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Physical factors affecting chloroquine binding to melanin

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

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

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ABSTRACT

Chloroquine is an antimalarial drug but is also prescribed for conditions such as rheumatoid arthritis. Long-term users risk toxic side effects, including retinopathy, thought to be caused by chloroquine accumulation on ocular melanin. Although the binding potential of chloroquine to melanin has been investigated previously, our study is the first to demonstrate clear links between chloroquine adsorption by melanin and system factors including temperature, pH, melanin type, and particle size. In the current work, two *Sepia* melanins were compared with bovine eye as a representative mammalian melanin. Increasing the surface anionic character due to a pH change from 4.7 to 7.4 increased each melanin's affinity for chloroquine. Although the chloroquine isotherms exhibited an apparently strong interaction with each melanin, isosteric heat analysis indicated a competitive interaction. Buffer solution cations competed effectively at low surface coverage; chloroquine adsorption occurs via buffer cation displacement and is promoted by temperature-influenced secondary structure swelling.

1. Introduction

The association between ingested drugs and melanin in the body has been the subject of many studies over the last 50 years. For example, melanin has a strong affinity towards compounds such as phenothiazines [1–6], antibiotics [1], antimalarials [2–6], antirheumatics [2], antifolates [1], as well as various illicit drugs [7–9] and herbicides [10], with each causing various adverse effects. Due to these interactions, various negative associations have been implicated with some compounds, resulting in outcomes unrelated to the main action of the drug. Chloroquine has been used widely for the treatment of malaria and other conditions, including rheumatoid arthritis, discoid lupus erythematosus and amoebic hepatitis, and has also been associated with delayed-onset retinopathy [11,12]. The present study focused on gaining a greater insight into the thermodynamics and mechanism of the melanin–chloroquine interaction. It is anticipated such knowledge would be connected to the associated pathologies in future research.

The accumulation of a particular drug and its mode of action ultimately determines its toxicity [13]. An irreversibly bound xenobiotic will not necessarily cause adverse effects, but rather depends on tissue or organ specificity. If no harm arises from the interaction, a substance such as melanin would simply act as a deactivating reservoir. However, the injury arises when accumulation (as localised adsorption) occurs in regions of the body where the released drug could cause damage [13]. The correlation between the percentage of tissue accumulation and the amount of pigment in the eye suggests a relationship may exist between retinopathy and melanin. It was proposed that retinopathy caused by chloroquine was either due to its adsorption to ocular melanin [14] or due to another pathophysiological pathway caused by chloroquine itself [15]. A third hypothesis suggested that chloroquine chemisorbs with melanin possibly leading to retinal toxicity through an alteration of the melanin surface, and subsequently undermining the protective role of melanin as a free radical scavenger [16]. Either way, melanin is perceived to play a role. The structural analogue of chloroquine, hydroxychloroquine, is less toxic and has not been linked to retinopathy. We have shown that this reduced toxicity might be interpreted in terms of its adsorption mechanism. Hydroxychloroquine adsorbs through weaker interactions than chloroquine to different types of melanin [17], suggesting that adsorption to melanin may be a deciding factor in determining toxicity.

Organic melanin production is highly regulated in melanosomes. The melanogenetic pathway begins with the hydroxylation of tyrosine to dopa through the action of the rate-limiting enzyme tyrosinase. Dopa progresses through a series of reactions from the oxidation to dopaquinone, which then cyclises to dopachrome. At this point, the intracellular conditions dictate the type of melanin produced. Dihydroxyindole (DHI) is formed

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

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by the decarboxylation of dopachrome, whereas the presence of dopachrome tautomerase (Dct – a tyrosinase related protein) structurally rearranges dopachrome to produce dihydroxyindole carboxylic acid (DHICA) [18–20]. DHI and DHICA are the primary building blocks (as monomers) of melanin. This process is well described by the Raper-Mason scheme [21–26].

Different classes of melanin are produced through the varied incorporation of the two monomers, DHI and DHICA. As dopachrome tautomerase concentration determines DHICA amounts, the ratio can vary depending on the location of the melanin production within the body, as well as being responsible for interspecies differences. Generally, naturally sourced (organic) melanin is lower molecular weight than synthetically produced, and thus a lower number of ionisable groups [27,28]. Additionally, there are differences between the ratios of DHI and DHICA that make up the primary structure of melanin. Generally, synthetic melanins are either all one monomer type, or the DHI:DHICA ratio is close to 1:1; variations can be apparent in organic melanins [29]. The actual ratio is difficult to ascertain. Most studies base the ratio on oxidative degradation of DHI and DHICA to pyrrole di- and tricarboxylic acid (PDCA and PTCA), respectively. However, interpretations of these outcomes are confused by the compounds also being found in the bound form in the native melanin [30]. Nevertheless, Sepia melanin appears to contain about double the amount of DHICA compared to bovine eye, while DHI is approximately at similar levels. A recent review suggests that the ratio is closer to 1:1 in most natural melanins [31]. However, this ratio appears unlikely considering the control of melanogenesis in melanocytes by dopachrome tautomerase [32]. When the enzyme is abundant, DHICA formation is favoured, suggesting the location of the melanocyte and the species involved will play a role in ultimately regulating the DHI:DHICA ratio [19]. The secondary structure is also very important as it contains a series of layers in a stacked, planar configuration. Each layer has been suggested to consist of monomers arranged in porphyrin-like structures [33] with the internal arrangement simulating graphene [34,35] (see Supplementary Information Fig. S1). Results from XRD and TEM studies have determined that the pigment has a multilayer 3D arrangement thought to comprise of dihydroxyindole rings and benzothiazine residues. These structure form highly cross-linked oligomers which aggregate through π-stacking in layers of 3–4 [31,19]. The spacing between each layer is approximately 3.7–4.7 Å, with the variation due to DHI:DHICA ratio (i.e., the type of melanin) and/or the method of analysis [27,36,37]. The range can be attributed to the specific location on the structure. The closer to the centre of the granule, the wider the spacing between each layer needs to be to accommodate the curvature of the oligomeric plates [37]. Following further hierarchical aggregation, these smaller units assemble to result in spherical structures of approximately 150 nm in diameter.

