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# Interaction of methotrexate with melanins and melanosomes from B16 melanoma

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It has been demonstrated that methotrexate forms stable complexes with melanin and melanosomes isolated from B16 melanoma. The number of binding sites and binding constants for methotrexate binding by intact melanosomes and melanin were $n = 0.046$ $\mu$mol/mg, $K = 0.32 \times 10^4$ $M^{-1}$ and $n = 0.063$ $\mu$mol/mg, $K = 1.08 \times 10^4$ $M^{-1}$, respectively. Binding of methotrexate to synthetic DOPA-melanin used for comparison also shows a single class of binding sites, $n = 0.060$ $\mu$mol/mg with binding constant $K = 2.34 \times 10^4$ $M^{-1}$. The possibility of side effects caused by methotrexate-melanin interactions after treatment of neoplasms is discussed.

## 1. Introduction

It is well known that melanin occurs in various tissues of humans and other animals including retinal pigment epithelium, choroid, iris and ciliary body of the eye, stria vascularis of the cochlea and planum semilunatum of the ampullae of the ear, skin, hair, hair follicles and substantia nigra and locus coeruleus of the brain. This pigment is found in the nerve cells of the brain of larger animal species including man and in macrophages within the superficial cervical lymph nodes [1]. Melanin is synthesized in melanosomes of the melanocyte. If an abnormally large amount of melanin is produced in the body, as with patients with malignant melanoma or hyperpigmentation of the skin, the migration of melanosomes from the skin seems to increase, leading to general melanosis.

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At present, many drugs are known to be markedly accumulated and retained for a considerable time by pigmented tissues of animals including humans and that the retention of these compounds is proportional to the degree of melanin pigmentation.

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, otic lesion and irreversible extrapyramidal disorders [2].

Among several other drugs, methotrexate (aminomethylpteroylglutamic acid), a synthetic inhibitor of dihydrofolate reductase, is useful in the treatment of human neoplasms. In addition to use in choriocarcinoma, osteogenic sarcoma, acute leukaemia, prophylaxis of meningeal leukaemia, methotrexate has been employed in dose arterial infusion in the treatment of liver cancer [3]. The interaction of methotrexate with either normal or neoplastic melanin-containing tissues is not known. The aim of the present study was to

determine whether methotrexate is able to form stable complexes with melanins obtained synthetically or isolated from melanin-containing tissues, as well as to demonstrate whether melanosomes from transplantable B16 melanoma are able to bind methotrexate. Both the number of methotrexate binding sites and the values of the binding constants were determined for melanins and melanosomes from B16 melanoma and compared with synthetic DOPA-melanin.

## 2. Materials and methods

### *2.1. Source and purification of melanosomes*

Melanosomes were isolated from B16 mouse melanoma transplanted on C57BL/6 mice using modified differential centrifugation methods as described previously [4]. 2 weeks after transplantation, the tumors were collected from 55 mice. 53 g of wet melanoma tissue were collected and homogenized in phosphate buffer (1 mM, pH 6.8), filtered through gauze and centrifuged at $600 \times g$ for 3 min. The supernatant was then centrifuged at $1000 \times g$ for 30 min and the resulting melanosomal sediment was resuspended in 0.25 M sucrose layered on an equal volume of 1.55 M sucrose and centrifuged at $1000 \times g$ for 1 h. This purification procedure was repeated three times and the melanosomes obtained were washed twice with phosphate buffer (1 mM, pH 6.8), suspended in distilled water and used for examination. The yield, 154 mg of melanosomes, amounted to 0.3% of the total tissue.

### *2.2. Disintegration of melanosomes*

The disintegration procedure includes removal of the outer membrane and surface constituents of melanosomes by treatment with 0.1% Brij 35 in sodium phosphate buffer (1 mM, pH 6.8) as described by Jimbow et al. [5]. Following centrifugation, the sediment was suspended in a chloroform/methanol mixture (2:1, v/v). After 24 h, the lipids were removed and discarded. The residual melanosome fragments were treated with 2% SDS-5% mercaptoethanol (ME) for 5 days at room temperature and then twice with fresh SDS-ME solutions for 30 min in a boiling water bath according to the method of Whittaker [6]. Both Brij 35 and SDS-ME supernatants were dialyzed against water and used for protein determination by the method of Sedmak and Grossberg [7] using Serva blue G. This method was recommended by Borovansky et al. [8] for assaying protein in the presence of melanins.

