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Chloroquine and hydroxychloroquine binding to melanin: Some possible consequences for pathologies

R.L. Schroeder, J.P. Gerber*

School of Pharmacy and Medical Sciences, The University of South Australia, North Terrace, Adelaide, South Australia 5000, Australia

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ABSTRACT

For many years chloroquine was used as a prophylactic agent against malaria, and more recently as a mild immunosuppressive. However, due to lengthy treatment periods, adverse effects have become apparent, which included retinopathy. The structurally related hydroxychloroquine is less toxic, thought to be owing to a lower tissue accumulation in melanin rich areas. This study primarily focused on quantifying melanin binding between chloroquine and hydroxychloroquine at physiological pH to investigate the potential link between binding and reported toxicity. In addition, for the first time this study quantified the actual extent of adsorption of chloroquine and hydroxychloroquine to melanin and examined the desorption profile of both drugs from melanin to demonstrate the affinity between the pigment and the solutes. The results suggest that there is a difference between the adsorption affinities of chloroquine and hydroxychloroquine, potentially explaining the differences in bioaccumulation in retinal tissue. In addition, both solutes displayed a strong physical attraction to the absorbent.

1. Introduction

The pigment melanin has been linked to a range of pathologies in association with drug binding and has consequently attracted much attention [26]. Skin and feather melanin have evolved over millennia and there is little doubt regarding its photoprotective role [18]. However, the reason for pigmentation elsewhere in the mammalian body remains speculative. Melanin has been suggested to act as a free radical scavenger and iron regulator in the brain [8]. Conditions such as Parkinson's disease are associated with the disappearance of melanin from relevant tissues, but whether this is a cause or consequence of the disease is unclear [10]. It therefore suggests that melanin may hold the key to at least a few pathologies. In this study, the focus was on the affinity of melanin for the antimalarial drug chloroquine, and if the difference in ocular toxicity between it and a related analogue, hydroxychloroquine, could be clarified.

The toxicity of a particular drug depends in part on its mode of action and whether it accumulates or not [17]. Due to tissue or organ specificity, an irreversibly bound xenobiotic will not necessarily cause adverse effects, but rather just act as a deactivated reservoir. However, melanin binding becomes dangerous when accumulation occurs in regions that the released drug could cause damage [17].

Many drugs have been examined over the last 50 years and include phenothiazines, antibiotics, antimalarials, antirheumatics, antifolates, illicit drugs and herbicides, which all have an affinity towards melanin [1,3,12,14,16,23–27,30]. Chloroquine (Fig. 1a) has been monitored for many years due to adverse effects caused through prolonged use. Discovered in 1934, through the

* Corresponding author. Tel.: +61 8 8302 2568; fax: +61 8 8302 1087.
E-mail addresses: rhiannon.schroeder@mymail.unisa.edu.au (R.L. Schroeder), Cobus.Gerber@unisa.edu.au (J.P. Gerber).

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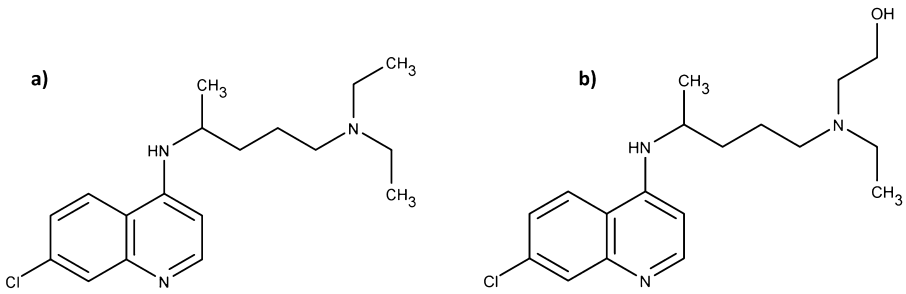

Fig. 1. (a) Chloroquine and (b) hydroxychloroquine.

modification of the antimalarial drug atabrine, and initially given the name resochin [15], chloroquine had high antimalarial activity without the side effects of other current drugs at the time. Despite initial toxicity concerns, it remained a first line defence for the treatment of malaria for many decades.

