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European Journal of Pharmacology 446 (2002) 7–13

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Melanin potentiates gentamicin-induced inhibition of collagen biosynthesis in human skin fibroblasts

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Received 10 December 2001; received in revised form 1 May 2002; accepted 6 May 2002

Abstract

One of the recognized side effects of gentamicin is ototoxicity. The mechanism underlying the organ specificity of this side effect of gentamicin has not been fully established. In view of the fact that a number of pharmacologic agents are known to form complexes with melanin and melanin is an abundant constituent of the inner ear tissues, we determined whether gentamicin interacts with melanin and how this process affects the biosynthesis of collagen in cultured human skin fibroblasts. Our results indicate that gentamicin forms complexes with melanin. The amount of gentamicin bound to melanin increases with increasing of initial drug concentration. The Scatchard plot analysis of drug binding to melanin showed that at least two classes of independent binding sites are implicated in gentamicin–melanin complex formation: one class with an association constant $K_1 \sim 4 \times 10^3\ M^{-1}$, and the second class with an association constant $K_2 \sim 3 \times 10^2\ M^{-1}$. The number of total binding sites ($n_1 + n_2$) was calculated as about 1.36 μmol gentamicin per 1 mg melanin. We have suggested that prolidase, an enzyme involved in collagen metabolism, may be one of the targets for gentamicin-induced inhibition of collagen biosynthesis. We found that gentamicin-induced inhibition of prolidase activity ($IC_{50} \sim 100$ μM) and collagen biosynthesis ($IC_{50} \sim 100$ μM). At this concentration of gentamicin, DNA biosynthesis in human skin fibroblasts was inhibited only by about 30%. Melanin at 100 μg/ml produced about 25% inhibition of DNA synthesis and about 30% inhibition of prolidase activity, but it had no effect on collagen biosynthesis in cultured fibroblasts. However, the addition of melanin (100 μg/ml) to gentamicin-treated cells (100 μM) augmented the inhibitory action of gentamicin on collagen and DNA biosynthesis and partially reversed its inhibitory effect on prolidase activity. A melanin-induced augmentation of the inhibitory effects of gentamicin on collagen and DNA biosynthesis may explain the mechanism for the organ specificity of gentamicin-induced hearing loss in patients administered this drug.

Keywords: Melanin; Collagen; Gentamicin; Prolidase; Fibroblast

1. Introduction

Gentamicin belongs to the aminoglycoside class of antibiotics that has wide spectrum of antibacterial activity. The use of gentamicin is accompanied by several untoward side effects (Huang and Schacht, 1990; Seligmann et al., 1996; Tran Ba Huy et al., 1983). One of them is ototoxicity (Govaerts et al., 1990). However, the precise mechanism underlying the organ specificity of gentamicin-induced toxicity is not understood.

It is known that several toxins and pharmacologic agents (antibiotics, psychotropic, antirheumatic and anesthetic agents) may undergo a specific interaction with melanin, leading to the accumulation of these agents in melanin-rich tissues and to an increase in their toxicity (Sarna, 1992). The symptoms of their accumulation in melanin-rich tissues may include neuropathy, retinopathy, skin hyperpigmentation and hearing loss (Larsson and Tjälve, 1979; Larsson et al., 1988).

In view of the fact that melanin is an abundant constituent of the inner ear, it seems reasonable to suspect that the specificity of gentamicin toxicity may result from its ability to form a complex with melanin. This phenomenon may contribute to the accumulation of the antibiotic in the inner ear and facilitate a toxic effect on surrounding cells. Fibroblasts, the main collagen synthesizing cells, may be a target

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0014-2999/02/$ - see front matter
PII: S0014-2999(02)01793-4

for gentamicin. Since collagen is the major constituent of the hearing organ, it seems possible that gentamicin-induced hearing loss 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 a C-terminal proline (Endo et al., 1989; Phang and Scriver, 1989). The biological function of the enzyme involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis (Chamson et al., 1989). The efficiency of proline recycling from imidodipeptides is about 90% (Jackson et al., 1975). Therefore, prolidase activity (despite collagen gene expression) may be a limiting factor in the regulation of collagen biosynthesis. Previously, it has been documented that doxorubicin inhibits prolidase activity in cultured human skin fibroblasts (Muszyńska et al., 1998), suggesting a mechanism for the anthracycline-induced reduction of collagen biosynthesis. Whether the same mechanism can be attributed to gentamicin remains to be determined.

