance given by CLSI M11-A7.

The clinical study was carried out at *Acne Unit-Section of Dermatology of the University of Ferrara, Italy*. Twenty-five patients affected by mild facial acne (Leeds score < 0.5 corresponding to approximately less than 10 comedones, less than 10 papules and pustules, no nodular or cystic lesions) [9] were enrolled in a prospective observational study.

We enrolled males and females regardless of the acne therapies previously carried out and of the duration of the disease. Pregnant or breastfeeding women were excluded from the study.

The studied product was topically applied on the face twice daily for 8 weeks during summer time.

No other oral or topical products were simultaneously prescribed. It was not recommended a specific cleanser but it was recommended to use the cleanser already in use.

The parameters used to evaluate the efficacy of the treatment were:

1) severity of the disease, assessed with the Leeds score before and after treatment,
2) psychological distress before and after treatment assessing by a score from a minimum of 0 to maximum of 10,
3) *Patient Global Assessment* (PGA) after treatment.

PGA was registered in order to obtain the patients' personal perception about the results of the treatment. "*great improvement*", "*moderate improvement*" and "*mild improvement*" were considered positive results, whereas "*no improvement*" and "*worsening*" were considered negative results.

Finally, patients were asked about tolerability and side effects such as burning, itching, erythema and desquamation during treatment.

Statistical analysis were performed by Wilcoxon test. Significance was accepted at $P < 0.05$.

Results

The MIC value observed on the different tested strains (sensitive, resistant C, resistant E, resistant C+E, resistant C+E+T) is the same, 0,156 % v/v, it means that 100 mL of solution contain dissolved 0,156 mL of *Diakon Krem*.

All *P. acnes* isolates irrespective of antibiotic genotype/phenotype or geographical origin were equally susceptible to *Diakon Krem*, hence no MIC change has been observed (Table 1D).

The clinical study including 25 patients, 21 females and 4 males, average age 18 years (range 13-30 ys), affected by mild facial *acne vulgaris*.

All the 25 patients completed the 8 weeks of treatment.

The medium Leeds score was reduced from 0.10 (range 0.01-0.25) to 0.03 (range 0-0.06), corresponding to a mean reduction of 70% ($P < 0.001$) (Figure 1).

Table 1D MIC data summary.				
P. acnes isolate	Phenotype	Mutation/ Gene	Source	MIC (% v/v)
EVO-2	Sensitive	None	United States	0.156
EVO-58	Resistant C	Unknown	Sweden	0.156
EVO-59	Resistant E	2057	England	0.156
EVO-33	Resistant C/E	erm (X)	Spain	0.156
EVO-44	Resistant C/E/T	1058/2059	United States	0.156

C = Clindamycin
E = Erythromycin
T = Tetracycline

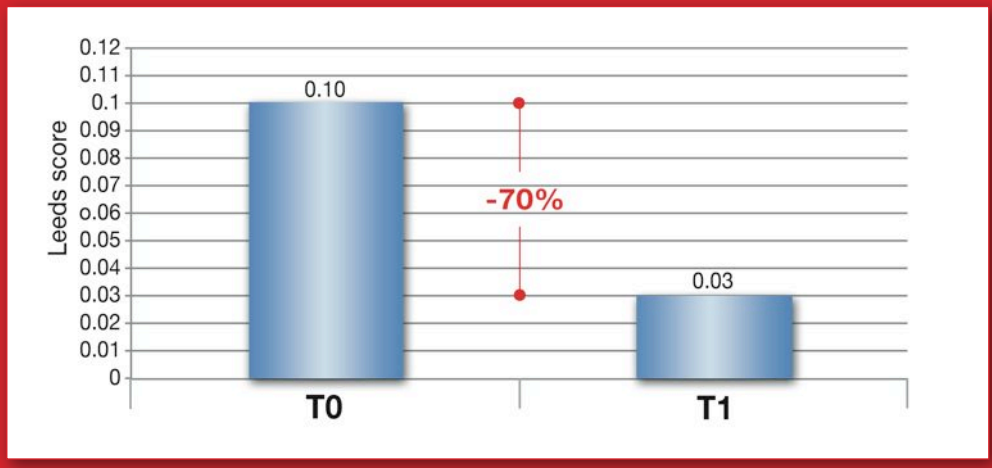

Figure 1. Leeds score before and after treatment.

