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Binding of doxycycline to keratin, melanin and human epidermal tissue

Thomas P. Banning, Charles M. Heard *

Welsh School of Pharmacy, Cardiff University, Cardiff CF10 3XF, UK

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Abstract

Doxycycline is licensed for the prophylaxis of malaria and recent research has indicated the feasibility of delivering this drug across the skin. The binding of doxycycline to keratin could influence skin permeation rates and it has been suggested that the interaction of anti-malarials with melanin may contribute to side effects, such as retinal damage. Doxycycline HCl was incubated with keratin (bovine horn), melanin (*Sepia officinalis*) and human epidermal samples (native and delipidised). Dose dependent binding of doxycycline to keratin and melanin was observed, and was of similar magnitude for each protein. However, the binding of doxycycline to melanin was lower by an order of magnitude relative to data previously reported for some other anti-malarials, and may indicate reduced side-effects. Doxycycline also demonstrated significantly greater affinity for native epidermal skin than for delipidised skin showing that doxycycline, a charged polar molecule, has affinity for the intercellular lipid matrix in addition to the proteinaceous domain. For native skin it was estimated that saturation would be reached at approximately 140 μg cm^{-2}; for delipidised skin it was estimated to be 60 μg cm^{-2}. Overall, the data suggested that the partition-diffusion steps that are involved in transcellular permeation are possible.

Keywords: Doxycycline; Malaria; Epidermis; Keratin; Melanin; Transdermal delivery

1. Introduction

There are approximately 90 million clinical cases of malaria each year, the disease being endemic in 101 countries worldwide, 2400 million people, therefore, live in areas where malaria is endemic (WHO, 1998). Malaria is caused by four species of plasmodial parasite, *Plasmodium vivax*, *P. falciparum*, *P. ovale and P. malariae*, transmission being by the female *Anopheles* mosquito. There are numerous commercial products available for the prevention and treatment of malaria (British National Formulary, 2001) and their use is influenced by the emergence of resistance by the plasmodial parasites in the area where the treatment is needed. Increasing resistance is presently a very large problem in many areas of the world and consequently, new drug regimens are of great interest.

Doxycycline, a semi-synthetic tetracycline antibiotic (Finch, 1996), is considered to be a safe

* Corresponding author. Tel./fax: +44-29-2087-5819.
E-mail address: heard1@cf.ac.uk (C.M. Heard).

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broad-spectrum antibiotic (Cunha, 1977) and is licensed for the prophylaxis and treatment of malaria (Tracy and Webster, 1996; Kotecka et al., 1996). In February 2001 its recommended use was substantially increased in many areas, mainly because at present the malarial parasite does not appear to be resistant to doxycycline (National Pharmaceutical Association Information Department, 2001). Recent research has considered the possibility of delivering doxycycline across the skin (Perkins and Heard, 1999).

However, to deliver a drug to the systemic circulation through the skin presents certain challenges, as the skin is a very effective barrier, generally thought to be attributable to the outermost layer of the skin, the stratum corneum (Downing and Wertz, 1989). In transdermal delivery there is a growing body of data demonstrating that interactions with proteins of the epidermis are of importance (Wurster and Dempski, 1961; Hwang and Bayne, 1984; Barry, 1990; Klimke and Schaefer-Horting, 1997). More recently, high concentrations of doxycycline located in skin, that could not be accounted for by lipid-only interactions, were observed in these laboratories (Perkins and Heard, 1999). Drug or solute partitioning into the corneocytes, which comprise 90% of the stratum corneum (Marks and Barton, 1983), will interact with or bind to keratin present within the corneocytes. Keratin is a complex mixture of proteins, comprised of coiled polypeptide chains that form super coils made up of a number of polypeptides linked by disulphide bridges between the cysteine amino acids. Most of the keratin found in the body is in the stratum corneum where it accounts for about 80% of corneocyte mass. Current theory suggests that keratin is not important in skin permeation and it is the lipid layers between the corneocytes that limit penetration. However, if doxycycline does have a high affinity for keratin it is logical to assume that, given sufficient contact, it will at least play a significant role in retention capacity within skin (Hasiguchi et al., 1998).

