

# In Search for New Antipsoriatic Agents: NAD<sup>+</sup> Topical Composition

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## Key Words

Nicotinamide adenine dinucleotide · Psoriasis · Antipsoriatic therapy

## Abstract

The aim of the study was to examine the effectiveness of the oxidized form of nicotinamide adenine dinucleotide (NAD<sup>+</sup>), adenosine precursor, in 37 patients suffering from psoriasis. As NAD<sup>+</sup> is known to be relatively unstable, the second goal was to establish the proper conditions for the satisfactory stability of topical NAD<sup>+</sup> composition. In each patient, two matching plaques were selected for the study. Topical treatment with 1 or 0.3% NAD<sup>+</sup> in Vaseline ointment administered twice daily was compared with overnight therapy with 0.1% anthralin applied for 12 h and placebo. The enzymatic method was applied to determine the stability of NAD<sup>+</sup> in Vaseline ointment. After a 4-week application, the reduction in erythema, infiltration and desquamation caused by 1 or 0.3% topical NAD<sup>+</sup> composition was similar to the reduction caused by 0.1% anthralin. It was demonstrated that NAD<sup>+</sup> underwent a considerable decomposition at room temperature, while it was sufficiently stable at 5°C; thus, for a longer use the agent should be stored at fridge temperature. NAD<sup>+</sup> therapy combines good efficacy, cosmetic acceptability and convenient twice-daily application.

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## Introduction

Psoriasis is a chronic disease observed in 1–3% of human population worldwide. However, the exact pathogenesis of this entity is still not completely understood. Both genetic and environmental factors are considered. The most common skin lesions are well defined indurated erythematous scaling plaques. Hyperproliferation of keratinocytes and inflammatory cell infiltrate with neutrophils and Th1 type T lymphocytes predominance, are responsible for clinical abnormalities. The currently available treatments include topical therapy, phototherapy, systemic agents and combined methods [1]. The most common topical therapies include anthralin (dithranol) [2], regarded by some authors as the gold standard of antipsoriatic therapy [3], vitamin D analogs [3] or topical corticosteroids [1]. As the drugs inhibit DNA synthesis, keratinocyte proliferation is decreased. Lack of adequate efficacy in some patients, adverse effects and the risk of toxicity limit the use of these methods of treatment, thus improved topical therapy for psoriasis is highly awaited.

Recently, it has been demonstrated [4] that adenosine and related nucleotides reversibly inhibit the proliferation of normal and transformed keratinocytes, but subsequent clinical observations have shown that adenosine-containing ointment is not effective in the treatment of psoriasis [Wozniacka et al., unpubl. data]. Xanthines, which are

structurally close to adenosine have also been demonstrated to inhibit fibroblast proliferation [5, 6], but no favorable effect was observed after topical administration [7]. The lack of the therapeutic effect of adenosine and xanthines can be attributed to their poor skin penetration [6].

It has been established that the oxidized form of extracellular  $\beta$ -nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), which is a very important cofactor for many redox reactions in living cells and a substrate for numerous enzymes [8–10], is effectively metabolized to purine and pyridine derivatives by enzymes located in human cells and tissues [11]. Nucleotide pyrophosphatase and  $\text{NAD}$  glycohydrolase located on the outer surface of human fibroblasts are engaged in degradation of extracellular  $\text{NAD}^+$  to nicotinamide, ADP ribose, nicotinamide mononucleotide and adenosine monophosphate [12]. ADP ribose and adenosine monophosphate are then catabolized to adenosine, which was found to be the only  $\text{NAD}^+$  hydrolysis product taken up by cells and further metabolized to ATP [13]. Thus, adenosine generated from adenine nucleotides should act in a different way than that applied exogenously. Those findings encouraged us to return to the concept of purines as antipsoriatic agents by employing  $\text{NAD}^+$  as their precursor. Our previous studies [14–16] on mechanistic aspects concerning oxidation of the reduced  $\beta$ -nicotinamide adenine dinucleotide ( $\text{NADH}$ ) analogs have led to favorable effects of  $\text{NADH}$  application in the treatment of some inflammatory dermatoses [17].

