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Effect of melanin on netilmicin-induced inhibition of collagen biosynthesis in human skin fibroblasts

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Abstract—It is known that various drugs form complexes with melanins and that melanins are abundant constituents of the inner ear. In this study, we determined whether the aminoglycoside antibiotic, netilmicin, interacts with melanin and how this process affects collagen biosynthesis in cultured human skin fibroblasts. The obtained results indicate that netilmicin forms stable complexes with melanin characterized by the association constants $K_1 \sim 10^6\ M^{-1}$ and $K_2 \sim 10^3\ M^{-1}$. We have suggested that prolidase, an enzyme involved in collagen metabolism, may be one of the targets for aminoglycoside-induced inhibition of collagen biosynthesis. We found that netilmicin strongly induced inhibition of prolidase activity ($IC_{50} < 5\ \mu M$) and collagen biosynthesis ($IC_{50} \sim 10\ \mu M$). At 10 μM concentration of netilmicin, prolidase activity in human skin fibroblasts was inhibited by about 80% and DNA biosynthesis—only by about 25%. Melanin at 100 μg/mL produced about 30% inhibition of collagen biosynthesis and about 30% inhibition of prolidase activity in cultured fibroblasts. However, the addition of melanin (100 μg/mL) to netilmicin-treated cells (10 μM) restored the prolidase activity in fibroblasts to almost 100% of control values and partially reversed the inhibitory action of the drug on collagen and DNA biosynthesis. The data suggest that the ability of netilmicin to form stable complexes with melanin may prevent its toxicity on prolidase activity and collagen biosynthesis.

1. Introduction

Netilmicin is an aminoglycoside antibiotic employed to treat severe infections caused by aerobic gram-negative bacteria; it interferes with protein synthesis in susceptible microorganism. Although most inhibitors of microbial protein synthesis are bacteriostatic, the aminoglycosides are bactericidal. Aminoglycoside antibiotics are widely used as important agents, but serious toxicity is a major limitation to the usefulness of these drugs. Most notable are nephro- and ototoxicity, which can involve both the auditory and vestibular function of the eight cranial nerve.[1,2] The precise mechanism underlying the organ specificity of aminoglycoside-induced ototoxicity is still discussed.

It is known that several toxins and pharmacologic agents may undergo specific interaction with melanins leading to the accumulation of these substances in melanin-rich tissues and to influence their toxicity.[3–5] In view of the fact that melanins are abundant constituents of the inner ear it seems reasonable to suspect that the specificity of aminoglycoside toxicity may result from their ability to form complexes with melanin. This phenomenon may contribute to the accumulation of the antibiotic in pigmented tissues of the inner ear and facilitate a toxic effect on surrounding cells. Fibroblasts, the main collagen synthetizing cells, may be a target for netilmicin. Since collagen is the major constituent of the hearing organ, it seems possible that netilmicin-induced hearing lesions may result from its ability to inhibit collagen biosynthesis in the inner ear.

One of the enzymes involved in collagen biosynthesis is prolidase [E.C.3.4.13.9]. The enzyme is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline.[6] The primary biological function of the enzyme

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involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis.[7] The efficiency of proline recycling from imidodipeptides was found to be about 90%.[8] It is evident that an absence of prolidase will severely impede the efficient recycling of collagen proline.[9] On the other hand, enhanced liver prolidase activity was found during the fibrotic process.[10] This suggests that prolidase, by providing proline for collagen biosynthesis, may regulate turnover of collagen and may be a rate limiting factor in the regulation of collagen production. Recently, the correlation has been found between collagen production and prolidase activity in cultured human skin fibroblasts treated with anti-inflammatory drugs,[11] anthracyclines,[12,13] during experimental aging of cells,[14] fibroblast chemotaxis,[15] and cell surface integrin receptor ligation.[16] Therefore, prolidase activity (despite the collagen gene expression) may be a step limiting factor in the regulation of collagen biosynthesis. Previously it has been documented that antineoplastic anthracyclines: doxorubicin,[12] and daunorubicin,[13] and aminoglycoside antibiotic-gentamicin,[17] inhibit prolidase activity in cultured human skin fibroblasts, suggesting a mechanism for the drug-induced reduction of collagen biosynthesis. Whether the same mechanism can be attributed to netilmicin remains to be determined.