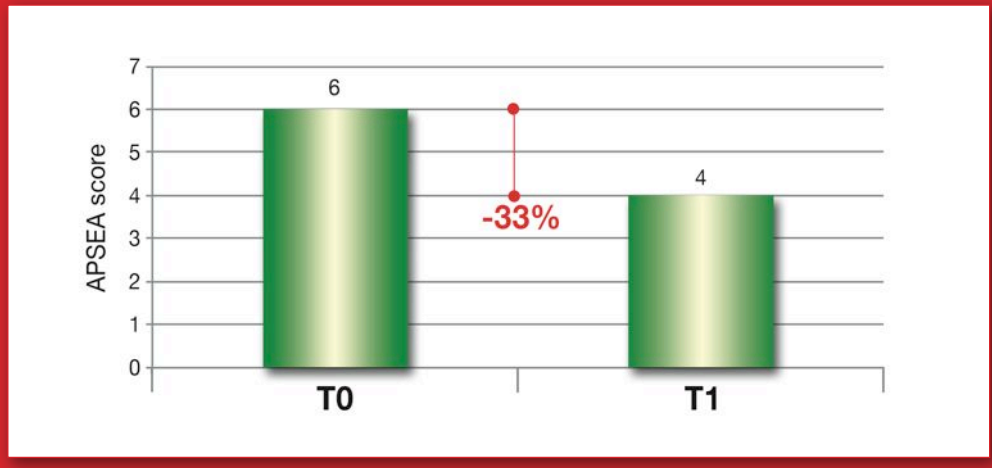

Figure 2. Psychological distress before and after treatment.

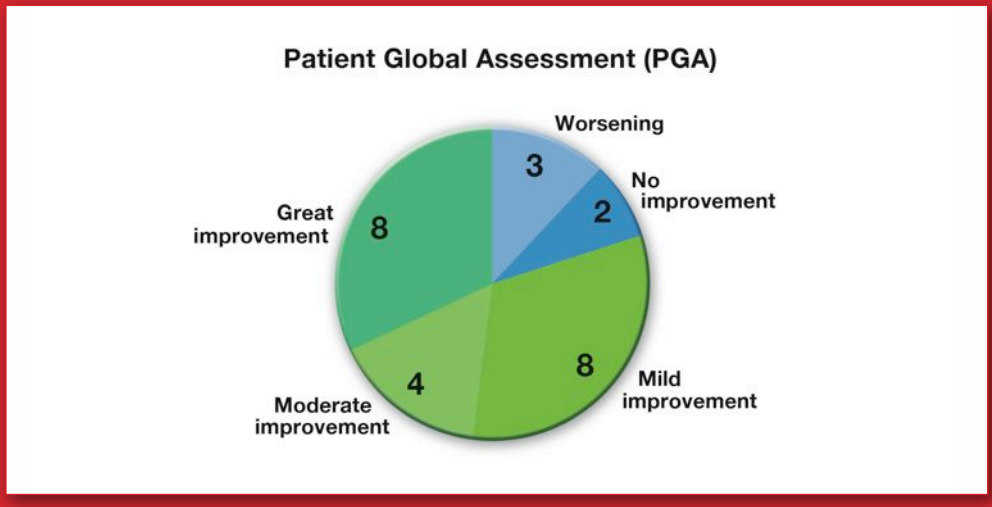

Figure 3. PGA after 8 weeks of treatment with Diakon Krem.

The medium psychological distress was reduced from 6 (range 0-10) to 4 (range 0-9), corresponding to a mean reduction of 33% (P = 0.055) (Figure 2).

