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ORIGINAL ARTICLE

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Preliminary approach to elucidate the role of pigment as a binding site for drugs and chemicals in anagen hairs: pigments as carriers for ^{3}H-haloperidol in HaCaT/Sk-Mel-1 co-cultures

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Abstract In view of the melanin-binding characteristics of haloperidol and its differential uptake by pigment- and non-pigment-producing cells, a co-culture of HaCaT with Sk-Mel-1 cell lines was performed to investigate whether melanosomes act as carriers for drug molecules associated with the pigments. Initially, HaCaT and Sk-Mel-1 cells were separately cultivated in the presence of ^{3}H-haloperidol (400 pmol/ml medium) for 28 days followed by subsequent co-cultivation in the absence of ^{3}H-haloperidol for 5 days. The transfer of pigments into the keratinocytes during co-culture was confirmed by transmission electron microscopy. After the co-culture experiments a striking increase (≥ 50%) of ^{3}H-haloperidol was observed in the pigmented HaCaT cells compared to the unpigmented keratinocytes. The present study proved the role of pigments as carriers for melanin-associated drug molecules. The results supported the hypothesis that hair pigment might be a factor affecting the outcome of hair assays for particular categories of commonly used licit and illicit substances. The chosen cell lines and the developed co-culture system may represent suitable in vitro models to study differential drug uptake into cell populations present in the skin or in the growing hair follicle as well as to elucidate drug uptake due to melanocyte-keratinocyte interactions.

Keywords Co-culture · Drug uptake · Melanin · Hair · HaCaT · Sk-Mel-1

Introduction

In recent years hair fibres have been shown to entrap and to store organic substances present in the organism during hair growth [19]. The detailed mechanisms of drug uptake into the various cell types present in the hair follicle, especially into the immature keratinocytes, mobile cells which interact with the melanocytes, a cell type that resides at the apex of the dermal papilla, needs to be clarified [12]. Drug localisation and binding sites in hair, neither in the transient nor in the permanent hair fibre, have yet been elucidated.

According to the biochemical concept of drug incorporation during hair fibre formation, it has been suggested that particular drug substances are bound during melanogenesis as well as onto the surface of mature melanin granules in addition to other binding sites such as intermediate filaments (IF, IFAPs) and the cell membrane complex [13].

Since it is extremely difficult to study drug uptake in the cells of the anagen phase hair root in vivo, there is a need for suitable in vitro models. The melanin pigment system of the human skin and hair is based on two cell types, melanocytes and keratinocytes, interacting within a functional unit. The spontaneously immortalised human keratinocyte cell line HaCaT with full epidermal differentiation capacity [2] and Sk-Mel-1, a cell line derived from a human malignant melanoma with melanin expression [10], represent readily available in vitro models and are frequently used as a model for research work in dermatology [4]. In a preliminary study HaCaT and Sk-Mel-1 cell lines have been already used to study differential drug uptake by pigment- and non-pigment-producing cells mimicking chronic drug consumption in forensic hair analysis [17]. The results obtained for ^{3}H-haloperidol were in accordance with previous observations for substances with high melanin affinity from animal studies [3, 5, 6, 14, 18, 25, 26] and in vitro binding experiments [1, 15, 16] which demonstrated that pigments act as scavengers and accumulate those substances.

The aim of the present study was to investigate whether melanin-associated drug molecules are transferred by keratinocyte-melanocyte interactions and which may result in an increase of ^{3}H-haloperidol due to the pigmentation of the keratinocytes during co-culture.

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Materials and methods

Cell lines and materials

The spontaneously immortalised cell-line HaCaT (passages 35–45) was kindly provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and grown at 5% CO_2/37 °C in 1 × MEM medium containing 10% fetal calf serum (FCS). For subcultures cells were disaggregated with a 0.1% trypsin/0.05% EDTA (1 : 1) solution and split out on a ratio of 1 : 10 every 10 days.

Sk-Mel-1 cells were obtained from the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) and cultured in 1 × MEM medium containing 10% FBS and 1 × non-essential amino acid mix.