The aim of this study was to examine the ability of netilmicin to form complex with melanin, to determine the stability constants of this complex, and to estimate the effect of melanin on netilmicin-induced inhibition of collagen and DNA biosynthesis in cultured human skin fibroblasts.

2. Results

The binding of netilmicin to DOPA-melanin is presented in Figure 1A. The obtained results demonstrate that the amount of netilmicin bound to melanin increases with increasing of initial drug concentration. It can be seen from the binding isotherm that the amount of netilmicin bound to the melanin polymer reaches a plateau at about 0.5 μmol netilmicin/mg melanin, which reflects the initial concentration of the drug equal to 7×10^{-4} M. The experimental data were analyzed by constructing the Scatchard plot (Fig. 1B) to determine the binding sites and the number of relevant binding classes. The analysis of netilmicin–melanin binding shows that the Scatchard plot is curvilinear with an upward concavity, indicating that at least two classes of independent binding sites must be implicated in netilmicin–melanin complex formation.[4] The calculated binding parameters for the interaction of netilmicin with melanin are the following: strong binding sites with the association constant $K_1 = 1.29 \times 10^6$ M^{-1} and weak binding sites with the association constant $K_2 = 7.12 \times 10^3$ M^{-1}. The number of binding sites is: $n_1 = 0.27$ and $n_2 = 0.35$ μmol netilmicin per mg melanin.

Confluent human skin fibroblasts were used to test the effect of netilmicin on collagen biosynthesis and prolidase activity. The rationale for the use of confluent cells

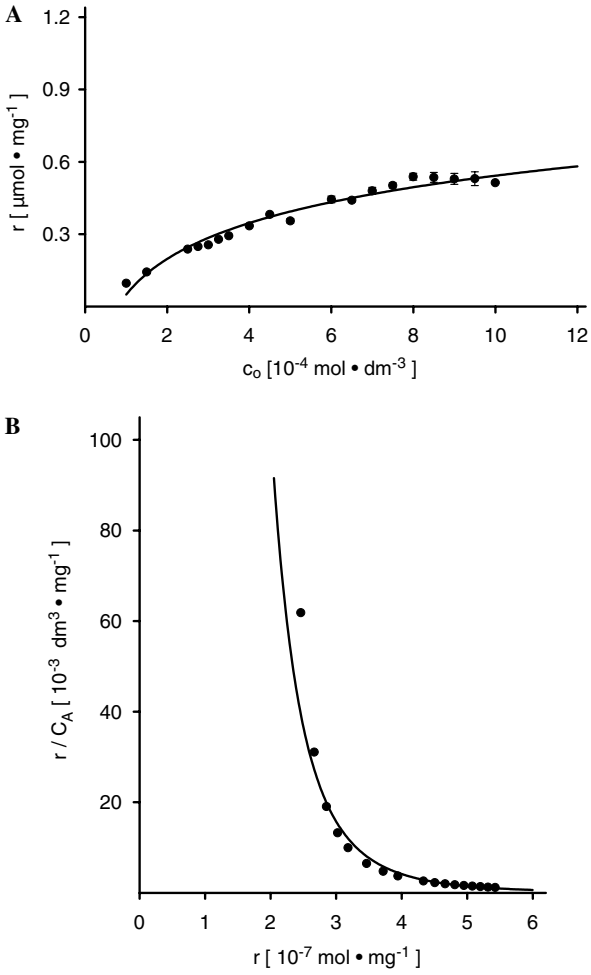

Figure 1. Binding isotherm (A) and Scatchard plot (B) for the netilmicin–melanin complexes obtained after 24 h incubation; c_0-initial drug concentration, r-amount of drug bound to melanin, and c_A-concentration of unbound drug. Mean values ± SD from three independent experiments are presented. Points without error bars indicate that SD was less than the size of the symbol.

