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Inhibition of Human Immunodeficiency Virus Type 1 Replication and Cytopathicity by Synthetic Soluble Catecholamine Melanins In Vitro

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Synthetic soluble melanins were synthesized by spontaneous oxidation of L-dopamine, norepinephrine or 5-hydroxytryptamine (serotonin) in weak alkaline solution. These three melanins inhibited infection of human $CD4^+$ lymphoblastoid cells (MT-2) by cell-free human immunodeficiency virus type 1 (HIV-1), without cell toxicity, at concentrations of 0.15-10 ug/ml. Also, syncytium formation and resulting cytopathic effects when uninfected cells were mixed with chronic HIV-1-infected cells were blocked by these melanins. Antisyncytial activity was greater when infected cells were preincubated with melanin than when uninfected cells were preincubated with melanin, thus suggesting that interaction of melanin with viral proteins is an important aspect of the antiviral mechanism. These results make synthetic soluble melanins interesting candidates for further study as possible anti-HIV-1 therapeutics.

Human immunodeficiency virus (HIV) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS) (1-3). This lentivirus selectively infects lymphocytes, monocytes and other cells that express the CD4 surface glycoprotein, which acts as receptor for the virus (4, 5). Infection leads to $CD4^+$ cell depletion and other immune deficiencies which make the host susceptible to opportunistic infections and neoplasms (6). At present, there is no cure and no vaccine for HIV infection.

Catecholamines, such as L-dopamine, norepinephrine and 5-hydroxytryptamine, undergo spontaneous oxidation in aqueous solution with the oxidation products forming a pigmented heteropolymer called melanin (7-10). Most natural melanins are insoluble products of the activity of the enzyme tyrosinase. This enzyme catalyzes the oxidation of tyrosine to dopa (3,4-dihydroxyphenylalanine) and the conversion of dopa to dopaquinone, which then spontaneously oxidizes and polymerizes to melanin (10, 11). Several structures for melanin have been proposed but none are definitive (12). Although structural analyses

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of melanins are incomplete (13), studies have shown melanins to contain substituted hydroxyindoles, indolequinones, pyrroles, free carboxylic acid groups, phenolic hydroxyls, carbon-based free radicals, and uncyclized aliphatic chains (12, 13). Biologically, melanins act as pigments in the skin, hair and eyes, and can participate in one and two electron exchange reactions (14-19). Here we describe potent antiviral activity of soluble catecholamine melanins against HIV-1 *in vitro*.

MATERIALS AND METHODS

Cells and viruses. The $CD4^+$ human lymphoblastoid cell lines MT-2 (20) and H9 (21) were cultured at 37°C in RPMI-1640 containing 12% heat-inactivated fetal bovine serum and 50 ug of gentamicin per ml. MT-2 cells carry the HTLV-1 genome and are highly susceptible to cytopathic infection with HIV (22). H9 cells resist HIV-induced cytopathic effect and are well-suited for HIV production (23). Stocks of the HIV-1 isolate $HTLV\text{-}III_B$ were harvested from chronically-infected H9 cultures. Virus-containing culture fluids were clarified of cells by low speed centrifugation and passed through 0.45 micron filters. Infectious virions were quantitated by microtitration on MT-2 cells using cytopathic effect as end point for infection (24); the reciprocal dilution at which 50% of wells showed cytopathic effect after 2 weeks incubation defined the infectious titer (1 $TCID_{50}$).

Melanins. Melanins were synthesized by a modification of procedures described previously (7, 25). One hundred micrograms of L-dopamine (3,4-dihydroxyphenyl-ethylamine), (±)-norepinephrine (arterenol) or 5-hydroxytryptamine (serotonin) (Sigma Chemical Company, St. Louis, MO) were dissolved in 40 ml of 0.05N NaOH and incubated for 2 days at room temperature with constant aeration. L-dopamine and 5-hydroxytryptamine melanins were precipitated from the dark brown solution by adding 0.1 ml of concentrated HCl. The precipitate was collected by centrifugation, dissolved in 40 ml of deionized water, and precipitated again with 0.1 ml of concentrated HCl. The melanin was washed this way a total of 3 times, then dissolved in 10 ml of phosphate buffered saline (PBS) adjusted to pH 10 with NaOH (the solution had a neutral pH at this time) and dried. Norepinephrine melanin was not precipitable with HCl and, therefore, was used without further processing. Stock solutions of L-dopamine and 5-hydroxytryptamine melanins were made in PBS, pH 7.4.