[^{3}H(G)]-Haloperidol (specific activity: 4,440,000 GBq/mmol) was obtained from NEN (Bad Homburg, Germany). The liquid scintillation cocktail (rotizint) was from Roth (Karlsruhe, Germany). Sterile 6-well and thermanox plates were purchased from NUNC (Wiesbaden, Germany). All other chemicals were of anaytical grade and were from Sigma (München, Germany), the cell media and medium supplements were from Gibco-BRL (Eggenstein, Germany).

Initial drug loading of the cell lines

Long term drug exposure of the cell lines was achieved as already reported [17]. Briefly, HaCaT and Sk-Mel-1 were incubated separately with ^{3}H-haloperidol (400 pmol/ml culture medium) in 250 ml flasks (Greiner, Frickenhausen, Germany) for 28 days. Long term drug exposure was achieved by ^{3}H-haloperidol liberation from osmotic pumps (Charles River, Sulzfeld, Germany). At the end of the experiment the cells were separated by centrifugation (1200 rpm, 8 min) and washed 4 times with cold PBS buffer. Media and washing solutions were collected and analysed in addition to the cell-bound ^{3}H-haloperidol by liquid scintillation spectrometry (LSS) using the Tricarb 1600 TR analyser (Canberra Packard, Dreieich, Germany).

Cell counting was performed by haemocytometry under the microscope and the cell viability was estimated by at least two independent observers by trypan blue exclusion. All experiments were run in duplicate, the measurements in triplicate. The mean of the assay runs (range ≤ 10%) was used for data analysis. Uptake values and total melanin content were standardised to 1×10^6 cells.

The total melanin content of the Sk-Mel-1 cells was determined by spectrophotometry (UV 240 spectrophotometer, Shimadzu, Duisburg, Germany) according to Ozeki et al. [11]. Calibration was performed with synthetic eumelanin and was linear (Sigma, München, Germany). The weight of HaCaT pellets (10×10^6 cells, $n = 10$) was determined after vacuum drying (Speed vac, Bachhofer, Reutlingen, Germany).

Co-culture experiments

The ^{3}H-haloperidol-loaded Sk-Mel-1 cells and the drug-loaded HaCaT cells (ratio 1.8 : 2) were co-cultivated in the absence of ^{3}H-haloperidol in 250 ml flasks for 5 days. At the end of the experiment the cells were separated by incubation with 0.1% trypsin at 37 °C. The detachment of the Sk-Mel-1 cells from the HaCaT monolayers was controlled microscopically. The reaction was stopped by addition of 1 × PBS/10% FCS (1 : 1 v/v). The Sk-MeL-1 cells were harvested from the supernatant by centrifugation. The keratinocytes remained in the flask, the desmosomes were disintegrated by incubation with 0.02% EDTA for 20 min at 37 °C followed by treatment with 0.05% trypsin for 1 min at 37 °C. Then the HaCaT cells were collected by centrifugation (1,200 rpm, 8 min).

After the co-culture, the cell-bound ^{3}H-haloperidol and total melanin were quantified for HaCaT as described above. Controls were run by separately cultured cell lines as well as by co-culturing ^{3}H-haloperidol loaded Sk-Mel-1 with native HaCaT cells. In addition native HaCaT cells were grown in the presence of low ^{3}H-haloperidol concentration (0.8 pmol/ml medium).

Transmission electron microscopy (TEM)

For transmission electron microscopy investigations 1.8×10^6 Sk-Mel-1 cells loaded with ^{3}H-haloperidol and 2×10^6 drug-loaded HaCaT cells were co-cultured in the absence of ^{3}H-haloperidol on thermanox 6-well plates for 5 days. Subsequently the thermanox plates were removed, rinsed with PBS buffer for 2 min and fixed with 2.5% glutaraldehyde solution in 0.05 M sodium cacodylate buffer (pH 7.4) for 4 h at 4 °C. After several washes the co-cultured cells were dehydrated in alcohol solutions of graded concentrations followed by routine Epon 812 (Glycidether 100, Serva, Heidelberg, Germany) embedding. Ultrathin sections (Ultramicrotome OM U2, Reichert-Jung, Heidelberg, Germany) were stained with 1% uranyl acetate and viewed in a Phillips 301 transmission electron microscope (Philips, Eindhoven, Netherlands). HaCaT and Sk-MeL-1 cells separately cultured on thermanox plates were used as controls.