in the experiments was that collagen biosynthesis,[18] and prolidase activity,[19] are dependent on cell density and rise when cell density increases. Collagen biosynthesis and prolidase activity were measured in fibroblasts treated for 24 h with different concentrations of netilmicin. It was demonstrated that netilmicin decreased collagen biosynthesis in confluent human skin fibroblasts in a dose-dependent manner (Fig. 2A). IC_{50} for collagen biosynthesis was found as about 10 μM. A similar effect of netilmicin on prolidase activity was stated. The drug strongly decreased fibroblast prolidase activity (Fig. 3A) in a dose-dependent manner. The concentration of netilmicin required for 50% inhibition (IC_{50}) of prolidase activity was below 5 μM. At 10 μM concentration of netilmicin, prolidase activity in human skin fibroblasts was inhibited by about 80% and DNA biosynthesis only by about 25% (Fig. 4A). Melanin also induced an inhibition of these processes. However, at 100 μg melanin/mL the collagen biosynthesis and prolidase activity were decreased only by about 30%

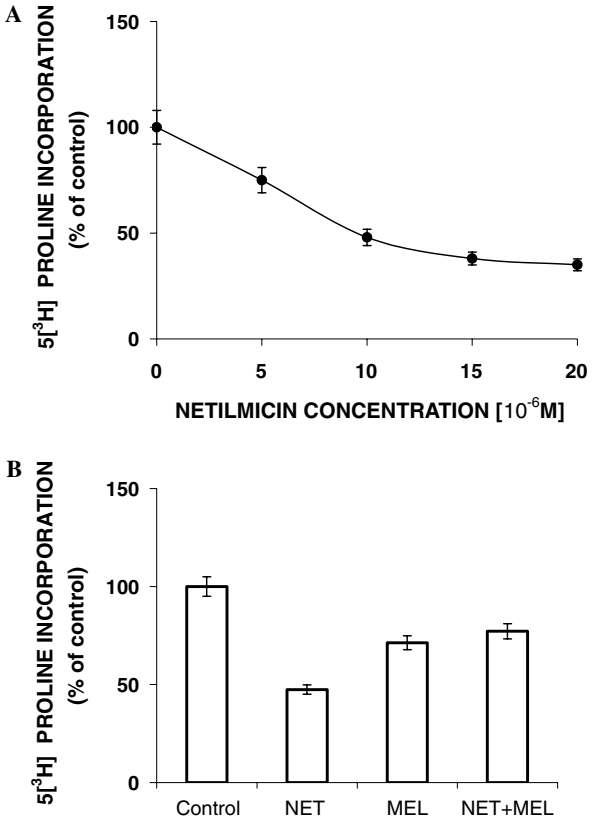

Figure 2. Collagen biosynthesis (measured as 5[^{3}H]proline incorporation into proteins susceptible to the action of bacterial collagenase) in confluent human skin fibroblasts incubated for 24 h with different concentrations of netilmicin (A) and with 10 μM netilmicin (NET), 100 μg/mL melanin (MEL) or both (NET + MEL) (B). Mean values ± SD from three independent experiments done in duplicate are presented.

