els in aged rats determined at two time-points during the night [18]. Reduction of the melatonin rhythm amplitude during aging does not occur only in rats, but in other species as well, e.g. in Syrian [19] and Djungarian [20] hamsters, in gerbils [19] and even in humans [21], and is in accordance with a decline of amplitude of other rhythms [1]. As the activity of NAT, which forms the melatonin precursor N-acetyl-serotonin, does not change with age, the reduction in melatonin levels may be due to the reported decline of another enzyme of the melatonin forming pathway during aging, i.e. of hydroxyindole-o-methyltransferase (HIOMT) [22]. This suggestion is in accordance with an earlier proposal that the NAT rhythm drives the melatonin rhythm but the maximum melatonin production may depend on HIOMT [10].

As during the fourth cycle after the 8-h advance shift the NAT and melatonin rhythm were expressed in the young, but still abolished in the old rats, the re-entrainment of both rhythms in the aged animals apparently proceeded at a slower rate. It appears that after travel over the longitudes, the rhythm in serum melatonin also adjusts more rapidly in younger than in older humans [23]. The slower rate of adjustment during aging may be due to a change in the entrainment or in the output pathway or to a change in the clock itself [2, 3]. Our data are in favor of the conclusion that re-entrainment of the circadian system proceeds more slowly in aged mammals. As melatonin itself can entrain the circadian system [24] and accelerate its re-entrainment [25, 26], low melatonin levels and a slow rate of re-entrainment of the melatonin rhythm in old individuals may further slow down the course of circadian rhythm re-entrainment in aged mammals.

1 Davis, F. C., in: Handbook of Behavioral Neurobiology, vol. 4, Biological Rhythms, p. 257. Ed. J. Aschoff. Plenum Press, New York 1981.
2 Pittendrigh, C. S., and Daan, S., Science *186* (1974) 548.
3 Morin, L. P., J. biol. Rhythms *3* (1988) 237.
4 Sollberger, A., Biological Rhythms Research. Elsevier, New York 1965.
5 Rosenberg, R. S., Winter, J. B., and Rechtschaffan, A., Sleep Res. *9* (1980) 105.
6 Peng, M. T., and Kang, M., Physiol. Behav. *33* (1984) 615.
7 Ehret, C. F., Groh, K. R., and Meinert, J. C., in: Aging and Biological Rhythms, p. 21. Eds H. V. Samis, Jr, and S. Capobianco. Plenum Press, New York, London 1978.
8 Quay, W. B., Trans. N. Y. Acad. Sci. *34* (1972) 239.
9 Klein, D. C., and Weller, J. L., Science *169* (1970) 1093.
10 Illnerová, H., Vaněček, J., and Hoffmann, K., Comp. Biochem. Physiol. *74A* (1983) 155.
11 Rusak, B., and Zucker, J., Physiol. Rev. *59* (1979) 449.
12 Klein, D. C., and Moore, R. J., Brain Res. *174* (1979) 245.
13 Illnerová, H., Pineal Res. Rev. *6* (1988) 173.
14 Illnerová, H., Vaněček, J., and Hoffmann, K., Brain Res. *417* (1987) 167.
15 Parfitt, A., Weller, J. L., Klein, D. C., Sakai, K. K., and Marks, B. H., Molec. Pharmac. *11* (1975) 241.
16 Deguchi, T., and Axelrod, J., Analyt. Biochem. *50* (1972) 174.
17 Frazer, S., Cowen, P., and Franklin, M., Clin. Chem. *29* (1983) 396.
18 Reiter, R. J., Craft, C. M., Johnson, J. E., King, T. A., Richardson, B. A., Vaughan, G. M., and Vaughan, M. K., Endocrinology *109* (1981) 1295.
19 Reiter, R. J., Johnson, L. Y., Steger, R. W., Richardson, B. A., and Petterborg, L. J., Peptides *1*, Suppl. 1 (1980) 66.
20 Hoffmann, K., Illnerová, H., and Vaněček, J., Neurosci. Lett. *56* (1985) 39.
21 Touitou, Y., Fèvre, M., Lagoguey, M., Carayon, A., Bogdan, A., Beck, H., Cesselin, F., and Touitou, C., J. Endocr. *91* (1981) 467.
22 Dax, E. M., and Sugden, D., J. Neurochem. *50* (1988) 468.
23 Wetterberg, L., J. neural. Transm., Suppl. *13* (1978) 289.
24 Redman, J. S., Armstrong, S. M., and Ng, K. T., Science *219* (1983) 1089.
25 Arendt, J., Aldhous, M., English, J., Marks, V., and Arendt, J. H., Ergonomics *30* (1987) 1379.
26 Redman, J. R., and Armstrong, S. M., J. Pineal Res. *5* (1988) 203.