In the present study, we examined the ability of gentamicin to form a complex with melanin, the stability constants of this complex and the effect of melanin on the gentamicin-induced inhibition of prolidase activity, DNA and collagen biosynthesis in cultured human skin fibroblasts.

2. Materials and methods

2.1. Materials

Glycyl–proline (Gly–Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin and L-3, 4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma (USA), as were most other chemicals used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum used in cell culture were obtained from Life Technologies (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals (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). Gentamicin was obtained from BUFA (Holland).

2.2. Preparation of melanin

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. (1970).

2.3. Preparation of gentamicin–melanin complex

Gentamicin–melanin complexes were obtained as follows: 5-mg amounts of melanin were 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 gentamicin ranged from 7×10^{-4} to 1×10^{-2} M. Control samples contained 5 mg of melanin and 5 ml of 0.067 M phosphate buffer without drug. All samples were incubated for 24 h at room temperature and then filtered.

2.4. Analysis of gentamicin binding to melanin

The concentration of gentamicin remaining in each filtrate after incubation with melanin was determined spectrophotometrically, using *p*-dimethylaminobenzaldehyde as a color reagent (Wang et al., 1993). The amount of gentamicin bound to melanin (calculated as the difference between the initial amount of the drug added to melanin and the amount of unbound drug in the filtrate after incubation) is expressed in micromoles of bound drug per 1 mg melanin.

A qualitative analysis of the gentamicin–melanin interaction was performed using the Scatchard plot of the experimental data according to Kalbitzer and Stehlik (1979). The number of binding sites (n) and the values of association constant (K) were calculated.

2.5. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from an 11-year-old male donor, were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 50 U/ml penicillin, 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 and 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/well in 2 ml of growth medium in six-well plates (Costar). Cells reached confluence at day 6 and in most cases, such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug (gentamicin), melanin or both added to the growth medium.

2.6. 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 containing various concentrations of gentamicin, melanin or both for 24 h as described previously (Oyamada et al., 1990). Incorporation of tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

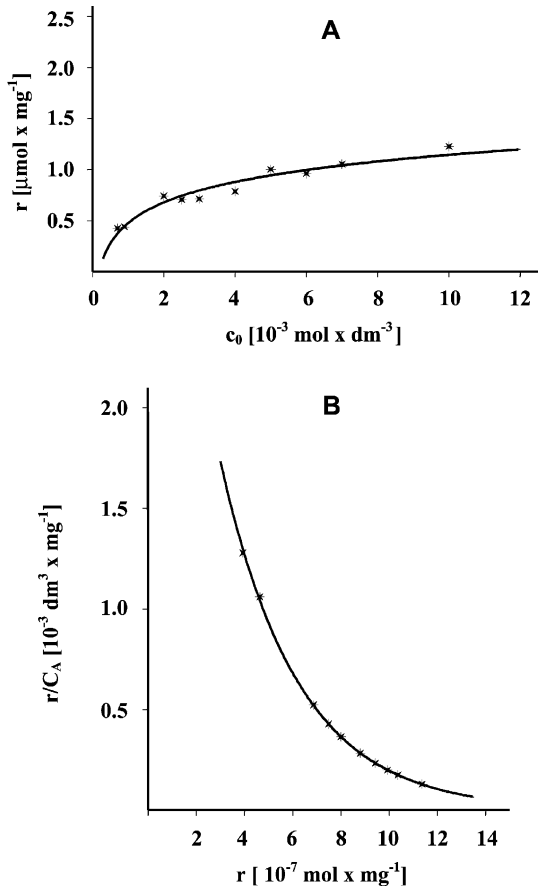

Fig. 1. Binding isotherm (A) and Scatchard plot (B) for the gentamicin–melanin complex; c_0—initial concentration of gentamicin, r—amount of gentamicin bound per 1 mg melanin, C_A—concentration of unbound gentamicin.