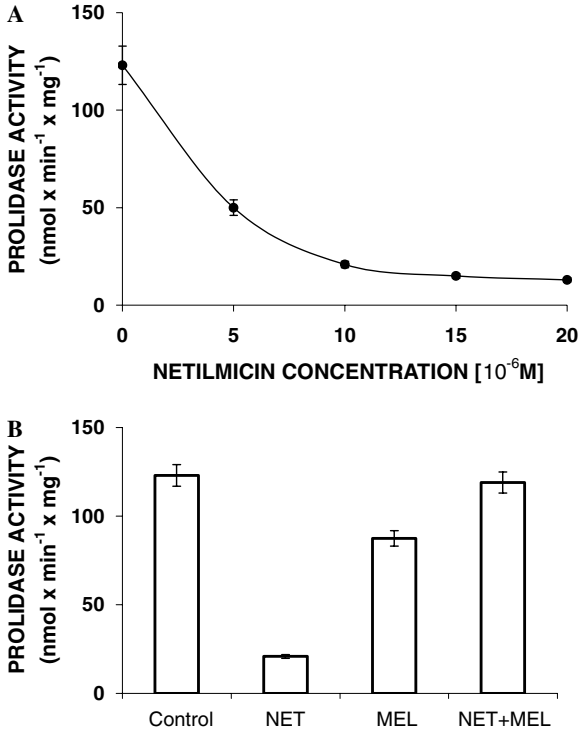

Figure 3. Prolidase activity in confluent human skin fibroblasts incubated for 24 h with different concentrations of netilmicin (A) and with 10 μM netilmicin (NET), 100 μg/mL melanin (MEL) or both (NET + MEL) (B). Mean values ± SD from three independent experiments done in duplicate are presented.

(Figs. 2B and 3B) and no large effect of melanin on DNA biosynthesis was demonstrated (Fig. 4B). Since netilmicin was found to form stable complexes with melanin, we determined the effects of both compounds added simultaneously on prolidase activity, collagen and DNA biosynthesis in confluent fibroblasts. In these experiments netilmicin was used at 10 μM together with 100 μg/mL of melanin. As can be seen from Figure 3B, the addition of melanin to netilmicin-treated cells restored the prolidase activity in fibroblasts from 20% to almost 100% of control values. Simultaneously, the addition of polymer partially reversed the inhibitory action of netilmicin on collagen biosynthesis (Fig. 2B) and DNA biosynthesis (Fig. 4B).

3. Discussion

The mechanism for ototoxicity of aminoglycoside class of antibiotics is still not well known. Although the histopathologic effects of aminoglycoside antibiotics have been well demonstrated,[2] the mechanism by which these agents induce cell damage has remained unclear. It has been hypothesized that uptake of the drug into the hair cells initiates a cascade of irreversible changes in intracellular processes, possibly through inhibition of the phosphoinositide second-messenger system.[20] More recently it has been proposed that toxic metabolites of aminoglycoside antibiotics are responsible for production of ototoxicity in mature hair cells.[21] It has been also suggested that free radicals generated after aminoglycoside treatment play an important role in aminoglycoside cytotoxicity.[1] Studies suggesting that radical scavengers demonstrate variable efficacy in protecting cochlear hair cells against a number of ototoxicants indirectly implicate radical oxygen species in aminoglycoside ototoxicity.[22]

It is known that melanin acts as a biochemical dustbin, mopping up free radicals and other potentially toxic agents.[4] Such properties would be important in protecting pigment cells from natural toxins, oxygen, and reactive oxygen species.[23] Many drugs are known to be markedly accumulated and retained for a considerable time by pigmented tissues and the retention of these compounds is proportional to the degree of melanin pigmentation.[4] This fact may explain the ability of melanin to bind some ototoxic drugs and the differences between the effect of these drugs on the inner ear of pigmented and albino animals.[24–26] It has been suggested that the absence of melanin was underlying cause for the differences in cell volume of the marginal and intermediate cells, which was reflected by differences in the size of endocochlear potential between albino and pigmented animals.[25,26]

