3H-Nicotine, 3H-Flunitrazepam, and 3H-Cocaine Incorporation Into Melanin: A Model for the Examination of Drug–Melanin Interactions*

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Abstract

To explore drug-melanin interactions, we examined the in vitro tyrosinase-mediated formation of melanin from tyrosine in the presence of the 3H-cocaine (3H-COC), 3H-flunitrazepam (3H-FLU), and 3H-nicotine (3H-NIC) at 10–100,000 ng/mL. Polymerization in the presence of 10 or 100 ng/mL of each drug resulted in almost complete drug incorporation into the melanin pellet. Only 12% (3H-NIC) to 28% (3H-FLU) of the pellet-associated radioactivity could be released upon treatment with 6M HCl. At 1000–100,000 ng/mL, between 20 and 50% of label became melanin-associated. In each case a significant percentage of melanin-associated radioactivity was resistant to treatment with 6M HCl. Nicotine-associated radioactivity in the polymer was subject to much greater quenching than was 3H-COC or 3H-FLU, suggesting a much tighter association with the melanin. The subsequent demonstration of a covalent adduct of a melanin intermediate and nicotine has demonstrated the utility of this polymerization system as a model for further chemical characterization of drug–melanin interactions.

Introduction

The validity of hair as an analytical substrate for drug analysis awaits answers to numerous mechanistic questions (1). In particular, the role of hair pigments in the deposition and retention of drugs in the hair matrix has received much attention (2). A number of investigators have found pigment-dependent differences in drug concentrations in hair following drug administration (3–9). We have recently demonstrated substantial pigment-dependent differences in the accumulation of radiolabeled serum constituents in mouse hair (10,11).

We have demonstrated, autoradiographically, the deposition of 3H-cocaine (3H-COC), 3H-flunitrazepam (3H-FLU), and 3H-nicotine (3H-NIC) in melanosomes of developing hair in pigmented mice (12) within minutes of administration. The labeled drugs continue melanin association in mature hair (13). Extensive digestion of hair in sodium hydroxide or sodium sulfide failed to completely liberate incorporated radio-labeled flunitrazepam and nicotine from the melanin matrix. The current study was undertaken to distinguish physical occlusion from possibly covalent drug–melanin interactions.

Extensive work on the structure of melanins (14–20) has demonstrated that eumelanin consists largely of oligomers of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, which in turn are generated through dopachrome from the oxidation of tyrosine by tyrosinase. Synthetic eumelanins can be generated via tyrosinase oxidation of tyrosine, dopa, or 5,6-dihydroxyindole-2-carboxylic acid (20–23). Because this process is the same as that occurring in the formation of pigmented hair, the in vitro polymerization reaction in the presence of drugs was viewed as a potentially useful model for drug incorporation into pigmented hair. In the case of nicotine, our recent demonstration (24) of adducts of both nicotine and cotinine with dopaquinone, a melanin precursor, has underscored the importance of this experimental approach in the characterization of drug–melanin interactions.

Methods

Methyl-3H-flunitrazepam (85 Ci/mmol), N-methyl-3H-nicotine (81 Ci/mmol), and benzoyl-3H-cocaine (25 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). Tyrosinase (mushroom) was purchased from Sigma Chemical Co. (St. Louis, MO).

Polymerization reactions were conducted as described by Bertazzo et al. (23). Drugs were included in the polymerization reaction at physiologically relevant (10–1000 ng/mL) as well as much higher (10,000 and 100,000 ng/mL) concentrations. Relative to the tyrosine in the polymerization reaction (5 μmol),
the mole ratio of tyrosine to drug was approximately 167,000 at a drug concentration of 10 ng/mL and decreased to 16.7 at 100,000 ng/mL. For each incubation, 11,100 dpm of tritiated drug was diluted with unlabeled drug as necessary to provide concentrations of 10–100,000 ng/mL in 0.1M sodium phosphate buffer at pH 6.8. Tyrosine solution (1 mL of 1 mg/mL in phosphate buffer pH 6.8) was then added, followed by tyrosinase (400 U in 100 μL of buffer). The solution was incubated at 37°C for 4 h under a 100% oxygen atmosphere. At the end of the incubation, a black precipitate had formed. The incubation mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was removed. The pellet was vortex mixed with 1 mL of deionized water and centrifuged again. The combined supernatants and pellets were counted separately in Fisher ‘Scintisafe 50% Plus’ cocktail using a Packard Liquid Scintillation Counter (Downers Grove, IL). The use of a 100% oxygen atmosphere is a standard procedure for this polymerization and gives rise to eumelanin identical to natural sources (23). We have found that the polymerization and adduct formation will proceed under normal atmospheric oxygen concentration, but at a much slower rate (24).