Malaria is a parasitic disease affecting erythrocytes and is spread by female *anopheles* mosquitoes. It has become one of the most devastating diseases in the tropics affecting millions of people annually [6]. Antimalarial medication, including chloroquine, has been the frontline choice in this battle. However, because of over- and inappropriate use, resistant strains of the malarial parasite started to evolve, diminishing the effects of the drug [6,15].

Nevertheless, alternative uses of chloroquine include treatment of rheumatoid arthritis, discoid lupus erythematosus and amoebic hepatitis [5]. Due to these conditions requiring on-going treatment, there is a risk that toxicity may occur through long term exposure. Similarly, exceeding dosage limits and patient age have been associated with side effects related to chloroquine therapy. One of the most severe adverse effects is retinopathy with symptoms including central vision loss, scotomata, an alteration in the field of vision, reading difficulties and reduced colour perception [21].

The structurally related hydroxychloroquine (Fig. 1b) is considered to cause a lower degree of retinal damage than seen with chloroquine [9]. It has been found that hydroxychloroquine has a lower tissue accumulation compared with chloroquine, which may translate into a decreased tendency for causing corneal change [7]. The correlation between the percentage of tissue accumulation and the amount of pigment in the eye suggests a relationship between retinopathy and melanin. Due to the apparent association between chloroquine accumulation in the eye and retinopathy, it was proposed that retinopathy caused by chloroquine was either due to its adsorption to ocular melanin [11] or due to another pathophysiological pathway caused by chloroquine itself [32]. An alternative hypothesis suggested that adsorption of chloroquine to melanin may alter the melanin granule which subsequently undermines the protecting role of melanin as a free radical scavenger, thus leading to retinal toxicity [4]. As the adverse effects of hydroxychloroquine use are not as severe, it has been speculated that the affinity of melanin for hydroxychloroquine is not as strong as for chloroquine [7].

To date, the binding potential of melanin for chloroquine has been examined quite extensively [22,28–31]. However, this study has gone one step further and made a direct comparison between chloroquine and its analogue, hydroxychloroquine, in an attempt to resolve physiological differences in toxicity. Although adsorption of chloroquine on one type of melanin has been reported before, no desorption data have been published. Therefore, the desorption profile has been examined for both compounds and compared in relation to the differences seen in drug toxicity.

2. Materials and methods

2.1. Melanin sample preparation

The melanin used was isolated from native Australian cuttlefish *Sepia apama* and *Sepia novaehollandiae* (Valente Seafoods, South Australia, Australia and SARDI, South Australia, Australia) through dissection of the ink sac of partially frozen animals. Freezing ensured granulation of the ink, facilitating easier recovery and minimising contamination. The contents of the ink sacs were emptied and treated once in 0.9% saline solution, and then centrifuged. The remaining pellet was frozen at −80 °C, freeze dried, ground into a fine powder and stored in a desiccator. This melanin will be referred to as *Sepia* melanin.

2.2. Chemicals

Chloroquine diphosphate was obtained from Merck Pty. Ltd (Australia), while potassium phosphate was purchased from The British Drug Houses Ltd (England) and hydroxychloroquine sulphate was sourced from Sigma–Aldrich (USA).

2.3. Preparation of stock solutions

A potassium phosphate (pH 7.4) buffer solution was prepared fresh at 20 mM concentrations prior to use. Stock solutions of 3.1 mmol/L chloroquine and 3.0 mmol/L hydroxychloroquine were prepared in the buffer solution. The phosphate buffer solution was used as a blank to correct for background in spectrophotometric measurements.

2.4. Instrumentation

A Varian Cary 50 ultraviolet/visible (UV/vis) spectrophotometer was used to determine chloroquine and

hydroxychloroquine concentrations. Solution pH was measured using a Hanna potentiometer (Model: pH211 microprocessor pH meter) fitted with a microelectrode.