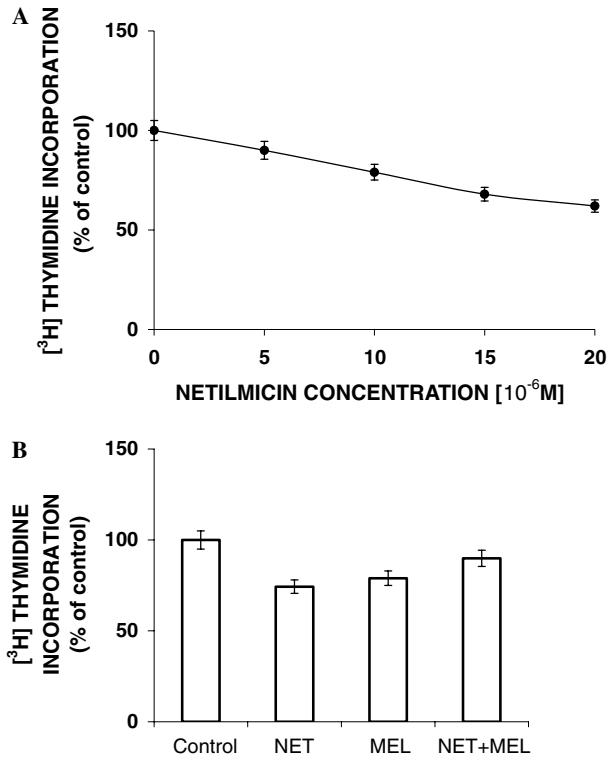

Figure 4. DNA biosynthesis (measured as [^{3}H]thymidine incorporation into DNA) in semiconfluent human skin fibroblasts incubated for 24 h with different concentrations of netilmicin (A) and with 10 μM netilmicin (NET), 100 μg/mL melanin (MEL) or both (NET + MEL) (B). Mean values ± SD from three independent experiments done in duplicate are presented.

The finding that aminoglycosides inhibit collagen biosynthesis[17] led to the hypothesis that the extent of the inhibition in tissues may depend on the abundance of tissue melanin. Our previous study documented that melanin augmented the inhibitory effect of gentamicin on collagen biosynthesis.[17] The results supplemented the data to the mechanism of gentamicin-induced ototoxicity. In fact, collagen represents important constituent of hearing organ. It plays not only structural function as a main extracellular matrix protein, but also it interacts with cell surface receptors that regulate cellular gene expression, differentiation and growth.[16] This mechanism is also required for regulation of prolidase activity that in turn regulates collagen biosynthesis at post translational level. Prolidase providing proline (from collagen degradation products) for collagen biosynthesis plays an important role in regulation of collagen turn-over and may be a rate limiting factor in regulation of collagen production.[8] In respect to melanin-dependent augmentation of gentamicin-induced ototoxicity, we postulated that the mechanism of the process is a consequence of accumulation of the drug in the melanin-rich tissue and constant, gradual release from the complex exerting permanent inhibitory action on the metabolism of surrounding cells. However, it is not universal mechanism for the ototoxicity of aminoglycoside antibiotics. The results of present study on another aminoglycoside antibiotic, netilmicin, suggest that melanin prevents netilmicin-induced collagen biosynthesis inhibition. It suggests that the nature of melanin-drug interaction is of great importance.

The heterogeneity of substances with melanin affinity is large—various drugs of different categories: psychotropic drugs and drugs for rheumatoid arthritis and malaria,[4] local anesthetics,[27] aminoglycoside antibiotics,[17] metal ions,[28] herbicydes, and alkaloids.[4] Melanins are polyanions with relatively high content of carboxy groups and *o*-semiquinones,[29,30] which are negatively charged at physiological pH. Substances with cationic properties (e.g., metal ions, some drugs) are thus bound to melanin mainly by ionic interaction, which also may be strengthened by other forces such as van der Waals attraction, charge-transfer reactions, and hydrophobic interactions.[4] Drug accumulation in pigmented tissues is of considerable interest and is generally believed to be the most important factor in the etiology of toxic retinopathy, hyperpigmentation of the skin, hair bleaching, irreversible extrapyramidal disorders, and some ocular and inner ear lesions.[31,32]

In view of this fact and taking into account that melanin is an abundant constituent of the inner ear, we determined whether netilmicin interacts with melanin and how this process affects the biosynthesis of collagen (an important structural and functional constituent of hearing organ) in cultured fibroblasts. The data presented here show that melanin forms stable complexes with netilmicin and that at least two classes of independent binding sites are implicated in complex formation. The two calculated binding sites in the netilmicin–melanin complex may be due to a different accessibility of the melanin binding sites. This may be explained by differences between the surface and the interior of the melanin polymer with regard to steric hindrances and physicochemical conditions, as was earlier suggested.[4] The biological impact of drug-melanin interactions has received considerable attention for the past years,[4,33] but the physiological meaning of melanin binding is still not fully understood.