### *2.3. Melanin from B16 melanoma*

Melanosomes from B16 melanoma tissues were used for hydrolytic deproteinization in the presence of 6 N HCl as described previously [4]. After hydrolysis the melanin was washed with distilled water, dried over $P_2O_5$ and used for preparation methotrexate complexes. The amino acids liberated in the resulting hydrolysate were analyzed in the standard manner by using an automatic amino acid analyzer (type AAA-81, Mikrotechna, Prague) and the data used for the determination of protein content.

### *2.4. Synthetic DOPA-melanin*

DOPA-melanin was obtained by oxidative polymerization of β-(3,4-dihydroxyphenyl)-L-α-alanine solution (1 mg/ml) in phosphate buffer (pH 8.0) for 48 h as described by Stępień et al. [9].

### *2.5. Methotrexate*

Pharmaceutical grade methotrexate (lot no. 149; 50 mg vials from Roger Bellon, France) was used for interaction with melanins and melanosomes. Preservatives: methyl- and propyl-*p*-hydroxybenzoate (1.6 and 0.4 mg, respectively) were present in each 50 mg methotrexate vial.

### *2.6. Methotrexate-melanin and -melanosome interactions*

The interaction of methotrexate with melanoma-melanin, synthetic melanin and intact or disintegrated B16 melanosomes was studied according to a procedure described previously [10]. Binding of methotrexate to melanin and melanosomes was

studied in 0.067 M phosphate buffer (pH 7.0). 5 mg of dry melanin or 1.2 ml of melanosomal suspension which was equivalent to 5 mg of dry melanosomes were placed in Erlenmeyer flasks. The buffer and methotrexate solution were added to each flask to a final volume of 10 ml. The initial concentration of methotrexate ranged from $1.0 \times 10^{-5}$ to $6.0 \times 10^{-4}$ M for melanins or to $5.2 \times 10^{-4}$ M for melanosomes. Control samples contained identical quantities of melanin or melanosomes in 10 ml buffer. Samples were kept at room temperature for 20 h. This incubation time was chosen on the basis of experiments described previously [11]. These suspensions were filtered and the absorbance of each filtrate with respect to the control was determined at 304 and 370 nm. The concentration of methotrexate remaining in each filtrate and the amount of methotrexate bound to melanin or melanosomes were then calculated, taking the molar absorption coefficient as $22.7 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 304 nm and $7.0 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 370 nm determined experimentally under the described conditions.

### 2.7. Analysis of methotrexate binding

A qualitative analysis of melanin- and melanosome-methotrexate interactions was performed via Scatchard plots of the experimental data according to Kalbitzer and Stehlik [12]. The number of binding sites and the value of the association constant were calculated.

## 3. Results and discussion

The interaction of methotrexate with intact melanosomes from B16 melanoma is represented in fig. 1 by plotting the amount of methotrexate bound as a function of the initial concentration of drug. It can be seen from the binding curve that the concentration of methotrexate bound to a constant amount of melanosomes (5 mg) reaches a plateau at about 0.12 μmol methotrexate/5 mg melanosomes, which reflects the initial concentration of methotrexate equal to $2.5 \times 10^{-4}$ M. The experimental data were analyzed by constructing Scatchard plots to determine the binding sites and

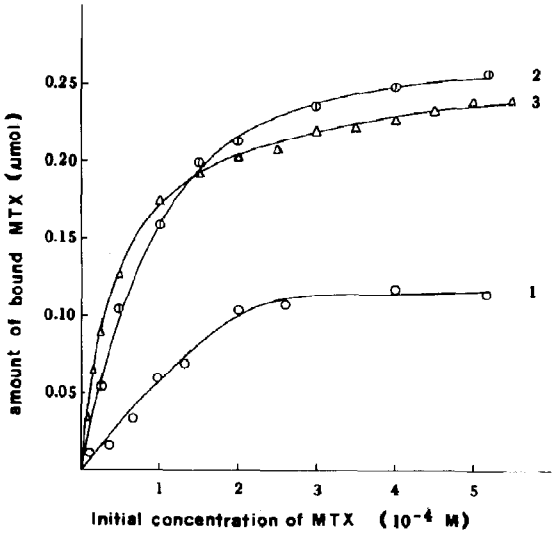


Fig. 1. Binding of methotrexate (MTX) to intact B16 melanosomes (1), protein-free melanin from B16 melanosomes (2) and synthetic DOPA-melanin (3).

the number of relevant binding classes. Plotting of the data results in a straight line with a negative slope. It was demonstrated by Kalbitzer and Stehlik [12] that in cases where there are no other indications of cooperative sites and when the slope of the Scatchard plot is negative throughout, the most reasonable procedure would be to assume that only independent sites are present in the macromolecule. Once the number and type of binding classes have been determined, the slopes and intercepts with the axes can be used in order to approximate the number of binding sites and magnitude of the binding constants.