To the best of our knowledge, this report presents the first application of  $\text{NAD}^+$  in topical treatment of psoriasis. As  $\text{NAD}^+$  is known to be relatively unstable [18, 19], the second goal of our work was to establish proper conditions for the satisfactory stability of topical  $\text{NAD}^+$  composition, i.e. a Vaseline ointment. The degree of  $\text{NAD}^+$  decomposition during storage of the ointment was determined by the enzymatic method. The simple spectrophotometric method that was previously applied [17] to determine  $\text{NADH}$  stability would be useless in the case of  $\text{NAD}^+$  as this compound and the products of its degradation absorb in the same spectral region.

## Materials and Methods

### Materials

Nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) free acid (98%) was purchased from NBS Biologicals, UK. Alcohol dehydrogenase (ADH; from baker yeast, >90%), albumin (bovine),  $\text{NaOH}$  and  $\text{Na}_2\text{HPO}_4$  were from Sigma. Acetic acid, n-hexane,  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_4\text{P}_2\text{O}_7$ ,  $\text{H}_3\text{BO}_3$  (all of analytical purity) and ethyl alcohol (99.9%) were from POCh, Poland. Phosphoric acid (85%, purity 99.999%) was from Aldrich. Water was deionized and had a specific con-

ductivity lower than  $0.1 \mu\text{S}/\text{cm}$ . Vaseline (vaselinum album) and paraffin oil (paraffinum liquidum) were purchased from Aflofarm Farmacja (Poland).

### Preparation of Vaseline Ointments Containing $\text{NAD}^+$

$\text{NAD}^+$  was powdered in a mortar, then dried over phosphorus pentoxide under reduced pressure at room temperature until the weight of the sample became constant, and stored at  $5^\circ\text{C}$ . To prepare the ointment, a sample of 0.902 g of the dried  $\text{NAD}^+$  was stirred with 15 g of Vaseline in a large mortar at room temperature until homogenized satisfactorily. Then 225 g of Vaseline and 60 g of paraffin oil were added, and the whole mixture was homogenized by stirring for 10 min to yield 300 g of the ointment containing 0.3% of  $\text{NAD}^+$ . Similar methodology was applied in preparation of the ointment containing 1% of  $\text{NAD}^+$ .

The degree of the nonhomogeneity of  $\text{NAD}^+$  microcrystals distribution in the freshly prepared ointment was estimated as follows: 20 samples (approximately 2 g each) were taken randomly from the ointment; the samples were dissolved in hexane (15 ml) and extracted with 10 ml of phosphate buffer (pH 6.9). The concentrations of  $\text{NAD}^+$  in the extracts were determined spectrophotometrically by measuring the absorbance at  $\lambda_{\text{max}} = 260 \text{ nm}$  and used to calculate  $\text{NAD}^+$  content in the ointment samples. We found out that  $\text{NAD}^+$  content in the analyzed samples followed the normal (Gaussian) distribution and the mean value determined was  $0.309 \pm 0.009\%$ .

### Extraction of $\text{NAD}^+$ from Samples of Vaseline Ointment

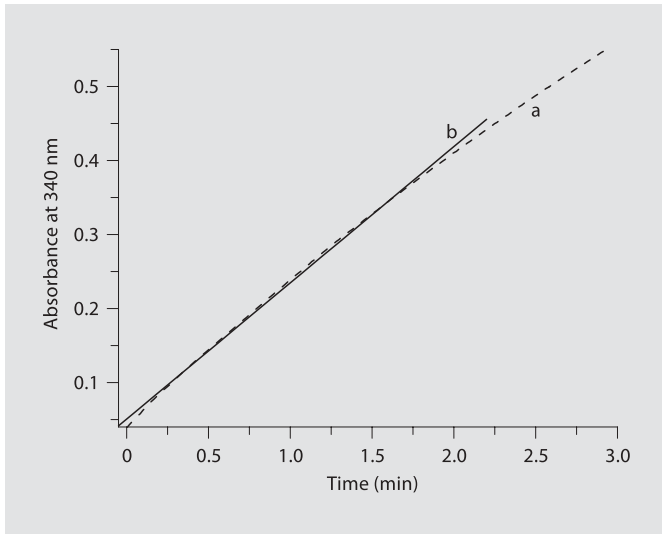
The ointment (approx. 3.5 g) was placed in 100-ml separatory funnel, dissolved in 30 ml of hexane and shaken with 10 ml of phosphate buffer (pH 6.9) for 10 min. The aqueous layer was separated and filtered through Wathman No. 1 filter paper.