The data presented here show that netilmicin induced a strong inhibition of prolidase activity in cultured human skin fibroblasts ($IC_{50} < 5$ μM). Simultaneously, the decrease in the enzyme activity was accompanied by a decrease in biosynthesis of protein susceptible to the action of bacterial collagenase, that is, collagen. The IC_{50} for collagen biosynthesis was about 10 μM. At this concentration of netilmicin prolidase activity in human skin fibroblasts was inhibited by about 80%. Therefore, it seems that the inhibition of collagen biosynthesis by netilmicin is mostly due to the inhibition of prolidase activity. Melanin reversed the inhibitory effect of netilmicin on collagen biosynthesis and prolidase activity in fibroblasts. One possible explanation for this phenomenon may be that the netilmicin–melanin complex (which has a relatively high stability constant) very slowly dissociates during the 24 h incubation of cultured cells and therefore has almost no effect on the metabolism of the cells. It seems possible that the mechanism of the melanin-dependent effect of netilmicin on cell metabolism involves: (i) the ability of melanin to form a

relatively stable complex with netilmicin, (ii) the accumulation of the drug in the extracellular matrix, and (iii) the relatively low release of netilmicin from the complex so that it no exerts an inhibitory action on collagen biosynthesis and prolidase activity.

Our previous and present studies suggest that melanin affects pharmacological activity of different drugs, including aminoglycoside antibiotics, and that the effect of melanin depends rather on the nature of melanin-drug interaction than on the amount of melanin in tissues. In some cases we found melanin as a depository for the compound without any discernible toxicity,[34] in others melanin augmented the pharmacological effect of drug.[35] In every specific case, possible adverse consequences of drugs may include: 1/high concentration of drug may produce damage to tissue accumulating the molecule; 2/binding of drugs to melanin may alter its role of free radical sink, thereby triggering a deleterious effect or 3/slow release of the drug from melanin may prolong the exposure of tissue to potentially adverse or beneficial effects.[36] Therefore, many factors (tissue melanin content, drug concentration, the nature of drug-melanin interaction, stability constant of the complex, duration of exposure of tissue to the drug, etc.) may determine tissue specific toxicity of drugs.

The data presented in this paper show that the high stability constant of netilmicin–melanin complex may be responsible for the relatively low netilmicin-induced hearing lesions seen in some patients administered this drug as compared with other aminoglycoside antibiotics.

4. Experimental

4.1. Materials

Glycyl-proline (Gly-Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin (BSA), and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma Chemical Co. (USA), as were most other chemicals used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were obtained from Life Technologies (USA). Glutamine, penicillin, and streptomycin were obtained from Quality Biologicals Inc. (USA). L-5[^{3}H]Proline (28 Ci/mmol) was purchased from Amersham (UK). [^{3}H]-Thymidine (6.7 Ci/mmol) was obtained from NEN (USA). Netilmicin (Netromycin) was obtained from Shering-Plough (Belgium).

4.2. Preparation of melanin

Model synthetic melanin was obtained by oxidative polymerization of L-DOPA solution (1 mg/mL) in 0.067 M phosphate buffer (pH 8.0) for 48 h, according to the method of Binns et al.[37]

4.3. Preparation of netilmicin–melanin complex

Netilmicin–melanin complexes were obtained as follows: 5 mg of melanin was placed in plastic test-tubes, to which drug solutions in 0.067 M phosphate buffer at pH 7.0 were added to a final volume of 5 mL. The initial concentration of netilmicin ranged from 1×10^{-4} M to 1×10^{-3} M. Control samples contained 5 mg of melanin and 5 mL of phosphate buffer without drug. All samples were incubated for 24 h at room temperature and then filtered.