An important consideration for adsorption studies is the available surface area to maximise the capacity for interacting with an adsorptive. Therefore, any porosity in an adsorbent would greatly increase the surface area to volume ratio and thus generate greater binding potential. Nitrogen gas adsorption studies have been conducted on melanin to determine porosity. Melanin is quite commonly compared to graphite in relation to the arrangement of the individual layers within the particle. It was found that *Sepia* melanin had a specific surface area of approximately 25 m^2/g [38]. To put this in perspective, graphite has a surface area approximating 4.8 m^2/g [39] whereas porous materials such as activated carbons can exceed 1000 m^2/g [40]. Therefore, it can be concluded that melanin is largely non-porous and thus the surface characteristics of the pigment are primarily of concern when conducting binding experiments.

To date, the binding potential of melanin for chloroquine has been examined quite extensively [6,41–44]. These previous studies demonstrated that the relationship between melanin and chloroquine could be fitted by the Langmuir isotherm, representing monolayer equivalent adsorption [41]. Interestingly, beyond simple adsorption, other factors associated with chloroquine adsorption such as changes in system pH and temperature or the composition of melanin have not been explored conclusively. From the previous studies, it is clear three main binding interactions occur between melanin and chloroquine: electrostatic, hydrophobic and Van der Waals [6,41–43]. As a general consensus, electrostatic interactions appeared to be primarily involved in chloroquine binding to melanin, occurring at both strong and weak adsorption sites. Relatively weak ionic interactions tended to exist between the carboxyl group on melanin and chloroquine. In addition, acidic solution phase pH caused phenolic group protonation and a consequent reduction of the surface concentration of available strong binding sites [43]. From a pharmacological perspective, Tsuchiya et al. interpreted their mechanism as a decrease in percentage adsorbed with increasing pH [44]. The melanin used in their study was prepared under extreme conditions, by soaking in 11 N HCl, which has been claimed to destroy the adsorption properties of the pigment [45].

In the previous studies examining the binding relationship between melanin and the compound of interest, the influence of the type and source of melanin was ignored. The review of numerous unrelated studies described by Derby [46] demonstrated that melanin properties can differ depending on the isolation method, the means of separation from tissue (when present as an integral part, for example neuromelanin) and, most significantly, its source [46,47]. Therefore, one of the primary aims of this study was to establish the effect of the type of melanin on chloroquine adsorption. Madaras et al. demonstrated how those proteins widely accepted to be part of melanin, are in all probability an artefact from surrounding tissue during isolation [48]. These proteins could therefore interfere with the adsorption of solutes onto the melanin surface [9,41,43,49]. To expand on this set of work, the current study further explored the affinity of melanin for chloroquine by examining adsorption as a function of organic melanin type, and also system pH and temperature. The DHI:DHICA ratios were explored as this is a much debated topic in the literature. No previous studies have investigated the effects of pH or temperature, or even the type of melanin. The pH values selected were 4.7 and 7.4 as these spanned the pKa values of each species to be considered: DHI; DHICA; and, chloroquine. The temperature range included a physiological value to promote applicability of the results. Together, the results provide a novel insight into the sorption characteristics of melanin for chloroquine.

2. Materials and methods

2.1. Melanin sample preparation

Two types of cuttlefish melanin were used in this study. The first was purchased as Tinta Calamar™ from SAMTAS, Adelaide, containing melanin from the European Common Cuttlefish, *Sepia officinalis* and was the result of the blending of multiple cuttlefish ink sacs, including the tissue, prior to packaging. This melanin will be referred to as *S. officinalis* melanin. A second melanin was extracted from the ink sacs of native Australian cuttlefish, primarily *Sepia apama* with a small proportion of *Sepia novaehollandiae* (Valente Seafoods, South Australia, Australia and SARDI, South Australia, Australia) and prepared according to Schroeder and Gerber [50]. Due to the low levels of *Sepia novaehollandiae* present, it is assumed that this melanin would behave like *Sepia apama* melanin, and thus will be referred to as *Sepia* melanin.