### Determination of $\text{NAD}^+$ Content in Vaseline Ointment

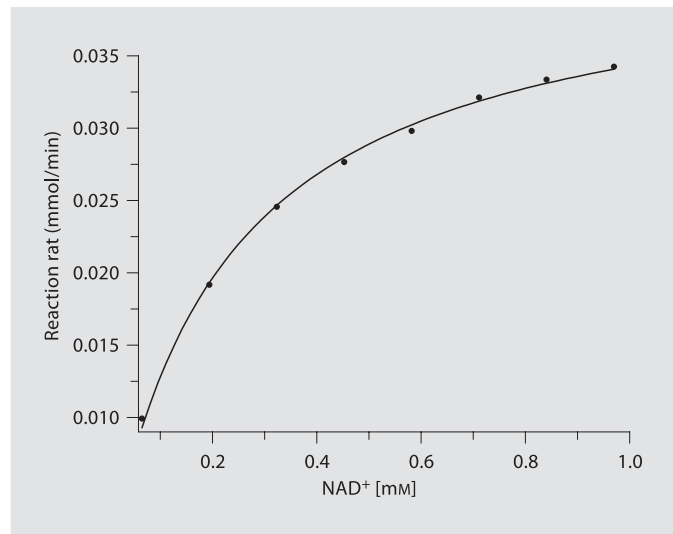
The enzymatic method was applied, which is based on the measurement of the reaction rate of ethanol oxidation by  $\text{NAD}^+$  catalyzed by ADH. The reaction rate was determined by monitoring spectrophotometrically the formation of  $\text{NADH}$  accomplished by determination of the absorbance at 340 nm.



The procedure was as follows. ADH (2 mg) was dissolved in 2 ml of  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer (10 mmol, pH 7.5). The solution (495 U/ml) was diluted to 5 U/ml with 0.1% bovine serum albumin dissolved in the same buffer and kept on ice (no longer than 10 h) to prevent loss of activity. To prepare the reaction mixture, the 5 U/ml enzyme solution (0.1 ml) was added to a cuvette (quartz, 3.5 ml, light path 10 mm) containing  $\text{NAD}^+$  solution (1.5 ml, various concentrations), ethanol (0.1 ml) and phosphate buffer pH 8.8 (1.3 ml, 50 mmol). Reference solution containing the same components and concentrations, except the enzyme, was prepared simultaneously in another cuvette. In order to determine the reaction rate, the changes in absorbance at 340 nm were measured within 3 min of the reaction carried out at  $25^\circ\text{C}$  and plotted against the reaction time (fig. 1). The initial reaction rate  $v$  was calculated from the data taken up to 2 min of the reaction. Before any measurements were undertaken, the new calibration curve (fig. 2) correlating the reaction rate and the concentration of  $\text{NAD}^+$  was independently generated. The concentration of



**Fig. 1.** Changes in NADH absorption at 340 nm with the reaction progress: a – experimental data, b – linear fitting of the data.



**Fig. 2.** Calibration curve (an example) correlating reaction rate of the enzymatic oxidation of ethanol with NAD<sup>+</sup> concentration.

NAD<sup>+</sup> in the extracts from Vaseline ointment was calculated from equation 1:

$$[\text{NAD}^+] = \frac{v \cdot l}{k - v}$$

where  $v$  is the reaction rate and  $k$ ,  $l$  parameters determined from the calibration curve by nonlinear regression method.

Relative errors for the enzymatic method of NAD<sup>+</sup> determination in the ointment was relatively high and estimated as close to  $\pm 20\%$ .

#### *Clinical Characteristic*

Thirty-seven patients (24 females and 13 males aged 22–61 years), Caucasian volunteers, were recruited after the study approval by the local ethics committee. The diagnosis was based on clinical presentation and confirmed by histopathological examination. All of them had similar, chronic psoriatic plaques.