Because melanin can quench low-energy tritium emission, an estimate of quenching of radioactivity for each sample was required. To accomplish this, samples were first subjected to liquid scintillation counting to determine drug-associated radioactivity. After this, a known increment of 3H-toluene (2.36 × 10⁶ dpm/mL) was added and the sample was re-counted. The efficiency of counting of the added radioactivity was calculated for that sample. It is clear that the 3H-toluene employed as a source of tritium may not interact with the melanin in the same fashion as the drugs, but would provide a frame of reference for more efficient quenching of tritium emission. To the extent that quenching correction based upon toluene standards underestimated the radioactivity known to be incorporated into the sample, the greater the quenching of the drug-associated radioactivity by melanin was apparent.

The acid reversibility of drug–melanin interactions was determined by resuspension of melanin pellets prepared in the presence of radiolabeled drugs in 6M HCl for 10 min. Subsequent centrifugation and liquid scintillation counting of the supernatant allowed quantitation of the percentage of melanin-associated drug removed from the pellet. Extract aliquots were also basified with sodium hydroxide and extracted with ethyl acetate to determine the amount of drug-associated radioactivity that could be attributed to parent drug. Each drug, if not chemically altered, should be transferred to ethyl acetate in this fashion. The effectiveness of acid digestion in reversing drug–melanin associations will be discussed in the next section.

Results and Discussion

The effect of drug concentration on the percentage of radioactivity remaining in the supernatant after melanin generation in the presence of radiolabeled drugs is shown in Figure 1. At 10 ng/mL, 4.4 ± 0.2% of 3H-COC-associated radioactivity remained in solution after removal of melanin. This value increased with increasing 3H-COC concentrations from 5.7 ± 0.4% (100 ng/mL) to 33.7 ± 2.3% at 100 μg/mL. Similar results were obtained with 3H-FLU. The percentage of radioactivity remaining in the supernatant after melanin formation increased from 4.8 ± 1.8% at 10 ng/mL to 53.4 ± 6.6% at 100 μg/mL 3H-FLU. The non-melanin-associated tracer concentration increased from 6.5 ± 1.7% at 10 ng/mL 3H-NIC to 29.3 ± 0.5% at 100 μg/mL 3H-NIC in the reaction mixture.

The percentage of radioactivity, after correction for quenching, associated with the melanin polymers following melanin generation in the presence of radiolabeled drugs is illustrated in Figure 2. The percentage of 3H-COC associated with the polymer decreased from 102.4 ± 9.6% at 10 ng/mL 3H-COC to 29.3 ± 0.5% at 100 μg/mL 3H-COC. The percentage of melanin-associated radioactivity observed at 10 ng/mL 3H-FLU (63.5 ± 12.7%) was significantly greater than that observed at 10 or 100 μg/mL (29.9 ± 2.0% and 17.1 ± 8.5%, respectively). At all concentrations of 3H-NIC employed in the polymerization reaction, a constant amount of pellet-associated radioactivity was observed (22.1–24.4%). The data demonstrate that, for 3H-COC and 3H-FLU, the maximum percentage of tracer associated with the melanin pellet is seen at very low (physiological) drug concentrations. At higher drug concentrations a smaller percentage of the total drug is associated with the pellet, suggesting an equilibrium between free and melanin-associated drug.

![Figure 1](https://academic.oup.com/jat/article-abstract/25/7/607/729597/2576072867)
Figure 3 illustrates the total quantity of melanin-associated drugs observed when melanin was polymerized in drug concentrations ranging from 10 ng/mL to 100,000 ng/mL (100 µg/mL). The results demonstrate that substantial drug accumulation in the melanin pellet can be achieved by polymerization in the presence of very high (non-physiological) drug concentrations (10–100 µg/mL). However, at low concentrations, the drugs are nearly completely associated with the melanin pellet.