2.7. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. (1982), which is based on the measurement of proline with Chinard's reagent (Chinard, 1952). Briefly, the monolayer was washed three times with 0.15 M NaCl. Cells were scraped off and 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, 10 min) at 4 °C. Supernatant was used for protein determination and for the prolidase activity assay. Activation of prolidase requires preincubation with manganese, and 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 this 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 an additional incubation for 1 h at 37 °C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes, the reaction was terminated at time "zero" (without incubation). The amount of proline released 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 a calibration curve for proline standards. Protein concentration was measured by the method of Lowry et al. (1951). Enzyme activity was calculated as nanomoles of proline released per minute per milligram of supernatant protein.

2.8. DNA biosynthesis assay

To examine the effect of the studied substances on fibroblast proliferation, the cells were seeded 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 melanin or gentamicin with or without melanin and 0.5 µCi of [^{3}H]thymidine for 24 h at 37 °C. Cells were rinsed three times with PBS and solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% sodium dodecyl sulfate (SDS). Scintillation liquid (9 ml) was added and radioactivity incorporated into DNA was measured in a scintillation counter.

2.9. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (S.D.) were calculated. The results were submitted to statistical analysis using Student's t-test (Figs. 2–4) or analysis of variance followed by a Student–Newman–Keuls test (ANOVA + SNK) to detect the difference between four studied groups (Figs.

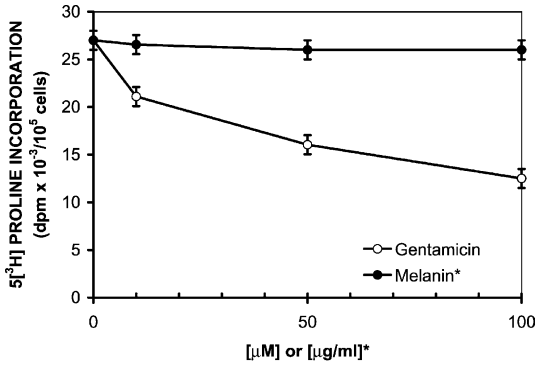

Fig. 2. Collagen biosynthesis (measured as 5[^{3}H]Proline incorporation into proteins susceptible to the action of bacterial collagenase) in confluent human skin fibroblasts incubated for 24 h with different concentrations of gentamicin or melanin. (a) Melanin concentration was reported as micrograms per milliliter of growth medium. Mean values ± S.D. from six independent experiments are presented.

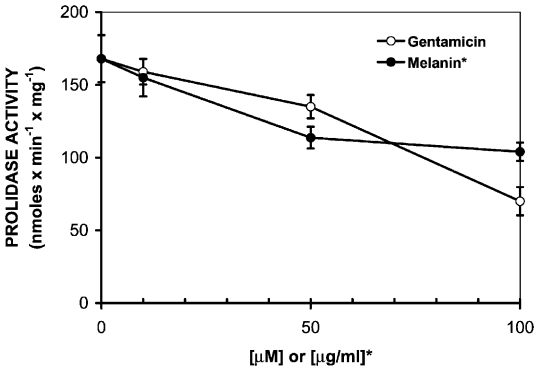

Fig. 3. Prolidase activity in confluent human skin fibroblasts incubated for 24 h with different concentrations of gentamicin or melanin. (a) Melanin concentration was reported as micrograms per milliliter of growth medium. Mean values ± S.D. from six independent experiments are presented.

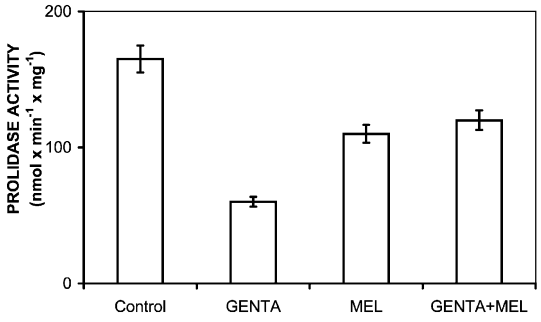

Fig. 5. Prolidase activity in confluent human skin fibroblasts (control) cultured for 24 h with 100 μM gentamicin (GENTA), 100 μg/ml melanin (MEL) or both (GENTA + MEL). Mean values ± S.D. from three independent experiments done in duplicate are presented. ANOVA followed by a Student–Newman–Keuls test showed significance at $P < 0.05$ between four studied groups except MEL/GENTA + MEL.

5–7), accepting $P < 0.05$ as significant. Statistical analysis was done with the Statistica 6.0 PL package.