Another important protein of the epidermis is melanin, a dark brown pigment that is mostly responsible for skin colour. Its main function is to protect the inner layers of the skin such as the basal cell layer, where the stem cells for the protection of the epidermis are located, from the damaging effects of ultra violet radiation. Melanin is also present in other tissues that are extensively exposed to light, e.g. hair, eyes (Tanenbaum and Tuffanelli, 1980). It has been demonstrated that some anti-malarials have an affinity for melanin, suggesting that they can accumulate in the melanin rich areas of the body such as the eye, skin and hairs (Kristensen et al., 1994). This could account for many of the side effects such as change in pigmentation of the skin, bleaching of the hair and retinal damage (retinopathy), sometimes observed following long term dosing or high accumulative doses of some anti-malarials. Although the binding of melanin with numerous anti-malarials has previously been reported (Kristensen et al., 1994; Lindquist, 1973) no such investigation has yet been carried out for doxycycline. Knowledge of the binding doxycycline to melanin could, therefore, be a possible indicator as to whether the drug is likely to accumulate in areas such as the eye.

This paper aims to determine the binding of doxycycline to models of epidermal keratin and melanin. In addition the binding of doxycycline to human epidermal tissue (native and delipidised) is examined to further elucidate drug/epidermis interactions.

2. Materials and methods

2.1. Materials

Melanin from *Sepia officinalis*, primaquine diphosphate, doxycycline hydrochloride and sodium oxalate was obtained from Sigma (Poole, UK). Keratin bovine powder was obtained from ICN (Thame, UK). Phosphate buffer was made using a standard method. Perchloric acid and acetonitrile HPLC grade were obtained from Fisher (Loughborogh, UK). Female breast skin was obtained post cosmetic surgery.

2.2. Binding of doxycycline to melanin

Solutions of doxycycline were made up in phosphate buffer saline pH 7.4 using serial dilution to make the following molar concentrations: 10^{-5}, 10^{-4}, 2×10^{-4}, 4×10^{-4} and 7×10^{-4} M. Melanin (5 mg) was weighed into Millipore Ultrafree Eppendorf tubes (Varian, USA) containing a 0.45 μm filter inserts. Drug solution (0.8 ml) was then added and the tubes placed on a blood tube rotator (Stuart Scientific SB1, Fisher, UK) inside an incubator at 37 °C for 1 h. The samples were then placed in a centrifuge (Centurion 8000 series) and spun at 14 000 rpm for 10 min. The filtrate was removed and the samples were spun again at 14 000 rpm for 10min. The filtrate was again removed and the outer casings of the Eppendorfs washed and dried. Next 0.3 ml of mobile phase (see Section 2.7) was added and the samples sonicated for 10 min. Following this they were centrifuged at 14 000 rpm for 10 min. This was repeated with a further 0.3 ml of mobile phase. Finally 0.5 ml of the filtrate was transferred to HPLC autosampling vials. The experiment was carried out in duplicate for each concentration and a control was used where phosphate buffer was used instead of the drug concentration.

2.3. Binding of doxycycline to keratin

This protocol was carried out as described in Section 2.2 except 5 mg of keratin were used instead of melanin. The keratin used to model binding to epidermal keratin was derived from bovine horn and has been used similarly by Uchida and Yamaguchi (1993). Hasiguchi et al. (1998) used a 50% aqueous methanol solution as the incubation medium, but we used PBS to avoid potential complications arising from the inclusion of the alcohol.

2.4. Isolation of human epidermal samples

Skin samples were thawed to room temperature then subcutaneous fat was removed using a scalpel. The skin sections were then placed in purified water at 60 °C for 60 s and the epidermis gently peeled away and placed back in the water (Pellet et al., 1997). After the epidermis had flattened it was removed using foil. One-centimetre diameter (0.785 cm^2) discs were then excised using a cork borer and stored at −20 °C prior to use.

2.5. Delipidisation of epidermal samples

The method used to remove the lipid was adapted from Wertz and Downing (1987). Skin discs were added to three different chloroform and methanol solutions (ratios 2:1, 1:1 and 1:2) for 2 h each on a blood tube rotator. Following this they were placed in 100% methanol for 16 h then dried using blotting paper and placed into Eppendorf tubes.