Infection Assays. Antiviral activities of melanins were measured in 96-well microdilution plates as described (24). Briefly, 2-fold serial dilutions of melanins were made in triplicate in a total of 100 ul growth medium per well. MT-2 cells (5×10^4) in 100 ul of growth medium were added to each well and incubated for 10 minutes. Fifty microliters of virus (5×10^4 $TCID_{50}$/50 ul) were then added to all wells except for 1 row of eight non-cytopathic control wells; these received growth medium in place of virus. Viral-induced cytopathic effect was quantitated 3 days later by vital dye (neutral red) uptake in remaining viable cells. Neutral red uptake is a linear function of cell viability where light absorption at 540 nm wavelength is linear from 0.025 to 0.85, which corresponding to 2×10^4 to 25×10^4 viable cells/well (24). Percent protection is defined as the difference in absorption between test wells (cells + melanin + virus) and virus control wells (cells + virus) divided by the difference in absorption between cell control wells (cells only) and virus control wells. Plates were harvested when cytopathic effect in virus control wells was greater than 90%.

Measurement of antisyncytial activity. Syncytium formation was induced by mixing chronic HIV-infected H9 cells with MT-2 cells in the presence and absence of melanins in

96-well microdilution plates. Serial dilutions of melanins were made in triplicate. MT-2 cells (1.5×10^5) in 100 ul of growth medium were added to each well. HIV-infected H9 cells (1.5×10^4) in 50 ul of growth medium were added to all wells except one row of 8 non-cytopathic control wells which received uninfected H9 cells. Syncytium formation leads to, and is directly proportional to, cytopathic effect in this assay (26). After incubation at 37°C for 20 hours, syncytium formation was observed microscopically while viable cells were measured by vital dye uptake as described above. Plates were harvested when cytopathic effect in control wells was greater than 50%.

RESULTS

Inhibition of infection by cell-free virus. Cytopathic infection of MT-2 cells with HTLV-III_B was inhibited in a concentration-dependent manner by all three melanins (Fig. 1). Protection was provided by L-dopamine and norepinephrine melanins at 0.078-10 ug/ml, and by 5-hydroxytryptamine melanin at 0.15-10 ug/ml; concentrations greater than 10 ug/ml were toxic to the cells (data not shown). Effective doses that provided 50% protection from infection (ED_{50}) were 0.09 ug/ml for L-dopamine melanin, 0.11 ug/ml for norepinephrine melanin, and 0.15 ug/ml for 5-hydroxytryptamine melanin. The beginning substrates, L-dopamine, norepinephrine, and 5-hydroxytryptamine, at concentrations of 0.78-100 ug/ml, had no antiviral activity in these assays even though spontaneous melanin formation occured during incubation in growth medium containing L-dopamine or norepinephrine (data not shown).

Inhibition of syncytium formation. All three melanins provided concentration-dependent protection from syncytium formation and resulting cytopathic effects at concentrations of 0.078-10 ug/ml (Fig. 2). Antisyncytial activities were optimal at melanin

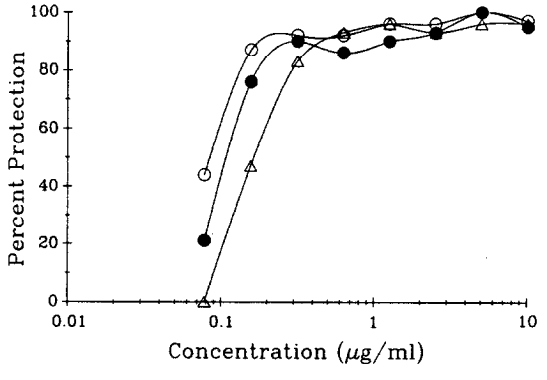

Figure 1. Anti-HIV-1 activity of catecholamine melanins in an MT-2 cell infection assay. Serial 2-fold dilutions of melanins were tested in triplicate at concentrations of 0.078 to 10 ug/ml where percent protection was a function of viable cells as measured by vital dye uptake. The range in vital dye absorption values at 540 nm wavelength was 0.491. All values had standard deviations of less than 8%. ○, L-dopamine melanin; ●, norepinephrine melanin; △, 5-hydroxytryptamine melanin.