Since our experimental data for the methotrexate-melanosome interaction fit a straight line with negative slope as shown in fig. 2, one must assume a single class of independent binding sites to be present, with a binding constant of $0.32 \times 10^4$ $M^{-1}$ and the number of binding sites being 0.046 μmol methotrexate/mg melanosomes. Even within the extrapolation range ($r > 3 \times 10^{-8}$ mol/mg), a change in slope of the straight line due to a further class of independent binding sites and/or to cooperativity of potential binding sites could take place, hence the binding constant for this additional

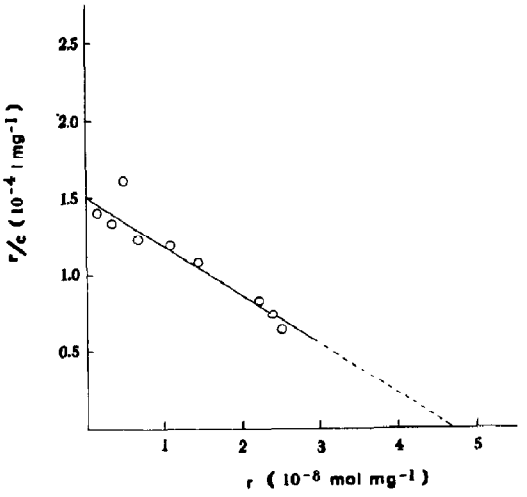


Fig. 2. Scatchard plot for methotrexate binding of B16 melanosomes. $r$ amount of methoxtrexate bound per mg melanosomes; $c$ concentration of free methotrexate.

binding class should be characterized by a very low value.

Binding parameters for the interaction of methotrexate with melanosomes and melanin are listed in table 1.

Aliquots of melanosomes were treated successively with 0.1% Brij 35 to remove protein from the surface of melanosomes and from their outer membrane [5], a chloroform/methanol mixture for removal of lipids and with SDS-ME to separate soluble matrix proteins. The amount of proteins extracted with the solubilizing agents was 13% on the basis of dry weight of melanosomes. The weight decrease of dry melanosomes treated with Brij 35 amounted to only 0.63%. These results are consistent with those in the report of Jimbow et al. [5], in which it was demonstrated that the surface of melanosomes from B16 and HP melanomas was poor in protein but rich in lipids and phospholipids. In our experiments, after treatment with Brij and lipid extraction with the chloroform/methanol mixture the disintegrated melanosomal pellets obtained were subjected to the action of SDS-ME and the release of melanosomal matrix proteins was shown to be quite effective. The protein-poor disintegrated melanosomes were then exposed to methotrexate. On inspection of the data in table 2, the methotrexate content of such melanosomes is observed to be 0.040 μmol/mg, i.e., almost double that in intact melanosomes (0.024 μmol/mg). One can assume that, following the removal of extractable proteins, the interaction of methotrexate with the melanosomal residue is more extensive than with intact melanosomes.

Table 1

Binding parameters for interaction of methotrexate with melanosomes and melanins

$K$, binding constant; $n$, number of binding sites

| Sample | $K$ ($M^{-1}$) | $n$ ($\mu$mol $mg^{-1}$) |
|---|---|---|
| Intact B16 melanosomes | $0.32\times10^4$ | 0.046 |
| Melanin from B16 melanosomes | $1.08\times10^4$ | 0.063 |
| DOPA-melanin | $2.34\times10^4$ | 0.060 |