3. Results

The ability of melanin to bind gentamicin is presented in Fig. 1A. The results demonstrate that the amount of gentamicin bound to melanin increased with increasing initial drug concentration. It can be seen from the binding isotherm that the amount of gentamicin bound to the melanin polymer reached a plateau at about 1 μmol gentamicin/mg melanin, which reflects an initial concentration of the drug equal to 5×10^{-3} M. The data were analyzed by constructing a Scatchard plot (Fig. 1B). Analysis of gentamicin–melanin binding showed that the Scatchard plot was curvilinear with an upward concavity, indicating that at least two classes of independent binding sites are implicated in complex formation (Larsson and Tjälve, 1979; Larsson, 1993). The calculated binding parameters for the interaction of gentamicin with melanin were as follows: one class of binding sites with an association constant $K_1 = 4.39 \times 10^3$ M^{-1} and second class of binding sites with an association constant $K_2 = 3.19 \times 10^2$ M^{-1}. The number of binding sites was $n_1 = 0.603$ and $n_2 = 0.754$ μmol gentamicin per milligram melanin.

Confluent human skin fibroblast was used to test the effect of gentamicin on prolidase activity and collagen biosynthesis. The rationale for using confluent cells in the experiments was that prolidase activity (Myara et al., 1985) and collagen biosynthesis (Makela et al., 1990) are dependent on cell density and increase when the cell density increases. Prolidase activity and collagen biosynthesis were measured in fibroblasts treated for 24 h with different concentrations of gentamicin and melanin. The drug decreased collagen biosynthesis in confluent human skin fibroblasts in a dose-dependent manner (Fig. 2). The IC_{50} for collagen biosynthesis was about 100 μM. Melanin had no significant effect on the process.

A similar effect of gentamicin on prolidase activity and DNA synthesis was found. The drug decreased fibroblast prolidase activity (Fig. 3) and DNA synthesis (Fig. 4) in a dose-dependent manner. The concentration of the drug required for 50% inhibition (IC_{50}) of prolidase activity

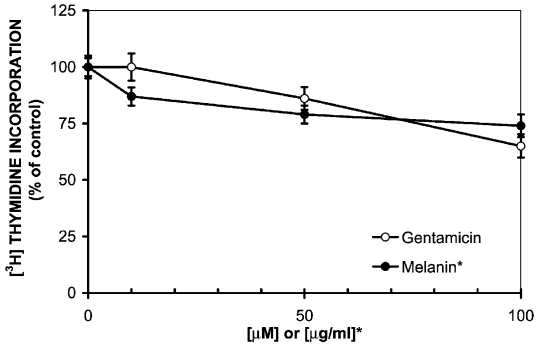

Fig. 4. DNA biosynthesis (measured as [^{3}H]thymidine incorporation into DNA) in semiconfluent human skin fibroblasts incubated for 24 h with different concentrations of gentamicin or melanin. (a) Melanin concentration was reported as micrograms per milliliter of growth medium. Mean values ± S.D. from six independent experiments are presented.

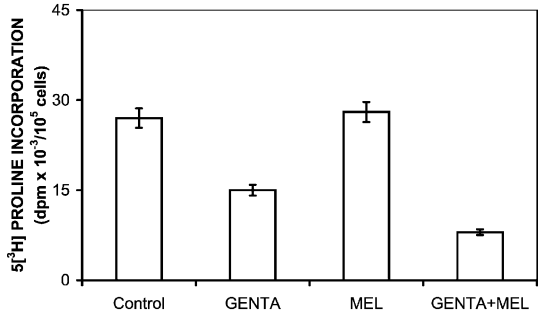

Fig. 6. Collagen biosynthesis in confluent human skin fibroblasts (control) cultured for 24 h with 100 μM gentamicin (GENTA), 100 μg/ml melanin (MEL) or both (GENTA + MEL). Mean values ± S.D. from three independent experiments done in duplicate are presented. ANOVA followed by a Student–Newman–Keuls test showed significance at $P < 0.05$ between four studied groups except CONTROL/MEL.