Results

Light microscopy

Co-cultures showed sheets of flattened HaCaT cells with irregularly distributed dentritic Sk-MeL-1 cells lying on or between the keratinocytes. (Fig. 1).

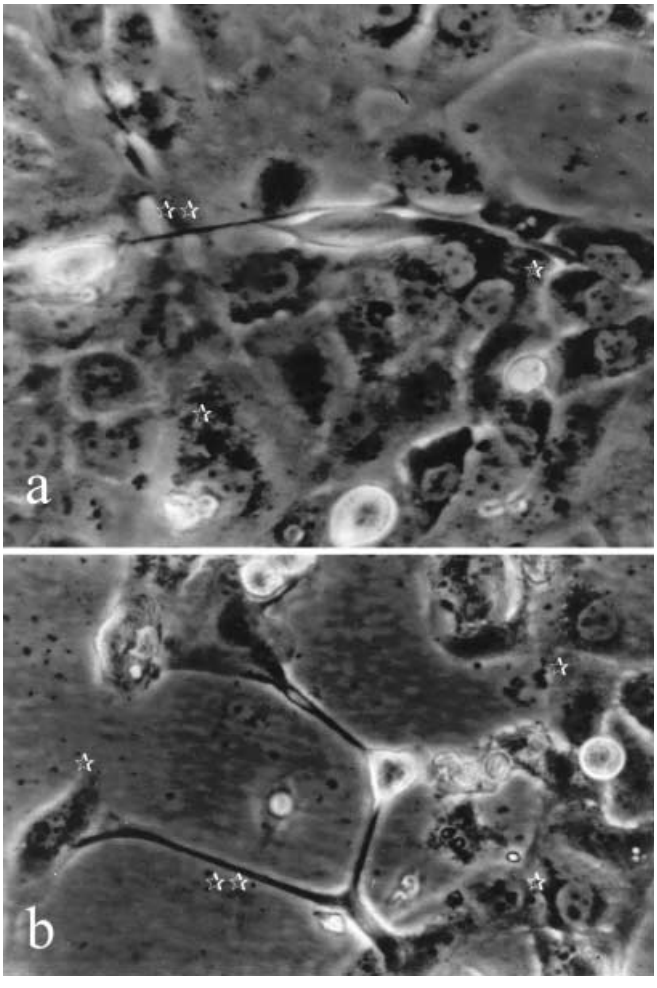

Fig. 1 Sk-MeL-1/HaCaT co-culture. Pigment-loaden dendrites (☆☆) of Sk-MeL-1 cells in co-culture located (a) on as well as (b) between HaCaT cells which already exhibit pigmentation (☆). Light microscopy. Magnification × 400