Exclusion criteria were as follows: topical steroid treatment within 2 weeks or systemic treatment for their psoriasis within 4 weeks before enrollment into the study. As NAD<sup>+</sup> is a physiological, nontoxic substance and the patients were in general good condition, only basic laboratory parameters were analyzed before and after the study (complete blood cell account, erythrocyte sedimentation rate, glucose level, urinalysis).

In each patient, two matching psoriatic plaques were selected for the study. Topical treatment with 1% (19 subjects – group 1) or 0.3% (7 subjects – group 2) NAD<sup>+</sup> Vaseline ointment administered twice daily was compared with 12-hour overnight therapy with 0.1% anthralin. Two percent salicylic acid was added to anthralin as an antioxidant and preservative agent, but not for its keratolytic action [20]. The rest of the affected skin was treated only with emollient. The double-blind study was impossible to perform because of a different color of the ointments (NAD<sup>+</sup> Vaseline ointment – white, 0.1% Anthralin – yellow).

To estimate the efficacy of 0.3% of NAD<sup>+</sup> versus vehicle in twice-daily application, we performed a double-blind right-left comparison study in additional 11 patients with similar plaque lesions.

Clinical analysis was based on the evaluation of erythema, infiltration and desquamation using a 5-point scoring system (0 = none to 4 = extremely severe). Therapeutic effects of NAD<sup>+</sup> ointment were estimated after 1, 2 and 4 weeks of follow-up, and global assessment score was calculated on each visit. At the baseline visit, the comparable plaques had the same initial score. Finally, the effect of NAD<sup>+</sup> ointment was classified as better, comparable or worse than the effect of conventional therapy with anthralin.

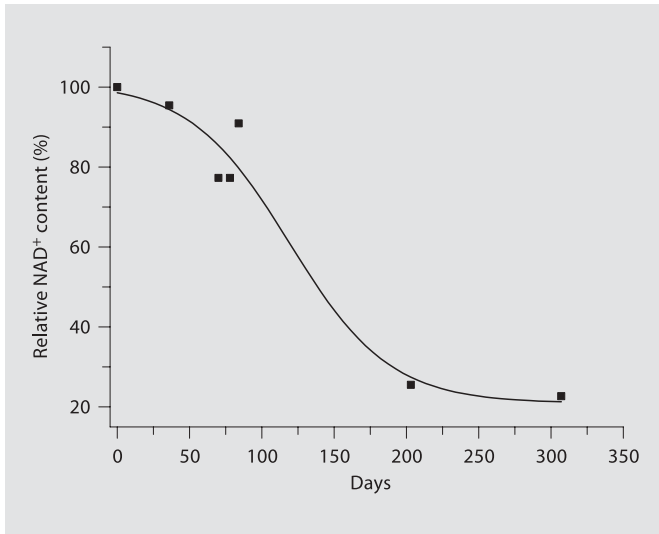
#### *Statistical Analysis*

For the statistical analysis of the obtained data, the mean arithmetic values and standard deviation (SD) have been calculated. The Shapiro-Wilk's test was used to evaluate the distribution. The mean values in the two groups were compared using a  $t$  test for independent samples; in the same group in two different time-using  $t$  test for dependent samples. Frequencies in the two groups were compared using Fisher's exact test. A  $p$  value  $< 0.05$  was considered statistically significant.

## **Results**

### *Stability of NAD<sup>+</sup> in Vaseline Ointment*

The Vaseline ointment containing 0.3% of NAD<sup>+</sup> was split into two parts. One of them was stored at room temperature and the other at 5°C for 10 months. The content of NAD<sup>+</sup> in the ointments was determined by the enzymatic method several times during the storage period, as