4.4. Analysis of netilmicin binding to melanin

The concentrations of netilmicin remaining in each filtrate after incubation with melanin were determined spectrophotometrically using chloranil as colored reagent.[38] All spectrophotometric measurements were made using the UV–vis spectrophotometer JASCO model V-530. The amounts of netilmicin bound to melanin, calculated as differences between the initial amounts of drug administered to melanin and the amounts of unbound drug (in filtrates after incubation), were expressed in μmol of bound drug per 1 mg melanin.

A qualitative analysis of netilmicin–melanin interaction was performed using the Scatchard plot of the experimental data according to Kalbitzer and Stehlik.[39] The number of binding sites (n) and the values of association constant (K) were calculated.

4.5. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from 11 years old male donor, were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C under 5% CO_2 in an incubator. The cells were used between 12th and 14th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) in calcium-free phosphate-buffered saline (PBS). For the experiments, cells were counted in hemocytometers and cultured at 1×10^5 cells per well in 2 mL of growth medium in six well plates (Costar). Cells reached confluency at day 6th and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug (netilmicin), melanin or both substances added to the growth medium.

4.6. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells with 5[^{3}H]proline (5 μCi/mL, 28 Ci/mmol) in growth medium with varying concentrations of netilmicin, melanin or both for 24 h, as described previously.[40] Incorporation of tracers into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al.[41] Results are shown as combined values for cell plus medium fractions.

4.7. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al.[42] which is based on the measurement of proline with Chinard's reagent.[43]

The monolayer was washed three times with 0.15 M NaCl. Cells were collected by scrapping, suspended in 0.15 M NaCl, and centrifuged at low speed (200*g*); the supernatant was discarded. The cell pellet (from two wells) was suspended in 0.3 mL of 0.05 M Tris–HCl, pH 7.8, and sonicated three times for 10 s at 0 °C. Samples were then centrifuged (18,000*g*, 30 min) at 4 °C. Supernatant was used for protein determination and the prolidase activity assay. Activation of prolidase requires preincubation with manganese, therefore 0.1 mL of supernatant was incubated with 0.1 mL of 0.05 M Tris–HCl, pH 7.8, containing 20 mM $MnCl_2$ for 2 h at 37 °C. After preincubation, the prolidase reaction was initiated by adding 0.1 mL of the preincubated mixture to 0.1 mL of 94 mM glycyl-proline (Gly-Pro) to a final concentration of 47 mM. After additional incubation for 1 h at 37 °C, the reaction was terminated with 1 mL of 0.45 M trichloroacetic acid. In the parallel tubes reaction was terminated at time 'zero' (without incubation). The released proline was determined by adding 0.5 mL of the trichloroacetic acid supernatant to 2 mL of a 1:1 mixture of glacial acetic acid/Chinard's reagent (25 g of ninhydrin dissolved at 70 °C in 600 mL of glacial acetic acid and 400 mL of 6 M orthophosphoric acid) and incubated for 10 min at 90 °C. The amount of proline released was determined colorimetrically at 515 nm and calculated by using calibration curve for proline standards. Protein concentration was measured by the method of Lowry et al.[44] Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

4.8. DNA biosynthesis assay

To examine the effect of the studied substances on fibroblast proliferation, the cells were plated in 24-well tissue culture dishes at 1×10^5 cells/well with 1 mL of growth medium. After 48 h ($1.6 \pm 0.1 \times 10^5$ cells/well), the plates were incubated with various concentrations of netilmicin with or without 100 μg/mL melanin for 24 h at 37 °C. Then 0.5 μCi [^{3}H]thymidine (6.7 Ci/mmol) was added to the wells and the cells were incubated in 37 °C for 4 h. After that time cell surface was rinsed three times with 1 mL of 0.05 M Tris–HCl and two times with 5% trichloroacetic acid. Then, the cells were lysed in 1 mL of 0.1 M NaOH containing 1% SDS. The cell lysate was added to 4 mL of scintillation liquid and radioactivity incorporation into DNA was measured in scintillation counter.

4.9. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (SD) were calculated, unless otherwise indicated. The results were submitted to statistical analysis using Student's *t*-test, accepting $p < 0.05$ as significant.

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