Figure 4 illustrates the ability of 6M HCl to solubilize drug-associated radioactivity from melanin pellets. For melanin associated with 3H-COC, 15–25% of the pellet-associated radioactivity could be removed, with no significant concentration dependence. In the case of melanin containing 3H-FLU, 20–40% of the polymer-associated radioactivity could be removed. In the case of 3H-NIC only 7 to 14% of the melanin-associated radioactivity could be removed by treatment of the melanin with 6M HCl.

3H-COC was almost completely removed from the supernatant of the polymerization mixture at low concentrations (10 and 100 ng/mL). The radioactivity lost from solution at these concentrations was accounted for by scintillation counting of the radioactivity in the pellet. The quench correction factor generated from recounting of the pellet after addition of a known toluene reference gave values for pellet content of 3H-COC which resulted in an accounting of total mass balance for cocaine. Incubation of the pellet with 6M HCl released only 20–25% of the pellet-associated radioactivity, indicating a tight association of the 3H-COC with the melanin. Simple binding interactions or ionic interactions of cocaine and melanin would not be expected to survive this treatment which would protonate carboxylic counterions. More vigorous chemical treatment of the pellet with acid or base was not possible as the cocaine was labeled on the benzoylester, which would be hydrolyzed. As would be expected, larger concentrations of cocaine resulted in a lower percent of the total tracer present being incorporated into the melanin pellet. At each concentration of 3H-COC a relatively constant percentage of radioactivity was releasable upon treatment with aqueous acid.

3H-FLU was also almost completely removed from the polymerization supernatant at physiologically relevant concentrations (10–100 ng/mL). The pellet from these incubations should contain sufficient radioactivity to account for that removed from solution. However, the radioactivity in the pellet was only approximately 70% of the total, indicating an underestimation of pellet radioactivity by the quench correction factor generated from addition of a 3H-toluene calibrator. Such a situation would occur if the flunitrazepam–melanin interaction was sufficiently close (spatially) as to result in increased quenching of radioactivity relative to 3H-toluene in solution. Approximately 25% of the melanin-associated 3H-FLU could be released from the pellet by...
treatment with aqueous acid, indicating a moderately irreversible drug–melanin interaction. Previous in vivo studies of \(^3\)H-FLU incorporation into mature hair following systemic administration (12,13) have also demonstrated the inability of aqueous sodium hydroxide and sodium sulfide digestion to completely release \(^3\)H-FLU (or \(^3\)H-NIC) from eumelanin-pigmented hair. The drug-associated radioactivity released upon digestion with these agents was to a great extent not extractable into ethyl acetate, indicating chemical modification of the drug in some fashion.

The more notable example of this was observed with \(^3\)H-NIC. At concentrations of 10–1000 ng/mL approximately 90% of the \(^3\)H-NIC was removed from the supernatant at 10 ng/mL; however, liquid scintillation counting of the pellets, and correction for quenching, accounted for only 22% of the total radioactivity in the reaction. The results suggest a substantial quenching of radioactivity by a melanin–nicotine association of such a nature that only 10–15% of the pellet-associated radioactivity could be solubilized by treatment with 6M HCl.

The nature of the drug–melanin associations could not be further explored chromatographically because of the insoluble nature of the polymers. However, the results presented are consistent with irreversible, possibly covalent, drug–melanin interactions that may model drug association with melanin in vivo. Certainly ionic and covalent drug–melanin interactions can be envisioned. The poly-quinone nature of melanin and the oxidative nature of the polymerization reaction (14–20) are conducive to many types of chemical interactions between melanin and drugs. Conjugate addition of nucleophilic drug functionality to melanin \(\alpha\)-quinones, as well as free radical generation and capture in the polymerization reaction could potentially give rise to numerous covalent drug–melanin interactions. The ionized carboxylates of the hydroxyindole carboxylate oligomers could give rise to ionic interactions. The large extended conjugated functionalities of the indole oligomers could serve as substrate for binding interactions.

These possibilities are strengthened by our recent demonstration by matrix-assisted laser desorption-ionization mass spectrometry of adducts of both nicotine and cotinine with dopaquinone (24).

The results suggest that although physical occlusion of drugs in hair matrix may occur, chemical interaction of drugs with melanin may represent a major mode of accumulation in pigmented hair, and that examination of melanin polymerization in the presence of radiolabeled drugs may serve as an important model of drug–melanin interactions.

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References