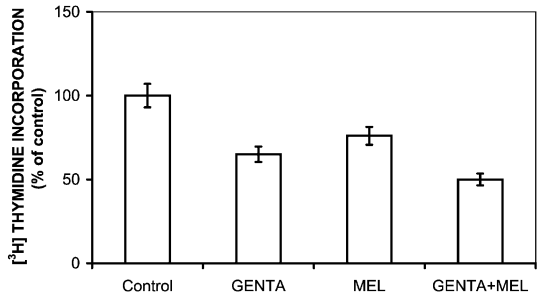

Fig. 7. DNA biosynthesis in semiconfluent human skin fibroblasts (control) cultured for 24 h with 100 μM gentamicin (GENTA), 100 μg/ml melanin (MEL) or both (GENTA + MEL). Mean values ± S.D. from three independent experiments done in duplicate are presented. ANOVA followed by a Student–Newman–Keuls test showed significance at $P < 0.05$ between four studied groups.

was about 100 μM. In both experiments, IC_{50} values were calculated on the basis of the gentamicin concentration in the medium of cultured cells. Melanin also induced inhibition of both processes. However, at 100 μg/ml of melanin, the prolidase activity was decreased only by about 30% and DNA synthesis by about 25% of control.

Since gentamicin was found to form a complex with melanin, we determined the effects of both compounds added simultaneously on prolidase activity, collagen and DNA biosynthesis in confluent fibroblasts. In these experiments, gentamicin was used at the IC_{50} concentration (100 μM) for the respective processes together with 100 μg/ml of melanin. As can be seen from Fig. 5, the addition of melanin to gentamicin-treated cells restored the prolidase activity in fibroblasts to about 80% of control values. In contrast, collagen biosynthesis (Fig. 6) and DNA biosynthesis (Fig. 7) were significantly decreased in these cells.

4. Discussion

One of the recognized side effects of gentamicin is ototoxicity. The mechanism for the organ specificity of this side effect of gentamicin has not been fully established. It has been suggested that eventual uptake of the drug into the hair cells of the inner ear initiates a cascade of irreversible changes in intracellular processes, possibly through inhibition of the phosphoinositide second-messenger system (Schacht, 1986; Williams et al., 1987). More recently, it has been proposed that a toxic metabolite of gentamicin is responsible for the production of ototoxicity in mature hair cells (Crann et al., 1992). Recent work has also suggested that free radicals generated after aminoglycoside treatment play an important role in aminoglycoside cytotoxicity. Studies have shown that radical scavengers have variable efficacy in protecting cochlear hair cells against a number of ototoxicants, which indirectly implicates radical oxygen species in aminoglycoside ototoxicity (Sha and Schacht, 1999; Lopez-Gonzalez et al., 1999; Forge and Schacht, 2000).

It was also postulated that melanin biopolymers act as a biochemical dustbin, mopping up free radicals and other potentially toxic agents (Larsson, 1993). Such properties may be important for protecting the pigment cells as well as surrounding tissues from the natural toxins, oxygen and reactive oxygen species (including free radicals) (Rozanowska et al., 1999). It is now well known that many drugs are markedly accumulated and retained for a considerable time by pigmented tissues and that the retention of these compounds is proportional to the degree of melanin pigmentation (Larsson, 1993). In vivo studies documented that the ototoxicity of another aminoglycoside, kanamycin, was different in pigmented and albino guinea pigs. In pigmented animals, a high kanamycin dose (200 mg per kilogram of body weight) resulted in hearing loss together with loss of both inner and outer hair cells. The albino animals in the same dose group showed significantly less hearing loss and hair cell degeneration (Wasterstrom et al., 1986). However, at four times lower doses, no difference in ototoxicity was found between the pigmented and albino animals.

In view of this fact and taking into account that melanin is an abundant constituent of the inner ear, we determined whether gentamicin interacts with melanin and how this process affects the biosynthesis of collagen (an important structural and functional constituent of the hearing organ) in cultured fibroblasts. The concentration of gentamicin used in our studies correlated with therapeutic doses of the drug used in severe infections (up to 2 mg of gentamicin per kilogram of body weight or up to 32 mg per liter of blood, which in both conditions may correspond to a blood concentration of the drug of about 50 μM).

The data presented here shows that melanin forms complexes with gentamicin and that at least two classes of independent binding sites are implicated in complex formation. The two calculated binding sites in the gentamicin–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 hindrance and physicochemical conditions, as was earlier suggested (Larsson and Tjälve, 1979; Larsson, 1993). Melanins are polyanions with a relatively high content of carboxyl groups and *o*-semiquinones (Ito, 1986; Prota, 1992), 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 (Larsson and Tjälve, 1979; Larsson et al., 1988).