**Fig. 3.** Changes in NAD<sup>+</sup> content in Vaseline ointments stored at room temperature.

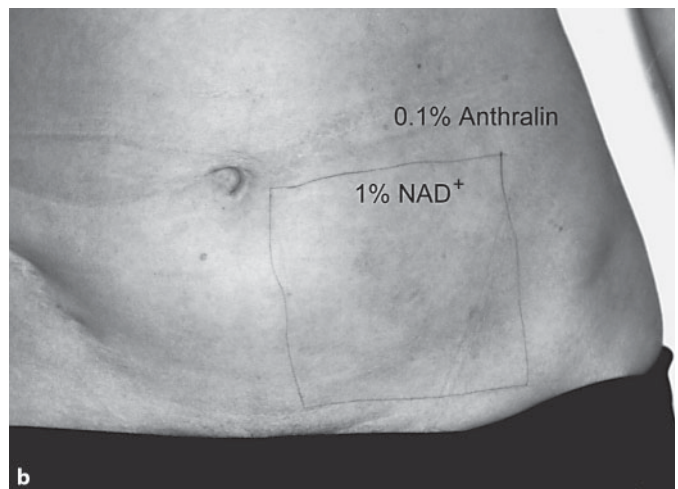
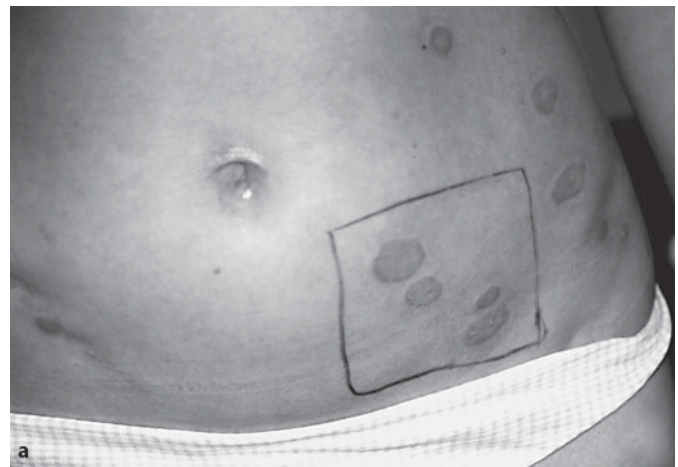
**Table 1.** Mean global assessment score during 4-week therapy

Week	Group 1		Group 2	
	1% NAD <sup>+</sup>	0.1% anthralin	0.3% NAD <sup>+</sup>	0.1% anthralin
0	10.4 ± 1.16	10.4 ± 1.16	9.9 ± 1.35	9.9 ± 1.35
1	9.1 ± 1.28	8.8 ± 1.01	8.7 ± 1.49	8.7 ± 1.49
2	7.8 ± 1.36	7.5 ± 1.22	7.3 ± 1.50	7.7 ± 1.11
4	5.6 ± 1.77	5.4 ± 1.57	5.7 ± 1.25	6.0 ± 1.53

a mean value of three independent measurements. The results obtained for the ointment stored at room temperature (fig. 3, relative content) indicate considerable decomposition of NAD<sup>+</sup>, up to 80% during 10 months of storage. NAD<sup>+</sup> content in the refrigerated ointment did not show any correlation with the storage time and the mean value of the determinations performed during the storage period was 0.30 ± 0.04% expressed as absolute content of NAD<sup>+</sup> in the ointment.

#### Clinical Data

The mean global score at the baseline was 10.4 ± 1.16 SD (group 1) and 9.9 ± 1.35 SD (group 2). The detailed results obtained during 4-week follow-up are presented in table 1. During the 4-week therapy, the mean value of the global score reduced gradually to 5.6 ± 1.77 SD in



**Fig. 4.** Psoriasis patient. **a** Before treatment. **b** After treatment.

group 1 and 5.7 ± 1.25 in group 2 (table 1). The differences between week 0 and 4 were statistically significant ( $p = 0.000000$ ;  $p = 0.000004$ , respectively). The similar reduction was also observed in comparable plaques treated with anthralin, 5.4 ± 1.57 SD and 6.0 ± 1.53 SD ( $p = 0.000000$ ;  $p = 0.000006$ , respectively). We did not find any statistical differences between the results obtained for patients treated with 1% NAD<sup>+</sup>, 0.3% NAD<sup>+</sup> or 0.1% anthralin ( $p > 0.05$  for all comparisons).

We have also compared the efficacy of each treatment method for an individual patient (an example is shown in fig. 4). The better improvement in skin sites treated with NAD<sup>+</sup> ointment was observed in 5 subjects, comparable with anthralin in 18 and weaker response in 3 (table 2).