2.5. Calibration curve

Chloroquine solutions ranging from 0.0031, 0.039, 0.078, 0.16 to 0.31 mmol/L were prepared in phosphate buffer at pH 7.4 to generate standard curves of absorbance at 255 nm against chloroquine concentration. Hydroxychloroquine concentrations over the range 0.0030, 0.037, 0.074, 0.15 and 0.30, were prepared as outlined above for chloroquine. However, analysis was conducted using the wavelength at 340 nm. All samples were diluted 40 times with phosphate buffer for analysis. Standard curves were produced in triplicate in order to determine the inter-day reproducibility.

2.6. Equilibration time for adsorption

An amount of 5 mg of melanin was spiked with 5 mL of 0.63 mmol/L chloroquine. Separate tubes were equilibrated for periods of 10, 30, 60, 120, and 240 min. After each equilibration time, the samples were centrifuged at $4770 \times g$ for 4 min and the chloroquine concentration in the supernatant was determined by comparison with the standard curve using UV/vis spectrophotometry at 255 nm. Solutions were measured against a melanin blank solution background. All experiments henceforth were produced in triplicate to assess the variability. Hydroxychloroquine equilibration was performed in the same manner using a concentration of 0.60 mmol/L and a wavelength of 340 nm.

2.7. Adsorption of chloroquine and hydroxychloroquine to Sepia melanin

To determine the adsorption capacity of melanin, a series of concentrations of chloroquine and hydroxychloroquine were prepared ranging from 0.16 to 2.2 mmol/L (chloroquine) and 0.089 to 1.2 mmol/L (hydroxychloroquine). A 5 mg portion of melanin was equilibrated with 5 mL of the specified concentration of chloroquine prepared in 20 mM potassium phosphate buffer for 2 h. The samples were centrifuged at $4770 \times g$ for 4 min and the supernatant removed and analysed using UV/vis spectrophotometry at 255 nm. Solutions of hydroxychloroquine were prepared in a similar manner. However, only 1 mg of melanin was used in a volume of 1 mL due to limited solute availability. The solutions were analysed at 340 nm.

2.8. Equilibration time for desorption

In order to investigate the rate of desorption, an appropriate amount of *Sepia* melanin was equilibrated with 5 mL of 1.9 mmol/L chloroquine or 1 mL of 1.5 mmol/L hydroxychloroquine for 2 h. Subsequently, the samples were centrifuged and the supernatant was drawn off. This was followed by the addition of the same volume of the buffer solution to the melanin pellet in separate tubes and allowed to equilibrate for periods of 0.5, 2, 4, 6 and 24 h. After centrifugation, the amount of chloroquine or hydroxychloroquine desorbed from melanin was determined using UV/vis spectrophotometry.

2.9. Desorption of chloroquine and hydroxychloroquine from Sepia melanin

Initially 5 mg of melanin was saturated by equilibration with 5 mL of 1.9 mmol/L chloroquine prepared in 20 mM potassium phosphate buffer at pH 7.4 for 2 h. The samples were then centrifuged at $477 \times g$ for 4 min and the supernatant removed. Five millilitres of fresh buffer solution was then used to resuspend the melanin and an additional 2 h was allowed for the samples to re-equilibrate. Centrifugation followed and the supernatant removed. This step was repeated another 7 times. The supernatant from each step was measured using UV/vis spectrophotometry at a wavelength of 255 nm to determine the concentration of chloroquine released from melanin, and therefore demonstrate the rate of solute release into or by the phosphate buffer. This was used as a measure of the affinity between melanin and chloroquine.

In addition, a desorption isotherm was examined. Five milligrams of melanin was equilibrated with 5 mL of a specified concentration of chloroquine ranging from 0.31 to 2.2 mmol/L. The samples were prepared in 20 mM potassium phosphate buffer at pH 7.4 and equilibrated for 2 h. After this time, the total volume of each individual buffer solution was increased by 1/3rd and re-equilibrated for a further 2 h. The samples were then centrifuged at $4770 \times g$ and the supernatant removed and measured using UV/vis spectrophotometry at 255 nm.