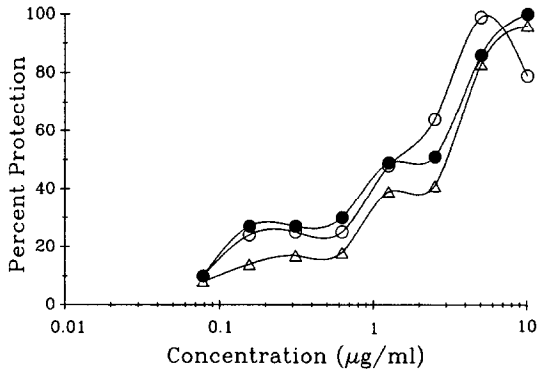

Figure 2. Inhibition of HIV-1-induced syncytium formation and cytopathicity. Catecholamine melanins were tested in triplicate at concentrations of 0.078 to 10 ug/ml for an ability to block syncytium formation and cytopathic effect resulting from mixing MT-2 cells with H9/HTLV-III_B cells. Percent protection was a function of viable cells as measured by vital dye uptake. The range in vital dye absorption values at 540 nm wavelength was 0.328. All values had standard deviations of less than 10%. ○, L-dopamine melanin; ●, norepinephrine melanin; △, 5-hydroxytryptamine melanin.

concentrations of 5-10 ug/ml. In these experiments, infected cells were added immediately after adding uninfected cells. In another set of experiments, the effect of preincubating infected cells with L-dopamine melanin was compared to the effect of preincubating uninfected cells (Fig. 3). Here, syncytium formation and cytopathic effects were inhibited optimally at melanin concentrations of 0.625-10 ug/ml when infected cells were preincubated with melanin prior to mixing with uninfected cells. In contrast, preincubation of uninfected cells had the same effect as no preincubations at all, where melanin concentrations below 5 ug/ml provided suboptimal protection.

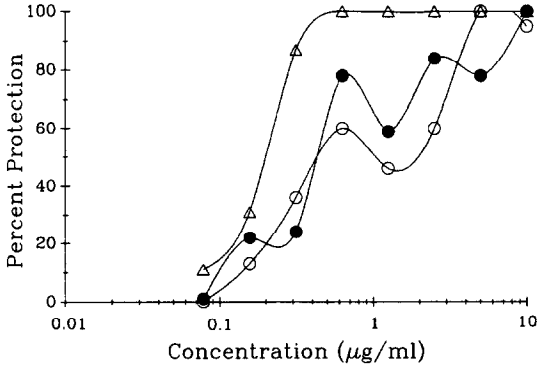

Figure 3. Antisyncytial activity after preincubation of cells with L-dopamine melanin. L-dopamine melanin was tested for antisyncytial activity as described in the legend to figure 2 with the exception that uninfected cells (○), infected cells (△) or neither cells (●) were preincubated with melanin for one hour before mixing. The range in vital dye absorption values at 540 nm wavelength was greater than 0.380. All values had standard deviations of less than 14%.

DISCUSSION

Knowledge of the synthesis and distribution of melanins in nature has been in existence for over 60 years (8) but no therapeutic applications for these substances have been found. In this study, melanins were synthesized from L-dopamine, norepinephrine and 5-hydroxytryptamine in weak alkaline solution. Unlike most natural melanins, these melanins were readily soluble in aqueous solution at neutral pH. Because of their solubility, it was possible to examine biological properties such as antiviral activity. In MT-2 cell cytopathic infection assays, the above melanins provided potent anti-HIV-1 activity at low concentrations (0.15-10 ug/ml) which were not toxic to the cells (Fig. 1).

A major mechanism of HIV-1 cytopathicity *in vitro* is the formation of multinucleated giant cells, or syncytia (1-3). Syncytia have also been observed in tissue sections from brains, lymph nodes and lungs of HIV-infected patients (27-31). The membrane binding and fusion events leading to HIV-1-induced syncytium formation involve interactions between the HIV-1 envelope glycoproteins, gp41 and gp120, and cell surface proteins, including the HIV-1 receptor, CD4, and possibly others (32-36). When chronically-infected cells (i.e., H9/HTLV-III_B) were mixed with $CD4^+$, HIV-1-permissive cells (i.e., MT-2) in the presence of melanins, syncytium formation and resulting cytopathic effects were blocked (Fig. 2). Thus, interfering with binding and/or fusion appears to be a possible mechanism of melanin's anti-HIV-1 activity *in vitro*. It cannot be ascertained from these studies what viral and/or cellular components melanin interacts with to block syncytium formation. However, the finding that antisyncytial activity was greater when infected cells were preincubated with melanin than when uninfected cells were preincubated with melanin (Fig. 3) suggests that interaction with the virus is important.

In conclusion, synthetic soluble melanins derived from L-dopamine, norepinephrine or 5-hydroxytryptamine appear to be unique anti-HIV-1 substances which have an ability to inhibit infection by cell-free virus and prevent viral-induced cytopathicity *in vitro*. Melanins are relatively innocuous substances that are inexpensive and simple to synthesize. Therefore, at present, they appear to possess several attractive features as potential anti-HIV therapeutics.

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