**Table 2.** Evaluation of the antipsoriatic effect of NAD<sup>+</sup> ointments

Therapeutic effect of NAD <sup>+</sup> ointment as compared to the conventional therapy with anthralin (0.1%)	1% NAD <sup>+</sup> ointment	0.3% NAD <sup>+</sup> ointment
Better	4	1
Comparable	12	6
Worse	3	0
Total	19	7

Figures represent numbers of patients.

NAD<sup>+</sup> ointments in both concentrations were well tolerated. No adverse events were recorded in any of the examined subjects. Anthralin treatment caused brownish staining of the surrounded skin in some patients, but not irritation.

At the baseline visit, the mean global score in the double-blind comparison study between the results obtained in patients treated with ointment containing 1% NAD<sup>+</sup> and without this agent was  $10.0 \pm 1.2$  SD for both groups. After 1 week, the mean global score decreased to  $8.8 \pm 1.49$  in patients treated with 1% NAD<sup>+</sup> versus  $9.5 \pm 1.35$  in the vehicle group. At this point, the differences were not statistically significant,  $p > 0.05$ . After 2 weeks, the respective values were  $7.6 \pm 1.51$  versus  $8.9 \pm 1.39$ , and the differences were statistically significant at  $p < 0.05$ . The greatest differences between the examined groups in mean global score were noted after 4 weeks and were as follows:  $5.8 \pm 1.32$  versus  $7.2 \pm 1.28$ ,  $p = 0.0294$ .

## Discussion

Anthralin, a synthetic derivate of chrysarobin – product of the bark of the araroba tree – was introduced in dermatological treatment by Unna in 1916 [21]. Since that time, many treatment modalities have been applied, but anthralin still plays a central part in psoriasis management as an effective [22] and inexpensive drug. Usually, this agent is applied in increasing concentrations from 0.05 to 4%. Comparative studies demonstrated its efficacy also in a low – 0.1% concentration [23]. Low-strength formulations are not only active, as also confirmed by light microscopy, transmission electron and scanning electron microscopy, but also less irritating and staining [24]. Our observations are in line with the literature data.

The results of our preliminary clinical observations undoubtedly showed that topical NAD<sup>+</sup> has an antipsoriatic potential. However, the double-blind comparison with vehicle demonstrated statistically significant differences after 2 weeks of treatment. The therapeutic effect of NAD<sup>+</sup> (tables 1 and 2) is apparently similar to the effect of 0.1% anthralin. Since no adverse or undesirable effects were observed, the topical NAD<sup>+</sup> therapy can be viewed as a potential alternative to the conventional treatment of psoriasis.

Anthralin, especially in higher concentrations, should be carefully applied to the plaque only, because of its irritant properties. Moreover, in many cases this substance can stain the surrounding normal skin, bathtubs, clothes and anything that comes in contact with it. This is the reason why this method is not acceptable for many patients. In contrast, NAD<sup>+</sup> produced not only a good therapeutic result but also was cosmetically more acceptable.

The only disadvantage of NAD<sup>+</sup> as the potential therapeutic agent seems to be related to its limited stability. As shown in figure 3, almost 30% of NAD<sup>+</sup> suspended in the Vaseline ointment underwent degradation while stored at room temperature for 3 months, and 80% of it was degraded after 10 months. Virtually, no degradation was demonstrated for the ointment stored at 5°C over the same period. Thus, in our opinion NAD<sup>+</sup> ointment can be regarded as an effective topical antipsoriatic agent but it should be stored at refrigerator temperature.

## Acknowledgments

This work was supported by grants from the Medical University of Lodz (503-119-1) and the Ministry of Science and Informationization (PBZ-KBN-101/T09/2003).