The same method was used to determine the desorption of hydroxychloroquine from melanin as for chloroquine. However, when determining the isotherm, 1 mg of melanin was equilibrated in 1 mL of 1.8 mmol/L hydroxychloroquine. All samples were analysed using UV/vis spectrophotometry at a wavelength of 340 nm.

2.10. Langmuir isotherms

The Langmuir adsorption isotherm was used to model chloroquine-melanin adsorption data. For Langmuir theory, sorption is assumed to take place at a specific homogeneous site and theoretically, a saturation point would be reached beyond which no further sorption would take place [1,3]. The Langmuir adsorption equation has the form,

$$q = \frac{q_{(m)} K C_{(eq)}}{1 + K C_{(eq)}}$$

where q is the amount of adsorbed solute (adsorbate) (mg/g), $q_{(m)}$ and K are related to monolayer adsorption capacity of adsorbent (melanin) and energy of adsorption respectively. $C_{(eq)}$ represents the equilibrium solution concentration of the solute and was found using the equation derived from the prior-generated standard curve. An adsorption equilibrium isotherm of q against $C_{(eq)}$ was generated to find the adsorptive capacity of melanin. The graph of $C_{(eq)}$ versus $C_{(eq)}/q$ was used to calculate $q_{(m)}$ from the slope of the line ($q_{(m)}$ = 1/slope).

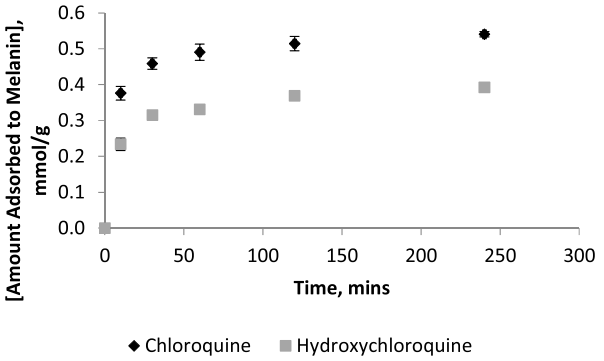

Fig. 2. Equilibration time of chloroquine and hydroxychloroquine at pH 7.4.

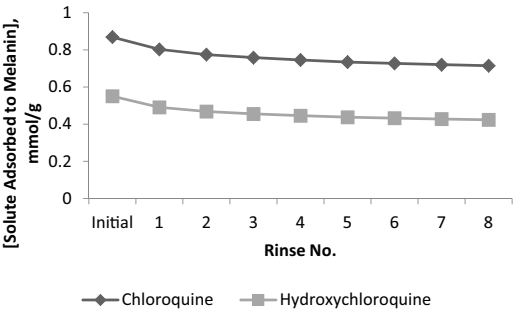

Fig. 4. Desorption profile for chloroquine and hydroxychloroquine on melanin.

3. Results

3.1. Analytical method development

Standard curves of absorbance against solute concentration were generated for pH 7.4 using both chloroquine and hydroxychloroquine. The coefficient of variation (CV) of the slopes for each standard curve ($n = 3$) was less than 1.05%, with each standard concentration having a CV of less than 3.00%. Each curve gave a linear fit of at least 99.9% for 5 data points. The lower limit of quantification was 0.0031 mmol/L and 0.0030 mmol/L for chloroquine and hydroxychloroquine respectively, with negligible variation.

3.2. Adsorption of chloroquine and hydroxychloroquine to Sepia melanin

The adsorption capacity was determined at pH 7.4 and an equilibration time of 2 h was found to be adequate for both chloroquine and hydroxychloroquine and was used henceforth (Fig. 2).

Chloroquine had an adsorption capacity of 0.87 mmol/g while hydroxychloroquine gave 0.56 mmol/g (Fig. 3).