2.6. Binding of doxycycline to skin samples

Based on a previously reported method (Monk et al., 1998), the skin samples were incubated in the Eppendorf tubes with 1 ml of the varying concentrations of doxycycline: 0.1, 0.5, 1, 2 and 5 mg ml^{-1}. The experiment was carried out in duplicate for both native and delipidised epidermal samples. These samples were placed in an incubator at 32 °C for 18 h, during this time they were rotated using a blood tube rotator. The epidermal samples were then transferred to Millipore Ultrafree Eppendorf tubes (Varian, USA) tubes and centrifuged at 14 000 rpm for 30 min. The bound doxycycline was then recovered from the epidermal samples by extracting three times with 1 ml of chloroform:methanol, 2:1. These extracts were then pooled, evaporated to dryness in a vacuum oven and the residue taken up in 2 ml of mobile phase.

2.7. HPLC analysis

HPLC was carried out using a Perkin–Elmer isocratic LC pump 250, with a Perkin–Elmer 100 auto sampler and a GBC LC 1205 UV/Vis detector, set at 346 nm. Results were reprocessed using the data acquisition program JCL Data 6000. The column used for all HPLC analysis was a Phenomenex Kingsorb C18 (250×4.60 mm, 5 micron). The mobile phase was aqueous 5% for doxycycline was aqueous 5% sodium oxalate and

acetonitrile HPLC grade, 70:30. The injection volume used was 20 μl and the flow rate used was 1 ml min^{-1}. The mobile phase was degassed before use using a 0.2 μm nylon filter. A standard curve was prepared in mobile phase, covering the range 10, 20, 30, 40, 50 μg ml^{-1} doxycycline HCl equating to 9.26, 18.5, 27.8, 37.1, 46.3 μg ml^{-1} $[doxycycline]^+$. The calibration coefficient was 4.92×10^3. Formula weight of doxycycline HCl = 480.9, calculations based on $[doxycycline]^+$ = 445.45.

3. Results and discussion

3.1. Doxycycline and keratin

It can been seen from Fig. 1 and Table 1 that the binding of doxycycline to keratin was dose dependent and approximately linear for melanin after 2×10^{-4} M. Although the binding sites are probably saturable, they were not saturated at the concentrations used. This, as a model for skin binding, is consistent with other research that has showed binding in the skin or layers of the skin to be generally linear (Artuc et al., 1980; Menczel et al., 1983, 1984). The data suggests that during the permeation process doxycycline can bind to epidermal keratin and could result in a reservoir of the drug being formed. Keratins are a complex group of compounds present either as a beta pleated sheet (β-keratin) or as an alpha helix (α-keratin) and their composition varies depending on their location. Binding probably involves a mixture of electrostatic and nonelectrostatic binding between the oxygen molecules of doxycycline and the hydroxyl groups present in the amino acids of keratin.

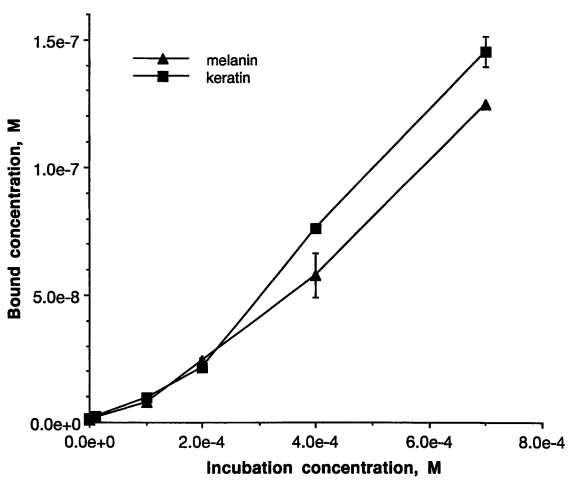

Fig. 1. The binding of doxycycline to melanin (*S. officinalis*) and keratin (bovine horn) ($\pm \Sigma\Delta$).

3.2. Doxycycline and melanin

Dose dependent binding of doxycycline to melanin was also demonstrated in this experiment. Many other anti-malarials are known to cause ocular damage such as corneal oedema, blurred vision and retinopathy, indicated by the development of a number of symptoms such as night blindness, tunnel vision, reduced visual acuity and eventually blindness (Weiss, 1991). It is possible that these serious ocular side effects may be as a result of the anti-malarials photosensitising effects, but this as yet has not been demonstrated conclusively (Kristensen et al., 1994; Moore and Hemmens, 1982).