## References

- 1 Ashcroft DM, Li Wan Po A, Griffiths CE: Therapeutic strategies for psoriasis. *J Clin Pharm Therap* 2000;25:1–10.
- 2 Müller K: Antipsoriatic and proinflammatory action of anthralin. *Biochem Pharmacol* 1997;53:1225–1221.
- 3 Fogh K, Kragballe K: Recent developments in vitamin D analogs. *Curr Pharm Design* 2000;6:961–972.
- 4 Brown JR, Cornell K, Cook PW: Adenosine and adenine-nucleotide-mediated inhibition of normal and transformed keratinocyte proliferation is dependent upon dipyridamole-sensitive adenosine transport. *J Invest Dermatol* 2000;115:849–859.
- 5 Levi-Schaffer F, Touitou E: Xanthines Inhibit 3T3 fibroblast proliferation. *Skin Pharmacol* 1991;4:286–290.
- 6 Levi-Schaffer F, Dayan N, Touitou E: Diethylene glycol monoethyl ether (Transcutol) displays antiproliferative properties alone and in combination with xanthines. *Skin Pharmacol* 1996;9:53–59.
- 7 Iancu I: Experimental treatment of psoriasis with compounds which increase the intracellular level of cAMP; thesis, Tel Aviv University, 1979.
- 8 Kim UH, Han MK, Park BH, Kim HR, An NH: Function of NAD glycohydrolase in ADP-ribose uptake from NAD by human erythrocytes. *Biochim Biophys Acta* 1993; 1178:121–126.
- 9 Lee HC, Aarhus R: ADP-ribosyl cyclase: an enzyme that catalyzes NAD<sup>+</sup> into a calcium-mobilizing metabolite. *Cell Regul* 1991;2: 203–209.
- 10 Travo P, Muller H, Shuber F: Calf spleen NAD glycohydrolase. Comparison of the catalytic properties of the membrane-bound and the hydrosoluble forms of the enzyme. *Eur J Biochem* 1979;96:141–149.
- 11 Zimmermann H: Extracellular purine metabolism. *Drug Dev Res* 1996;39:337–352.
- 12 Aleo MF, Sestini S, Pompuci G, Preti A: Enzymatic activities affecting exogenous nicotinamide adenine dinucleotide in human skin fibroblasts. *J Cell Physiol* 1996;167:173–176.
- 13 Aleo MF, Gindici ML, Sestini S, Pompuci G, Preti A: Metabolic fate of extracellular NAD in human skin fibroblasts. *J Cell Biochem* 2001;80:360–366.
- 14 Gębicki J, Marcinek A, Adamus J, Paneth P, Rogowski J: Structural aspects and rearrangement of radical cations generated from NADH analogues. *J Am Chem Soc* 1996;118: 691–692.
- 15 Gębicki J, Marcinek A, Zielonka J: Transient species in the stepwise interconversion of NADH to NAD<sup>+</sup>. *Acc Chem Res* 2004;37: 379–386.
- 16 Marcinek A, Adamus J, Huben K, Gębicki J, Bartczak TJ, Bednarek P, Bally T: Hydrogen-transferred radical cations of NADH model compounds. 1. Spontaneous tautomerization. *J Am Chem Soc* 2000;122:437–443.
- 17 Wozniacka A, Sysa-Jędrzejowska A, Adamus J, Gębicki J: Topical application of NADH for the treatment of rosacea and contact dermatitis. *Clin Exp Dermatol* 2003;28:61–63.
- 18 Ganti T, Fodor J: Studies on the kinetics of NAD-decomposition. *Acta Physiol Acad Sci Hung* 1965;26:199–205.
- 19 Lawry OH, Passonneau JV, Rock MK: The stability of pyridine nucleotides. *J Biol Chem* 1961;236:2756–2759.
- 20 Braun-Falco O, Plewig G, Wolf HH, Burgdorf WHC: *Dermatology*. Heidelberg, Springer, 2000, p 597.
- 21 Unna PG: Cignolin als Heilmittel der Psoriasis. *Dermatol Wochenschr* 1916;62:116–137.
- 22 Griffiths CE, Camp RD, Barker JN: Psoriasis; in Burns T, Breathnach S, Cox N, Griffiths C (eds): *Rook's Textbook of Dermatology*. Oxford, Blackwell Science, 2004, pp 35.1–35.69.
- 23 Lowe NJ, Breeding J: Anthralin. Different concentration effects on epidermal cell DNA synthesis rates in mice and clinical responses in human psoriasis. *Arch Dermatol* 1981; 117:698–700.
- 24 Montes LF, Wilborn WH, Brody I: Low strength anthralin in psoriasis. *J Cutan Pathol* 1979;6:445–456.