3.3. Desorption of chloroquine and hydroxychloroquine from Sepia melanin

To determine the relationship between the solute and the adsorbent, a desorption isotherm was used. Known concentrations of chloroquine were diluted after the initial equilibration with melanin and allowed to re-establish a new equilibrium. It was observed that both the adsorption and desorption isotherms coincided with each other, indicating that there is a physical attraction between melanin and chloroquine.

A series of sequential rinses were performed to assess the affinity strength between chloroquine and melanin. The initial rinse displaced the highest percentage of solute at 7%, with each sequential rinse removing less chloroquine. The total amount of chloroquine lost from the surface of melanin was 0.15 mmol/g (18%) (Fig. 4).

A similar pattern was apparent for hydroxychloroquine. However, a higher percentage was initially lost from melanin (11.5%) and resulted in a greater amount of solute displacement overall at 0.13 mmol/g in relation to initial adsorbed concentration.

4. Discussion

The primary aim of the study was to compare chloroquine and hydroxychloroquine adsorption to determine if that could account for toxicity differences between the two

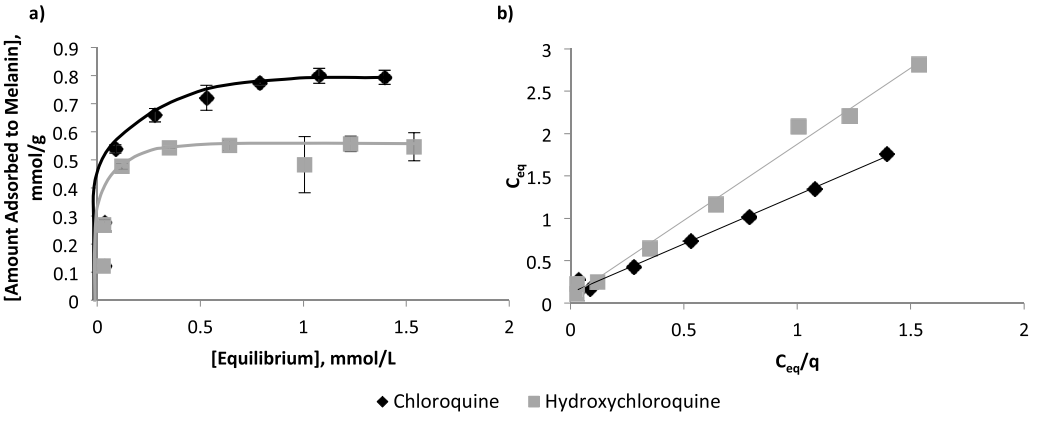

Fig. 3. (a) Adsorption isotherm of chloroquine and hydroxychloroquine for melanin at pH 7 4. (b) Linear form of the Langmuir expression.

compounds at physiological pH. To avoid complications, melanin was carefully extracted from two native South Australian cuttlefish; *Sepia apama* (Australian giant cuttlefish) and *Sepia novaehollandiae*. In a previous study, it was demonstrated that proteins which have been widely accepted to be part of melanin are in all probability adsorbed from surrounding tissue during isolation [20]. For this study, *Sepia* melanin was used as it is more readily available and required very little treatment, whereas the preparation procedure of mammalian melanin is a lot more rigorous and raises concerns about preserving the structural integrity of the pigment [19]. Synthetic melanins have been widely employed to investigate adsorption [2,22,27,29]. However, there is clear evidence that such melanins are not structurally representative of native melanins and therefore any data gathered is of debatable significance. Results have shown that dopamine melanin desorbs substantial amounts of free base after numerous attempts to clean the pigment [20]. Though this observation is intriguing in itself, dopamine being such an unstable compound under experimental conditions, it confirmed the need for careful consideration of the type of melanin employed in our studies.

The risk of developing retinopathy is reduced with hydroxychloroquine use compared with chloroquine [33]. Therefore, the adsorption capacity of melanin for chloroquine and hydroxychloroquine was investigated and compared to determine whether there was any difference in the extent of binding. *Sepia* melanin isolated from Australian cuttlefish was used to examine the relationship between the solutes and melanin. This melanin was carefully extracted from the animals to minimise protein contamination.