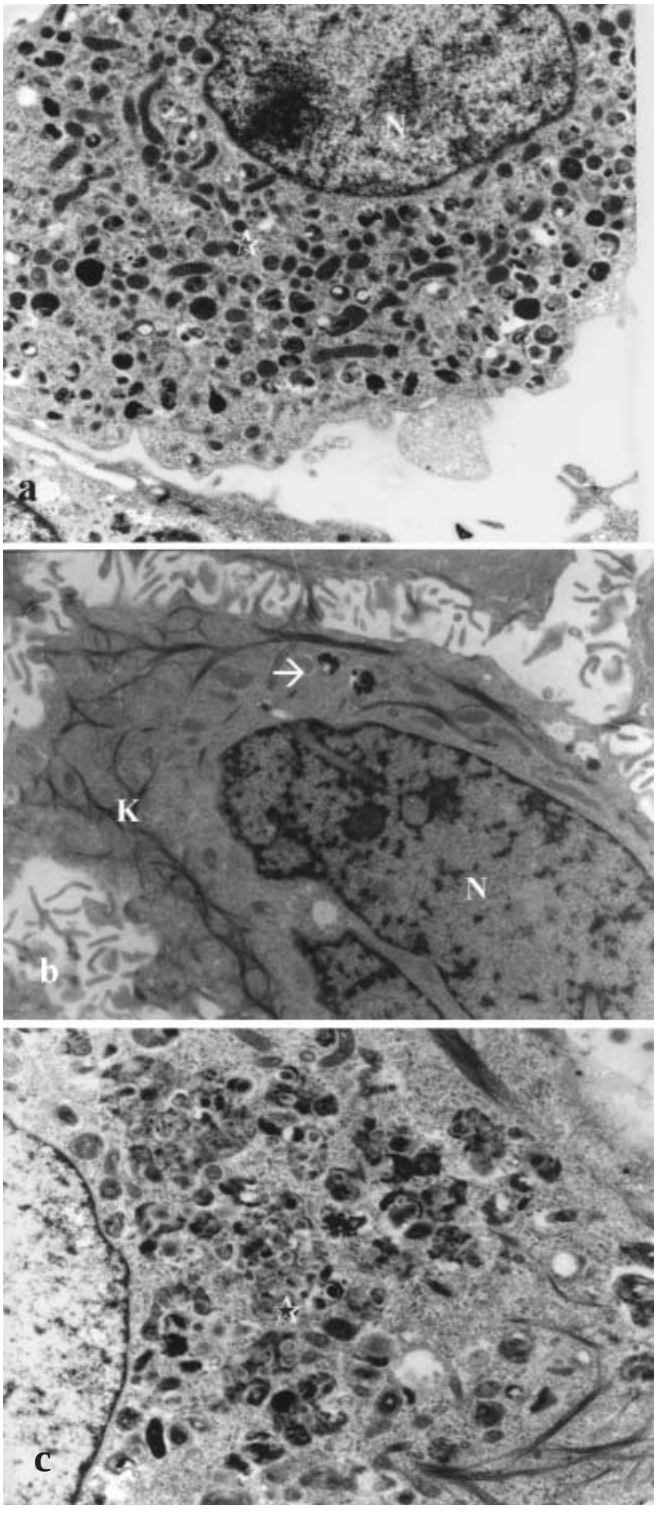

Fig. 2 a Sk-Mel-1 with melanosomes in different stages of maturation. Magnification × 10,500. **b, c** TEM confirmation of the pigment/melanosome transfer by Sk-MeL-1–HaCaT interactions during co-culture. Ultrastructure of HaCaT cells after co-culture. Melanosomes transferred into keratinocytes. **b** HaCaT cell after co-culture with single melanosomes (→, magnification × 8,250). **c** HaCaT cell with numerous melanosomes, mostly in early and intermediate stages (magnification × 14,000) (*N* nucleus, *K* keratin fibrils, ☆: melanosomes)

TEM

Melanosomes in different stages of maturation (stages I–IV) were observed in Sk-MeL-1 cells. Transmission electron microscopy confirmed the transfer of melanin/melanosomes from the Sk-Mel-1 to the HaCaT cells during co-culture and thus melanocyte-keratinocyte interaction in vitro. After co-culture single melanosomes as well as numerous melanosomes in the various stages of maturation, mainly stages I–III, were found with some tendency to cluster in the perinuclear areas of the HaCaT cells (Fig. 2).

Co-culture experiments

After 28 days drug exposure, the ^{3}H-haloperidol concentrations in Sk-Mel-1 (20 pmol/10^{6} cells) were found to be approximately tenfold higher than in HaCaT (2.1 pmol/10^{6} cells). As already reported [17] drug concentrations in the keratinocytes reached a plateau within a short time period whereas no tendency to saturation was observed for the pigment-producing cell line.

After the co-culture experiments with drug-loaded cells, the ratio of Sk-Mel-1 to HaCaT cells had changed (2.5 : 10) due to their different proliferation rates. The ^{3}H-haloperidol concentration was 0.8 pmol/ml medium and had declined to 4.5 pmol/10^{6} cells for Sk-MeL-1, whereas an increase was found for HaCaT (3.5 pmol/10^{6} cells). Overall, pigmentation of the keratinocytes and transfer of melanin-associated drug molecules resulted in a ≥ 50% increase of ^{3}H-haloperidol concentrations compared to the non-pigmented HaCaT cells prior to the co-cultures (Fig. 3). The control experiments confirmed this result in general. After co-culturing of drug-loaded Sk-