By comparing the binding of doxycycline to melanin with work that has previously been carried out by Kristensen et al. (1994) it is apparent that doxycycline has much less affinity for melanin than the other anti-malarials. For example, at an incubation concentration of 7 M the bound concentration of doxycycline was 1.3×10^{-7} M, compared to 1×10^{-6} M for proguanil, 1.6×10^{-6} M for primaquine, 1.8×10^{-6} M for hydroxychloroquine and 2.1×10^{-6} M for chloro-quine. Although there were some differences in the protocols used, the data suggests that the interaction between doxycycline and melanin may not be as strong as in the other anti-malarials, possibly due to the different core structure to the other anti-malarials studied which were all quinones. To serve as a positive control in this experiment we examined primaquine diphosphate alongside doxycycline hydrochloride. However, under the conditions used primaquine was found to decompose, although this was not noted in the earlier paper. The conclusion from this experiment is that, using melanin binding as an indica-

Table 1
Binding of doxycycline to melanin and keratin

Incubation concentration, dox HCl (M)	Incubation concentration, $[dox]^+$ (M)	Boundto melanin (M)	se	g Dox bound per mg melanin	Bound to keratin (M)	se	g Dox bound per mg kelanin
1×10^{-5}	9.3×10^{-6}	1.87×10^{-9}	1.92×10^{-10}	1.00×10^{-4}	2.28×10^{-9}	6.69×10^{-11}	1.22×10^{-4}
1×10^{-4}	9.3×10^{-5}	7.69×10^{-9}	4.41×10^{-10}	4.10×10^{-4}	9.79×10^{-9}	4.66×10^{-10}	5.24×10^{-4}
2×10^{-4}	1.86×10^{-4}	2.45×10^{-8}	4.85×10^{-10}	1.31×10^{-3}	2.13×10^{-8}	9.01×10^{-10}	1.14×10^{-3}
4×10^{-4}	3.72×10^{-4}	5.77×10^{-8}	8.61×10^{-9}	3.00×10^{-3}	7.63×10^{-8}	8.42×10^{-10}	4.08×10^{-3}
7×10^{-4}	6.51×10^{-4}	1.25×10^{-7}	0	6.68×10^{-3}	1.45×10^{-7}	5.86×10^{-9}	7.76×10^{-3}

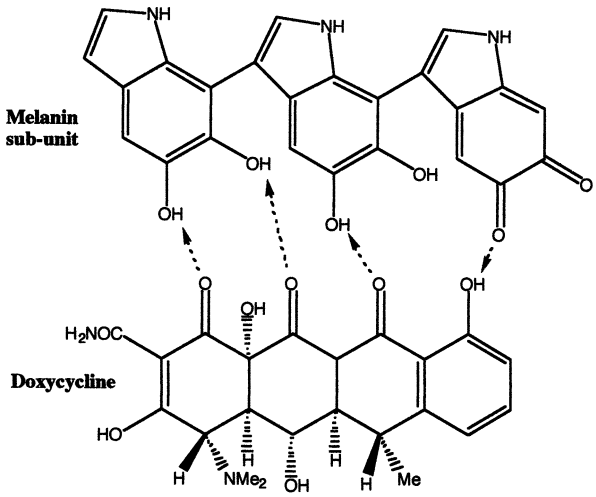

Fig. 2. Hypothesised complementary hydrogen bonding between doxycycline and a sub unit of melanin.

tor of phototoxicity, these data suggest that there may be less propensity for phototoxic side-effects from the use of doxycycline than from quinone anti-malarials. However, it is recognised that cases of doxycycline-induced photosensitivity exist (Vassileva et al., 1998).

It can also be seen from Fig. 1 that the affinity of doxycycline for melanin and keratin is similar, probably involving a similar population of binding sites per unit mass of protein. However, binding to keratin will be of far greater significance than melanin to the transdermal delivery of doxycycline because it is present in the skin in much larger amounts. This, therefore, presents a much larger number of keratin binding sites and that the reservoir effect that melanin may have would be significantly lower than keratin.