Hydroxychloroquine had a substantially lower affinity towards *Sepia* melanin than chloroquine at pH 7.4 (45% difference). This difference was thought to be the consequence of the small variation in the structure of hydroxychloroquine compared to chloroquine. Hydroxychloroquine has three basic functional groups with pKa values of <4.0, 8.3 and 9.7, two of which would be protonated at pH 7.4. Similarly, chloroquine has three pKa values of 4.0, 8.4 and 10.2 [34]. Thus the difference between the two compounds is the presence of the additional hydroxyl group on hydroxychloroquine which would impart a greater aqueous solubility [13]. Therefore, the adsorption equilibrium between the surface of melanin and the hydroxychloroquine in solution may be expected to favour the solvent compared to chloroquine. Intriguingly, hydroxychloroquine does not result in extensive tissue damage. The result therefore suggests that the toxicity may be a consequence of the reduced binding capacity of hydroxychloroquine compared to chloroquine, meaning it does not bioaccumulate to the same extent.

Adsorption experiments demonstrated that at physiological pH there was a significant difference in the adsorption capacity between chloroquine and hydroxychloroquine for melanin, potentially explaining the differences in bioaccumulation in retinal tissue. However, how the two drugs are released from the pigment and what this may mean for long term side effects, as seen with the development of retinopathy, also needs to be considered.

Therefore, the desorption profile of both chloroquine and hydroxychloroquine were examined at pH 7.4. As there was very little difference between the adsorption profile and the established desorption profile for both compounds, it was concluded that a physical attraction exists between the solute and the absorbent. This signifies a reversible ionic interaction, rather than chemical binding.

The next important aspect was to assess the strength of the relationship between melanin and chloroquine. This was examined by conducting sequential rinses with the specified bathing solution at 2 h intervals. The first rinse resulted in the largest loss of compound from the surface of the melanin, at 7%. Each subsequent rinse removed correspondingly less material, with the final amount being only 0.8% after 7 treatments. A total of 82% of the original bound solute was still present on melanin (Fig. 4). The trend highlights the strong affinity between chloroquine and melanin, potentially indicating the role chloroquine plays in retinopathy. Once melanin is bound, it is not readily released, as demonstrated by the failure of successive rinses to wash any appreciable amount off the surface.

Similar results were apparent for hydroxychloroquine. However, hydroxychloroquine desorbed at a slightly faster rate than chloroquine, again indicating that the binding capacity in aqueous medium is less (hydroxychloroquine showed an overall loss of 26% compared to chloroquine at 18%). However, whether this small difference is enough to explain the less toxic nature of hydroxychloroquine remains unclear and warrants further investigation.

5. Conclusion

The relationship between melanin and hydroxychloroquine was examined for the first time and compared to the adsorption of chloroquine on melanin. Hydroxychloroquine was found to have a lower affinity than chloroquine, potentially explaining the differences in bioaccumulation in retinal tissue. Furthermore, desorption profiles for both chloroquine and hydroxychloroquine were derived, a novel result, and it was concluded that these solutes have a strong physical interaction rather than chemical. Further studies will be needed to examine the surface relationship between these two compounds and melanin in order to gain a full appreciation of the binding mechanisms.

Transparency document

The Transparency document associated with this article can be found in the online version.

References

[1] M. Bridelli, A. Ciati, P. Crippa, Binding of chemicals to melanins re-examined – adsorption of some drugs to the surface of melanin particles, Biophys. Chem. 119 (2) (2006) 137–145, http://dx.doi.org/10.1016/j.bpc.2005.06.004.
[2] M. Bridelli, P. Crippa, Theoretical analysis of the adsorption of metal ions to the surface of melanin particles, Adsorption 14 (1) (2008) 101–109, http://dx.doi.org/10.1007/s10450-007-9059-8.
[3] M.G. Bridelli, A. Ciati, P.R. Crippa, Binding of chemicals to melanins re-examined: adsorption of some drugs to the surface of melanin particles, Biophys. Chem. 119 (2) (2006) 137–145.