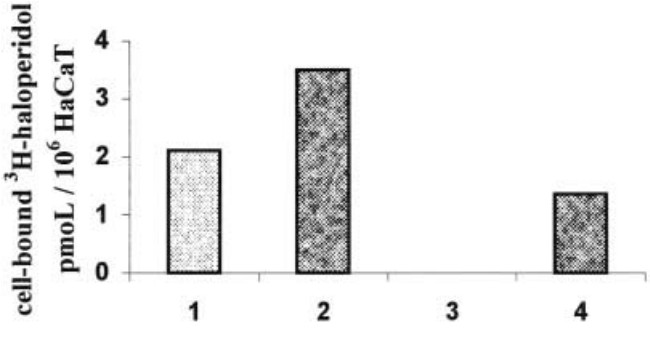

Fig. 3 Comparison of cell-bound ^{3}H-haloperidol/10^{6} drug loaden HaCaT prior to and after co-culture with drug-loaded Sk-MeL-1 cell line for 5 days. After co-culture, pigmentation of HaCaT cells was present and associated with an increase of the ^{3}H-haloperidol content of the keratinocytes. *1* cell-bound ^{3}H-haloperidol prior to co-culture of drug-loaded Sk-MeL-1 and drug-loaded HaCaT, *2* cell-bound ^{3}H-haloperidol after co-culture of drug loaden Sk-MeL-1 and drug-loaded HaCaT, *3* native HaCaT cells prior to co-culture, *4* cell-bound ^{3}H-haloperidol after co-culture of drug loaden Sk-MeL-1 and native HaCaT cells

Mel-1 with native HaCaT cells the cell-bound ^{3}H-haloperidol was 1.35 pmol/10^6 HaCaT cells. The drug uptake for HaCaT cells after culturing in the presence of low ^{3}H-haloperidol concentration (0.8 pmol/ml medium) revealed a concentration plateau of 0.03 pmol/10^6 cells.

For the co-culture experiments a total recovery of ≥ 92% of the initially radioactivity inserted by the drug-loaded cell lines could be determined.

Cell mass and melanin content

The mean value for dried, native HaCaT cells was 0.75 ± 0.02 mg/10^6 cells. The HaCaT cell pellets were initially white in colour. However, light brown HaCaT pellets were obtained after the co-culture experiments and melanin uptake was 0.50 ± 0.08 μg/10^6 HaCaT cells. Thus normalised melanin uptake averaged 0.6 μg melanin/mg dried keratinocytes. The melanin content of the pigment-producing cell line was 25 μg/10^6 Sk-MeL-1 cells.

Discussion

Since the pioneering work of Rheinwald and Green in 1975 [21] many versions of co-cultures have been developed to mimic the in vivo situation of normal skin. Such models are increasingly employed as in vitro test systems in pharmaco-toxicological studies [20]. In primary cell cultures the material is usually limited and standardised quality is crucial due to the wide individual biological variations. Working with cell lines is advantageous due to their independence of donor variation and the availability in unlimited quantity. The major disadvantages of immortalised cell lines are possible alterations in cell proliferation and in their cellular metabolism compared to the primary cells. However, the immortalised human keratinocyte cell line HaCaT has been demonstrated to exhibit a high differentiation potential under in vivo and in vitro conditions and to be a suitable substitute for normal human keratinocytes [2]. In addition HaCaT cells are free of contaminating melanocytes, a bias if using interfollicular keratinocytes in primary cell cultures. The Sk-MeL-1 cell line is one of the rare human cell lines that express melanogenesis [10].

For these reasons HaCaT and SkMeL-1 were chosen as models for the cell populations present in the human anagen hair follicle. In previous experiments it has been observed that single Sk-MeL-1 cells exhibited dendrite formation which encouraged the development of a simplified co-culture model. For the first time HaCaT/SkMel-1 co-culture is reported in the present paper.

In separate cultures Sk-MeL-1 were far less dentritic than in the co-cultures. Obviously keratinocyte-derived factors modulated their morphology and led to formation of a dendritic melanocyte cell shape (Fig. 1). This ability ensured the transfer of pigment to the keratinocytes as verified by the TEM investigations (Fig. 2). The present study proved the role of pigments as carriers for melanin-associated drug molecules. A striking increase of cell-bound ^{3}H-haloperidol for HaCaT cells was found after pigmentation during co-culture (Fig. 3) indicating that for substances with high melanin affinity a minor component in a specimen such as pigment in hair fibres may contribute to or even determine the analytical results. However, melanin production in a pigment-producing cell is genetically regulated, which defines its limit and potential to synthesise melanin, as well as by the cellular environment which determines and modifies the expression of that potential [9]. Therefore, due to the complexities of melanins, their physico-chemical properties and inherent heterogeneity, for hair analysis it seems difficult, at least at the present time, to reach specific or even general opinions from the surprising influence of the melanin-associated portion of ^{3}H-haloperidol molecules in pigmented HaCaT cells (Fig. 3). Nevertheless, some useful conclusions can be drawn from the experiments.