Larsson and Tjälve (1979) showed that melanin has many sites to which a drug may bind. Fig. 2 shows a sub unit of melanin which may show how doxycycline and melanin interact, involving reciprocal hydrogen binding between the oxygen groups on doxycycline and the hydroxyl groups present in the sub unit of melanin. Melanin is present in the body as two different forms: eumelanin and pheomelanin, existing as co-polymers of both types. The chemicals which are present in the human are probably copolymers of both these types. The chemical composition of melanin will, therefore, show differences between both different tissues and different individuals (Sarna, 1992). In this experiment the melanin isolated from *S. officinalis* was used as a model so is not particularly representative of human skin. However, there will be many structural similarities and electrostatic interactions between doxycycline and melanin are of high importance, as melanin has strong polyanionic properties.

As for keratin, melanin can act in the body as a chemical depot from which compounds are slowly released, although in much lower in capacity. As there is no degradation pathway for melanin in the skin, it is removed (peeled) along with the rest of the skin from the stratum corneum and can lead to a build up of the drug and may increase its half-life. The number of melanocytes between individuals is relatively similar (in the skin of the thigh there are ca. 1000 cm^{-2}), independent of sex, race or exposure. The difference that is observed between different skin colorations e.g. racial differences are dependent on the amount of melanin granules produced. Differences in skin tone could potentially lead to different absorption of a transdermally delivered product for doxycycline if binding to melanin is high.

3.3. Doxycycline and native and delipidised epidermal samples

The results for binding of doxycycline to native and delipidised epidermal samples are shown in Table 2 and Fig. 3. More doxycycline bound to native skin than delipidised skin, by a factor of between 2.5 and 3. Delipidised skin produced a more linear profile. However, the binding isotherm for native skin was curved and by extrapolation it is estimated that saturation would be reached at a bound concentration of 1.2×10^{-7} M, or 2.4×10^{-4} mol per 1 cm diameter disc (3.06×10^{-4} mol cm^{-2}). The effects of potential damage to epidermal proteins by the process of heat-separation and metal ion complexation in the incubation medium are unclear.

Fig. 3 shows that there is a great difference in the binding of doxycycline to native and deli-

Table 2
Binding of doxycycline to native and delipidised epidermal tissue

Skin type	Incubation concentration, $[dox]^+$ (g ml^{-1})	Bound concentration (g ml^{-1})	SD	Tissue bound g, 1 cm diameter	SD	%	Tissue bound (g cm^{-2})	SD
Native	9.26×10^{-5}	1.89×10^{-6}	5.16×10^{-7}	3.78×10^{-6}	1.03×10^{-6}	5.20	4.815×10^{-6}	$1.31\times10-6$
	4.63×10^{-4}	10.01×10^{-6}	9.90×10^{-7}	20.22×10^{-6}	1.98×10^{-6}	4.37	25.76×10^{-6}	$2.52\times10-6$
	9.26×10^{-4}	15.54×10^{-6}	2.97×10^{-7}	31.08×10^{-6}	5.94×10^{-7}	3.36	39.59×10^{-6}	$7.57\times10-7$
	1.85×10^{-3}	27.9×10^{-6}	4.41×10^{-6}	55.8×10^{-6}	8.82×10^{-6}	3.02	71.08×10^{-6}	$1.12\times10-5$
	4.63×10^{-3}	45.74×10^{-6}	9.64×10^{-6}	91.48×10^{-6}	1.93×10^{-5}	1.98	116.54×10^{-6}	$2.46\times10-5$
Delipidised	9.26×10^{-5}	1.73×10^{-6}	1.41×10^{-8}	3.46×10^{-6}	2.82×10^{-8}	4.76	4.41×10^{-6}	$3.59\times10-8$
	4.63×10^{-4}	3.94×10^{-6}	4.60×10^{-7}	7.84×10^{-6}	9.20×10^{-7}	2.16	9.99×10^{-6}	$1.17\times10-6$
	9.26×10^{-4}	6.13×10^{-6}	6.36×10^{-7}	12.26×10^{-6}	1.27×10^{-6}	1.67	15.62×10^{-6}	$1.62\times10-6$
	1.85×10^{-3}	9.11×10^{-6}	2.34×10^{-6}	18.22×10^{-6}	4.68×10^{-6}	1.25	23.21×10^{-6}	$5.96\times10-6$
	4.63×10^{-3}	18.52×10^{-6}	0	37.04×10^{-6}	0	1.02	47.18×10^{-6}	0