[4] L.K. Charkoudian, K.J. Franz, Fe(III)-coordination properties of neuromelanin components: 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, Inorg. Chem. 45 (9) (2006) 3657–3664, http://dx.doi.org/10.1021/ic060014r.
[5] R.G. Cooper, T. Magwere, Chloroquine: novel uses & manifestations, Indian J. Med. Res. 127 (4) (2008) 305–316.
[6] U. D'Alessandro, H. Buttiëns, History and importance of antimalarial drug resistance, Trop. Med. Int. Health 6 (11) (2001) 845–848, http://dx.doi.org/10.1046/j1365-3156.2001.00819x.
[7] M. Easterbrook, The ocular safety of hydroxychloroquine, Semin. Arthritis Rheum. 23 (2) (1993) 62–67.
[8] W. Enochs, T. Sarna, L. Zecca, P. Riley, H. Swartz, The roles of neuromelanin, binding of metal ions, and oxidative cytotoxicity in the pathogenesis of Parkinson's disease: a hypothesis, J. Neural Trans. Park. Dis. Dement. Sec. 7 (2) (1994) 83–100, http://dx.doi.org/10.1007/bf02260963.
[9] D.F. Farrell, Retinal toxicity to antimalarial drugs: chloroquine and hydroxychloroquine: a neurophysiologic study, Clin. Ophthalmol. 6 (2012) 377–383, http://dx.doi.org/10.2147/OPTH.S27731.
[10] L.S. Forno, Neuropathology of Parkinson's disease, J. Neuropathol. Exp. Neurol. 55 (3) (1996) 259–272.
[11] L.M. Gonasun, A.M. Potts, In vitro inhibition of protein synthesis in the retinal pigment epithelium by chloroquine, Investig. Ophthalmol. 13 (2) (1974) 107.
[12] R.M.J. Ings, The melanin binding of drugs and its implications, Drug Metab. Rev. 15 (5–6) (1984) 1183–1212, http://dx.doi.org/10.3109/03602538409033561.
[13] C. Knox, V. Law, T. Jewison, P. Liu, S. Ly, A. Frolkis, A. Pon, K. Banco, C. Mak, V. Neveu, Y. Djoumbou, R. Eisner, A.C. Guo, D.S. Wishart, DrugBank 3.0: a comprehensive resource for 'omics' research on drugs, Nuclei Acids Res. 39 (2011) D1035–D1041.
[14] M. Koeberle, P. Hughes, G. Skellern, C. Wilson, Binding of memantine to melanin: influence of type of melanin and characteristic, Pharm. Res. 20 (10) (2003) 1702–1709, http://dx.doi.org/10.1023/A:1026116208008.
[15] K. Krafts, E. Hempelmann, A. Skórska-Stania, From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy, Parasitol. Res. 111 (1) (2012) 1–6, http://dx.doi.org/10.1007/s00436-012-2886-x.
[16] B. Larsson, A. Oskarsson, H. Tjälve, On the binding of the bisquaternary ammonium compound paraquat to melanin and cartilage in vivo, Biochem. Pharmacol. 27 (13) (1978) 1721–1724, http://dx.doi.org/10.1016/0006-2952(78)90546-4.
[17] B. Leblanc, S. Jezequel, T. Davies, G. Hanton, C. Taradach, Binding of drugs to eye melanin is not predictive of ocular toxicity, Regul. Toxicol. Pharmacol. 28 (2) (1998) 124–132.
[18] Q. Li, J.A. Clarke, K.-Q. Gao, C.-F. Zhou, Q. Meng, D. Li, L. D'Alba, M.D. Shawkey, Melanosome evolution indicates a key physiological shift within feathered dinosaurs, Nature 507 (7492) (2014) 350–353, http://dx.doi.org/10.1038/nature12973.
[19] Y. Liu, V.R. Kempf, J. Brian Nofsinger, E.E. Weinert, M. Rudnicki, K. Wakamatsu, S. Ito, J.D. Simon, Comparison of the structural and physical properties of human hair eumelanin following enzymatic or acid/base extraction, Pigment Cell Res. 16 (4) (2003) 355–365, http://dx.doi.org/10.1034/j.1600-0749.2003.00059.x.