The ability of melanin to bind gentamicin was accompanied by the melanin-dependent potentiation of the inhibitory action of gentamicin on collagen and DNA biosynthesis. One possible explanation for this phenomenon is that the gentamicin–melanin complex (which has a relatively low stability constant) gradually dissociates during the 24-h incubation of cultured cells and that the

released gentamicin constantly affects the metabolism of the cells. It seems possible that the mechanism of the melanin-dependent potentiation of the effects of gentamicin on cell metabolism involves: (i) the ability of melanin to form a complex with gentamicin, (ii) an accumulation of the drug in the melanin-rich extracellular matrix, and (iii) the gradual release of gentamicin from the complex (due to its low stability constant), thereby exerting a permanent inhibitory action on collagen and DNA biosynthesis. Since melanin is an abundant constituent of the inner ear, this phenomenon may explain the organ specificity of the hearing lesions seen in patients administered this drug.

Another interesting point arises from the data, namely, whether the melanin–gentamicin complex serves as a prodrug with a prolonged duration of action. If this is true, one would expect that similar therapeutic effects of gentamicin would be achieved with lower doses of the melanin–gentamicin complex.

Although melanin potentiated the inhibitory effect of gentamicin on collagen biosynthesis, it partially reversed the inhibitory effect of gentamicin on prolidase activity in fibroblasts. The biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resynthesis (Chamson et al., 1989; Jackson et al., 1975; Yaron and Naider, 1993). It is evident that an absence of prolidase will severely impede the efficient recycling of collagen proline (Goodman et al., 1968). However, enhanced liver prolidase activity was found during the fibrotic process (Myara et al., 1987). This suggests that prolidase, by providing proline for collagen biosynthesis, may regulate the turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Recently, collagen production and prolidase activity were found to be associated in cultured human skin fibroblasts treated with anti-inflammatory drugs (Miltyk et al., 1996), anthracyclines (Muszyńska et al., 1998), during experimental aging of these cells (Pałka et al., 1996), fibroblast chemotaxis (Pałka ka et al., 1997) and cell-surface integrin receptor ligation (Pałka and Phang, 1997). However, unlike collagen biosynthesis, the inhibition of prolidase activity by gentamicin was not augmented but rather partially reversed by melanin. The discrepancy between the melanin-dependent potentiation of the inhibitory effect of gentamicin on collagen biosynthesis and the lack of an effect on prolidase activity is being intensively studied in our laboratory. A possible explanation of the phenomenon is that gentamicin inhibits collagen biosynthesis and prolidase activity independently, through different mechanisms. Collagen biosynthesis can be inhibited intracellularly at transcriptional or posttranscriptional levels (Wu and Danielsson, 1994). In contrast, intracellular prolidase activity may be inhibited extracellularly. It is known that prolidase is regulated by signals mediated by the activated β1 integrin receptor (Pałka and Phang, 1997) and that this process involves the phosphorylation of prolidase (Surażyński et al., 2001). Whether gentamicin differentially affects these processes remains to be explored. Nevertheless, the mechanism of the gentamicin-induced decrease in collagen biosynthesis may involve the inhibition of processes that are downstream of prolidase in the synthesis of this protein. Another mechanism of gentamicin action may be responsible for the inhibition of prolidase activity (possibly at the integrin level). The melanin–gentamicin complex with its very low association constant possibly dissociates slowly, and the released gentamicin may not reach a high enough concentration to affect the extracellular part of the integrin receptor that transmits the signal for prolidase activity. Therefore, the ability of melanin to bind gentamicin may prevent the gentamicin-induced inhibition of prolidase activity. In respect to collagen biosynthesis, gentamicin released from the melanin reservoir may enter the cells constantly, during the period of incubation (24 h), leading to an increase in its intracellular concentration and inhibition of the process.

We postulate that the ability of gentamicin to form complexes with melanin may contribute to the accumulation of the drug in melanin-rich tissues (e.g. inner ear). Since the stability constant of the complexes is rather low, it seems that the drug slowly dissociates from the complex, leading to augmentation of its inhibitory action on collagen and DNA biosynthesis in fibroblasts. The phenomenon may be one of the factors responsible for the organ specificity of gentamicin-induced hearing lesions seen in patients administered this drug.

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