In 1998 Henderson et al. [7] already demonstrated race to be a factor affecting hair analysis by incorporation of isotopically labeled cocaine into human hair. Meanwhile there is a growing interest in studying the major factors for drug uptake and how drug substances are entrapped in the hair follicle during hair growth, melanin still being a matter of controversial debate.

Pigmentation is genetically determined. It has been known for a long time that hair from non-Caucasians contain a higher amount of pigments than hair from Caucasians [22]. Pigmentation of hair is mainly related to four biological processes:

1. The production of melanosomes in the melanocytes
2. The melanisation of the melanosomes
3. Their transfer within secondary lysosome-like organelles to the keratinocytes
4. The degradation of the secondary lysosomes, that are known to contain acidic phosphatase [23], followed by the distribution within the keratinocytes [8].

The mechanisms advocated for melanosomal transfer from the melanocytes to the keratinocytes include cytophagocytosis, endocytosis and direct inoculation. The melanosomal distribution inside the keratinocytes is still poorly understood. There appears to be a good correlation between melanosome size and distribution pattern either singly as in Negroid and Mongoloid skin and hair (≥ 1 μm) or in complexes as in Caucasians (< 1 μm).

The granular nature of hair pigment differs from the melanosomes transferred to the keratinocytes during Sk-MeL-1/HaCaT co-culture as confirmed by the TEM investigations (Fig. 3). In addition to the high sensitivity of the radiotracer technique used, this finding may predominantly contribute to the results obtained for the ^{3}H-haloperidol content in pigmented HaCaT cells. Seiji et al. [24] termed the natural pigment granules “the melanosomes” and delineated four stages in their maturation. Regular and periodic deposition of electron opaque melanin is evident on the matrix by stage III, in late stage IV the melanosomes are completely melanised and the melanin that is deposited within the pigment granules obscures all

internal structures. TEM investigations revealed that most melanosomes taken up by the HaCaT cells were in an early or intermediate stage of maturation (Fig. 2).

At the ultrastructural level the presence of some spiral elements was regularly observed suggesting that melanised protein sheets or lamellae are rolled upon themselves like a "jelly roll" at the end of melanosome maturation. It has already been reported for particular substances that drug accumulation occurs onto the surface of the melanin biopolymers and that those melanin-associated molecules can be easily removed by routine extraction procedures [16]. The present results support these findings. In the co-culture experiments, which were performed in the absence of drug substances, a release of ^{3}H-haloperidol molecules from the drug-loaden Sk-MeL-1 cells due to a concentration gradient was observed resulting in a decrease of cellular-bound substance for this cell type and in low extracellular ^{3}H-haloperidol concentrations in the media at the end of the experiments.

An excellent access might be gained to pigment-associated drug molecules for immature or degraded melanosomes. However, extraction of melanin-associated drug molecules from mature, intact melanosomes such as the pigment granules in natural human hair fibres might be restricted to the drug portion bound onto the outer surface of the melanin granules.

In conclusion, the present findings at the cellular level imply that research concerning the influence of pigmentation on hair analysis has just begun. It is likely that continued progress will be made eventually resulting in a thorough understanding of drug uptake and drug binding in human hair.