As far as PGA is concerned, 20 patients (80%) referred a positive result: 8 patients referred a "*great improvement*", 4 a "*moderate improvement*" and 8 a "*mild improvement*". "*No improvement*" was reported by 2 patients and "*worsening*" by 3 patients (Figure 3).

None of the patients complained any side effects and the topical was well tolerated by all the subjects.

Discussion

The role of *P. acnes* in the pathogenesis of acne is well established. The evidence suggests a gradual increase of strains of *P. acnes* resistant to the most commonly topical antibiotics used in acne treatment over the last 20 years.

Obviously the presence of *P. acnes* resistant to antibiotic reduce the efficacy of the acne treatment increasing the psychological discomfort and reducing the patient's compliance.

Moreover, in order to act also against strains of *P. acnes* resistant to antibiotic new substances with antimicrobial properties have been developed.

The study of bactericidal substances is particularly timely considering the recent increase of new resistant strains of bacteria to the most potent antibiotics [10, 11].

This has promoted research in the well known activity of silver ions and silver-based compounds, including micronized silver.

The medical properties of silver have been known for over 2,000 years.

Micronised silver, due to their unique properties, find use in many day-to-day applications in human life and most importantly in the medical field as a bactericidal and as a therapeutic agent.

Though micronised silver find use in many antibacterial applications, the action of this metal on microbes is not fully known. It has been hypothesized that micronised silver can cause cell lysis or inhibit cell transduction.

There are various mechanisms involved in cell lysis and growth inhibition. Micronised silver have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell.

There is formation of '*pits*' on the cell surface, and there is accumulation on the cell surface [12].

Several studies have shown the beneficial effect of salts of zinc in the treatment of inflammatory acne lesions of mild and moderate type, so that in some European countries (France) this compounds have been used in the last 30 years as a real drug.

The mechanisms of action of zinc in the treatment of mild to moderate inflammatory acne lesions are not yet fully understood.

It has been shown that zinc exerts a bacteriostatic activity against *P. acnes*.

Zinc also inhibits the chemotaxis of polymorphonuclear cells, the activity of natural killer cells (NK) and the phagocytic ability of granulocytes.

Moreoveor, zinc has a protective effect against the development of bacterial resistance, however, mechanisms by which zinc acts are unknown.

In addition to this, zinc salt have a specific inhibitory action on 5α reductase type I, as demonstrated in vitro, resulting in anti-androgen activity and anti-hyperseborrhoea [13].

The strong bactericidal properties of lauric acid (C12:0), a middle chain-free fatty, have been shown in a number of studies.

Incubation of the skin bacteria *P. acnes*, *Staphylococcus aureus* (*S. aureus*), and *Staphylococcus epidermidis* (*S. epidermidis*) with lauric acid yielded minimal inhibitory concentration (MIC) values against the bacterial growth over 15 times lower than those of benzoyl peroxide (BPO).

The lower MIC values of lauric acid indicate stronger antimicrobial properties than that of BPO.

The detected values of half maximal effective concentration (EC50) of lauric acid on *P. acnes*, *S. aureus*, and *S. epidermidis* growth indicate that *P. acnes* is the most sensitive to lauric acid among these bacteria [14].

Conclusion

In vitro, *Diakon Krem* has shown not only to be active against *P. acnes* resistant to the common antibiotic used in acne therapy (erytromycin, clyndamycin and tetracyclines), but also to have the same MIC both in the sensitive strain and in all the resistant strains. So *Diakon Krem* seems not to be influenced by *P. acnes* antibiotic-resistant.
In vivo, the topical seems efficacy to reduce acne severity and well tolerated.

This clinical study presents some limitations: the small number of patients enrolled and the period of treatment limited to 8 weeks.
It should be considered as a pilot study.
Further studies including a high number of patients for a longer period of time are needed. It should also be interesting to assess the efficacy of this treatment in association with other classical acne treatment such as retinoids.

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