[20] F. Madaras, J. Gerber, F. Peddie, M. Kokkinn, The effect of sampling methods on the apparent constituents of ink from the Squid *Sepioteuthis australis*, J. Chem. Ecol. 36 (11) (2010) 1171–1179, http://dx.doi.org/10.1007/s10886-010-9869-0.
[21] M. Michaelides, N.B. Stover, P.J. Francis, R.G. Weleber, Retinal toxicity associated with hydroxychloroquine and chloroquine: risk factors, screening, and progression despite cessation of therapy, Arch. Ophthalmol. 129 (1) (2011) 30–39, http://dx.doi.org/10.1001/archophthalmol.2010.321.
[22] C. Ono, M. Tanaka, Binding characteristics of fluoroquinolones to synthetic levodopa melanin, J. Pharm. Pharmacol. 55 (8) (2003) 1127–1133, http://dx.doi.org/10.1211/002235703322277168.
[23] P.N. Patil, Cocaine-binding by the pigmented and the nonpigmented iris and its relevance to the mydriatic effect, Investig. Ophthalmol. Vis. Sci. 11 (9) (1972) 739–746.
[24] P.N. Patil, K. Shimada, D.R. Feller, L. Malspeis, Accumulation of (-)-14c-ephedrine by the pigmented and the nonpigmented iris, J. Pharm. Exp. Ther. 188 (2) (1974) 342–352.
[25] A. Potts, The reaction of uveal pigment in vitro with polycyclic compounds, Investig. Ophthalmol. Vis. Sci. 3 (4) (1964) 405–416.
[26] A.M. Potts, The concentration of phenothiazines in the eye of experimental animals, Investig. Ophthalmol. Vis. Sci. 1 (4) (1962) 522–530.
[27] K. Shimada, R. Baweja, T. Sokoloski, P.N. Patil, Binding characteristics of drugs to synthetic levodopa melanin, J. Pharm. Sci. 65 (7) (1976) 1057–1060, http://dx.doi.org/10.1002/jps.2600650725.
[28] K.B. Stepień, J.P. Dworzanski, S. Imielski, T. Wilczok, Study of chloroquine binding to melanins by pyrolysis-gas chromatography and electron spin resonance spectroscopy, J. Anal. Appl. Pyrolysis 9 (4) (1986) 297–307, http://dx.doi.org/10.1016/0165-2370(86)80003-1.
[29] K.B. Stępień, T. Wilczok, Studies of the mechanism of chloroquine binding to synthetic dopa-melanin, Biochem. Pharmacol. 31 (21) (1982) 3359–3365.
[30] H. Tjälve, M. Nilsson, B. Larsson, Studies on the binding of chlorpromazine and chloroquine to melanin in vivo, Biochem. Pharmacol. 30 (13) (1981) 1845–1847, http://dx.doi.org/10.1016/0006-2952(81)90021-6.
[31] M. Tsuchiya, S. Hayasaka, K. Mizuno, Affinity of ocular acid-insoluble melanin for drugs in vitro, Investig. Ophthalmol. Vis. Sci. 28 (5) (1987) 822–825.
[32] R. Tzekov, Ocular toxicity due to chloroquine and hydroxychloroquine: electrophysiological and visual function correlates, Doc. Ophthalmol. 110 (1) (2005) 111–120.
[33] D. Wallace, The use of chloroquine and hydroxychloroquine for non-infectious conditions other than rheumatoid arthritis or lupus: a critical review, Lupus 5 (Suppl. 1) (1996) S59.
[34] D.C. Warhurst, J.C.P. Steele, I.S. Adagu, J.C. Craig, C. Cullander, Hydroxychloroquine is much less active than chloroquine against chloroquine-resistant *Plasmodium falciparum*, in agreement with its physicochemical properties, J. Antimicrob. Chemother. 52 (2) (2003) 188–193.