Table 2

Amounts of methotrexate bound to melanosomes and melanins

Initial concentration of methotrexate $5.2\times10^{-4}$ M; mass concentration of melanosomes or melanins, 5.0 mg/10 ml

| Sample | μmol methotrexate/mg |
|---|---|
| Intact B16 melanosomes | 0.024 |
| Disintegrated B16 melanosomes | 0.040 |
| Protein-free melanin from B16 melanosomes | 0.052 |
| Melanosomes (calculated per melanin content) | 0.056 |
| Synthetic DOPA-melanin | 0.048 |

Another portion of purified melanosomes was subjected to acid hydrolysis without initial treatment with the above-mentioned chemicals. It was found that the protein content of B16 melanosomes amounted to 39% (w/w) of the dry weight of melanosomes. The melanin content estimated from the dry weight of insoluble melanosomal residue was about 43%. The interaction of methotrexate with melanin isolated from B16 melanoma is depicted in fig. 1. The amount of methotrexate bound to melanoma-melanin was determined to be 0.052 μmol/mg, as demonstrated in table 2 for an initial concentration of

methotrexate of $5.2 \times 10^{-4}$ M. This value is almost identical to that obtained by calculation on the basis of the melanin content of dry melanosomes (0.056 μmol/mg). As one can see from table 2, the removal of proteins from melanosomes is accompanied by an increase in methotrexate binding to melanosomes. This is in support of the assumption that methotrexate binds mainly to the melanin biopolymers in melanosomes. In order to verify the above hypothesis, synthetic melanin from L-DOPA was obtained by oxidative polymerization as described above. The values obtained for the amount of methotrexate bound following the interaction with synthetic melanin fell within a concentration range similar to that of natural melanoma-melanin. The binding of methotrexate to these melanins is also shown in fig. 1. In order to evaluate the binding constants from experimental data on the interaction of methotrexate with melanin, Scatchard plots were constructed and, similarly to intact melanosomes, the data yielded a linear plot with a negative slope. This suggests that binding of methotrexate to natural or synthetic melanins shows only one class of independent binding sites. The highest values for the amount of methotrexate bound to melanin from B16 melanoma and DOPA-melanin were found to be 0.063 and 0.060 μmol/mg, respectively (table 1); however, the binding constants were determined as $1.08 \times 10^4$ $M^{-1}$ for natural melanin and $2.34 \times 10^4$ $M^{-1}$ for synthetic melanin. Differences in the values of the binding constants are mainly due to variations in the structure of melanin. It was shown by Ito [13] that treatment with hot HCl may lead to typical decarboxylation phenomena in melanins, being also responsible for liberation of free ammonia.

The effects of the preservatives, methyl- and propyl-*p*-hydroxybenzoates, present in the methotrexate preparation used for investigation of the methotrexate-melanin interaction should be negligible due to their low concentrations. If preservative-melanin interactions do take place and if the binding to melanin or melanosomes were stronger or weaker than for methotrexate, two binding classes would be expected to be shown on the Scatchard plots. However, we observed no such phenomena in our experiments.

Although drug-melanin binding appears to be associated with many types of ocular toxicity, ototoxicity, and pigment and extrapyramidal disorders, only a relatively small number of drugs known to bind to melanin show any of these effects at therapeutic dosages. In contrast, it has been shown by Bassett et al. [14] that, during treatment of experimental bladder cancer, methotrexate may be administered at a dose of 30 mg/kg. Such high dosages may result in severe side effects, and therefore the possibility cannot be excluded that some of the side effects may also be correlated with the methotrexate-melanin interaction. Our attempts to find literature data that clearly indicate methotrexate to be the likely source of the side effects associated with melanin were unsuccessful.

Freise et al. [15] also demonstrated that, after administration of methotrexate in liposomes to mice, the level of methotrexate in several tissues was 20-fold greater than free injected methotrexate. After methotrexate administration, the concentration of the drug was high in blood, spleen, liver and kidney as shown by Colley and Ryman [16]. For methotrexate, its levels and integrity within the plasma, uptake by both red blood cells and tissues, and renal excretion have been detailed by Kimelberg [17]. Unexplained phototoxic responses have been recently reported for patients treated with high doses of methotrexate [18]. Methotrexate is also used in the treatment of severe skin diseases such as psoriasis. In the latter case, synergistic effects have been observed on using methotrexate in conjuction with PUVA therapy [19].

Finally, it should be borne in mind that, even if no indications of methotrexate exerting an antitumor action on B16 melanoma are obtained, its accumulation in melanin-containing tissues may occur and result in unexpected side effects.

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