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References

1. Atlasik B, Stepien K, Wilczok T (1980) Interaction of drugs with ocular melanin in vitro. Exp Eye Res 30:325–331
2. Boelsma E, Verhoeven MC, Ponec M (1999) Reconstruction of a skin equivalent using a spontaneously transformed keratinocyte cell line (HaCaT). J Invest Dermatol 112:489–498
3. Dürsch I, Meyer HHD, Karg H (1995) Accumulation of the β-agonist clenbuterol by pigmented tissues in the rat eye and hair of veal calves. J Anim Sci 73:2050–2053
4. Fusenig NE, Graf H (eds) (1994) Cell culture in pharmaceutical research. Springer, Berlin
5. Green S, Wilson JF (1996) The effect of hair color on the incorporation of methadone into hair in the rat. J Anal Toxicol 20:121–123
6. Gygi SP, Joseph RE, Cone EJ, Wilkins DG, Rollins DE (1996) Incorporation of codeine and metabolites into hair. Role of pigmentation. Drug Metab Dispos 24:495–501
7. Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P (1998) Incorporation of isotopically labeled cocaine and metabolites into hair:1. Dose-reponse relationship. J Anal Toxicol 20:1–12
8. Jimbow K, Fitzpatrick TB, Quevedo WC (1986) Formation, chemical composition and functions of melanin pigments in mammals. In: Maltoltsy AM (ed) Biology of the integument. Springer, New York, pp 278–296
9. Mishima Y (1994) Molecular and biological control of melanogenesis through tyrosinase genes and intrinsic and extrinsic regulatory factors. Pigment Cell Res 7:376–387
10. Oettgen HF, Aoki T, Old LJ, Boyse EA, Harven E de, Meills GM (1968) Suspension culture of a pigment-producing cell line derived from a human malignant melanoma. J Natl Cancer Inst 41:827–843
11. Ozeki H, Ito S, Wakamatsu K, Thody A (1996) Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res 9:265–270
12. Pötsch L (1996) A discourse on human hair fibers and reflections on the conservation of drug molecules . Int J Legal Med 108:285–293
13. Pötsch L, Skopp G, Möller MR (1997) Biochemical approach on the conservation of drug molecules during hair fiber formation. Forensic Sci Int 84:25–35
14. Pötsch L, Skopp G, Möller MR (1997) Influence of pigmentation on the codeine content of hair fibers in guinea pigs. J Forensic Sci 42:1093–1096
15. Pötsch L, Skopp G, Rippin G (1997) A comparison of 3H-cocaine binding on melanin granules and human hair in vitro. Int J Legal Med 110:55–62
16. Pötsch L, Emmerich P, Skopp G (2000) In vitro experiments on melanin association and drug desorption of ^{3}H-cocaine/BZE and ^{3}H-haloperidol. Abstract. TIAFT, 38th International Meeting, 13–17 August, Helsinki, Finland, p 214
17. Pötsch L, Emmerich P, Skopp G (2002) Preliminary approach to elucidate the role of pigment as a binding site for drugs and chemicals in anagen hair: differential uptake of ^{3}H-haloperidol by pigment-producing compared to non-pigment-producing cell lines. Int J Legal Med 116:58–61
18. Pötsch L, Skopp G, Zörntlein S, Becker J (1997) Zum Suchtmittelnachweis in Haaren. IV. Einfluß der Pigmentierung auf den Ofloxacingehalt in Haaren bei Meerschweinchen. Rechtsmedizin 7:147–151
19. Pragst F, Rothe M, Spiegel K, Spokert F (1998) Illegal and therapeutic drug concentrations in hair segments – A timetable of drug exposure? Forensic Sci Rev 10:81–111
20. Regnier M, Asselineau D, Lenoir MC (1990) Human epidermis reconstructed on dermal substrates in vitro: an alternative to animals in skin pharmacology. Skin Pharmacol 3:70–85
21. Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–344
22. Robbins CR (1988) Chemical and physical behavior of human hair. Springer, New York
23. Seiji M, Kikuchi A (1969) Acid phosphatase activity in melanosomes. J Invest Dermatol 52:212–216
24. Seiji M, Fitzpatrick TB, Birbeck MSC (1961) The melanosome: a distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis. J Invest Dermatol 36:243–252
25. Slawson MN, Wilkins DG, Rollins DE (1998) The incorporation of drugs into hair: relationship of hair color and melanin concentration to phencyclidine incorporation. J Anal Toxicol 22:406–413
26. Wilkins DG, Valdez AS, Nagasawa PR, Gygi SP, Rollins DE (1998) Incorporation of drugs for the treatment of substance abuse into pigmented and nonpigmented hair. J Pharm Sci 87: 435–440