pidised epidermal samples. Again like the binding to keratin and melanin it was dose dependent and saturation was again not reached. However, by extrapolation it is estimated that saturation of the delipidised skin would occur at 60 μg cm^{-2} while, for native epidermis, saturation would occur at 140 μg cm^{-2}. The latter figure compares reasonably well with the 460 μg cm^{-2} reported earlier (Perkins and Heard, 1999), and the difference can be attributed to the enhancing vehicle (ethanol/Miglyol 840) used on the earlier occasion. These estimations could be confirmed using higher doxycycline solutions, although insufficient tissue did not allow so during this work. As both the native and delipidised epidermal samples were evidently saturable there is a limit to the amount of doxycycline that can be retained within the epidermis. When saturation is reached the permeation rate will also increase as the drug is free to pass through the skin with a smaller amount of resistance. The time taken for binding to become saturated may contribute to the lag time. Effects of the long term binding to protein are not known but after dosing is stopped (the patch removed) doxycycline could potentially continue to be released into the systemic circulation.

When presented with intact epidermal tissue, both lipid and epidermal protein would be available for interaction with doxycycline, although the latter would be in much greater abundance. In the delipidised skin there would be easier accessibility for doxycycline to interact and bind to the epidermal proteins. The data suggests that protein binding alone is not the prime interactive process, as there was significantly greater binding to intact tissue than when the lipids were absent. This was not anticipated because doxycycline, as the hydrochloride salt, is charged and very polar.

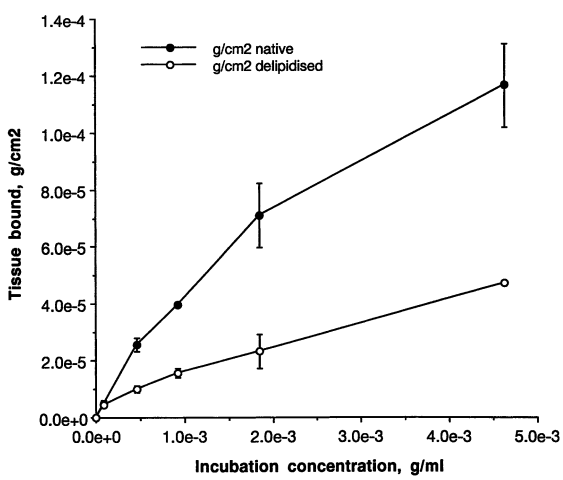

Fig. 3. The binding of doxycycline to native and delipidised epidermal samples ($\pm \Sigma\Delta$).

Recent research has suggested that doxycycline interacts significantly with monolayers of ceramides IV (Courtois et al., 2001, submitted not yet accepted). This paper suggests that the doxycycline molecule interacts with both the head groups and the acyl chains of the ceramides. This can not be explained using either log *P* or solubility parameters. A possibility is that doxycycline can interact using hydrogen bonds to ceramide groups present in the lipid matrix of the stratum corneum. This significant binding to both lipid and keratin suggests that doxycycline in vivo would have a high affinity for both the keratin rich proteinaceous component of the skin, the corneocytes and the intercellular lipid matrix (Courtois et al., 2001 submitted not yet accepted). This means that the partition–diffusion–partition steps that are involved in transcellular permeation may be possible. Doxycycline, therefore, probably permeates the stratum corneum via the inter and transcellular routes or, more likely, a mixture of both.

4. Conclusions

This work has demonstrated that doxycycline appears to have considerably less affinity for melanin than the other anti-malarials previously studied, suggesting that the deleterious side effects associated with the other anti-malarial drugs may be substantially reduced. Doxycycline was shown to have a higher affinity for native epidermal tissue than for the delipidised samples. This would, therefore, imply that doxycycline would pass via the intercellular route, but doxycycline also showed affinity for keratin, which would mean that a significant proportion would bind to the keratin in the corneocytes of the stratum corneum as well as the lipoidal domains.

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