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Calculation of drug-melanin binding energy using molecular modeling

P. R. Raghavan, P. A. Zane and S. L. Tripp

Preclinical Drug Metabolism, Ciba-Geigy Corp., Ardsley (New York 10502, USA)
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Summary. Conformational analysis and molecular graphics are used to model a representative melanin structure to estimate a chemical's in vitro affinity for melanin. The modelling data fit to a simple linear equation relative to a logarithmic transformation of the experimentally-derived binding data (r = 0.901). The goodness of fit, as evidenced by the F-statistic, $F_{(1,14)} = 60.09$ (p = 0.000002), for the regression indicates that this technique gives an accurate representation of the interaction of these chemicals with melanin in vitro.
Key words. Conformational analysis; molecular modelling; melanin.

Knowledge of the interactions of compounds with biological macromolecules is critical to the design of new drug candidates and to the prediction of adverse reactions for those and other compounds. However, while the binding parameters for some systems (e.g., stable proteins, DNA) are readily accessible through in vitro binding assays, it is frequently necessary to examine binding in systems which are not amenable to such direct

experimental observation. One way to circumvent this problem is to model the binding site and then to use molecular modelling techniques to predict the nature of the interactions. This paper represents a report of an example, binding to melanin, in which this technique has been successfully applied.

"Melanin" is a purely descriptive term which merely denotes a black pigment of biological origin. Melanins occurring naturally in animals are known as "eumelanins", which are usually black or brown, contain nitrogen and are derived from tyrosine, dopa and dopamine. Typical examples are the black and brown pigments of skin, hair and eyes [1]; in higher mammals, such as man, eumelanins are also present in the substantia nigra of the central nervous system. The exact structure of eumelanins remains elusive, but they appear to consist of irregularly structured polymers of 5,6-dioxoindole, frequently with conjugation to proteins.

The observation that chronic administration of phenothiazines [2] or long-term, high-dose chloroquine therapy [3] produced chorioretinopathy, led to the awareness of an association between the toxic effects of some drugs and their high affinity for the pigment melanin. Since these early observations, melanin binding of drugs has been implicated not only in ocular toxicity, but also in ototoxicity and pigment disturbances of the skin and hair [4]. To date, however, the nature of the interaction between melanin and drugs still has not been fully characterized. Studies have indicated that electrostatic forces play an important role in the binding of drugs to melanin [5]. However, these studies also suggested that non-electrostatic contributions must be added to form the binding sites for such drugs as chlorpromazine and chloroquine.

Many previous studies have focused on the ability of a compound to bind to melanin in vitro as a measure of its affinity for melanin-containing structures in vivo. The experimental approach often entails the isolation of melanin from biologic sources or the preparation of synthetic melanin from its precursors tyrosine and/or dopa. Potts [6] showed that for a number of different compounds, extent of binding to both isolated and synthetic melanin in vitro corresponded to their in vivo affinities for melanin-containing structures of the eye. However, the poor solubility and complex structure of natural melanin make it difficult, if not impossible, to probe directly the exact nature of its interactions with other molecules.

An alternative, indirect approach would involve the use of conformational analysis and molecular graphics to model a representative melanin structure produced by assembling a limited number of monomeric units. Then the structural elements involved in the interaction of a compound with melanin can be characterized and hypotheses formulated regarding the possible biologically relevant conformation of drug molecules [7]. In the present study, this method was used to estimate the in vitro affinity of a chemical for melanin based on 16 of the 39 compounds compiled by Potts [6]. The compounds studied were randomly selected so as to cover the range of melanin binding from 0% to 87%.

Materials and methods

Determination of the percentage bound to melanin in vitro. The experimental procedure employed by Potts is as follows: Pigment granules, containing choroidal melanin, was prepared from the uvea of bovine eyes. In a 10-ml nylon centrifuge tube was placed the following: 1.0 ml of a pigment suspension containing 10 mg of pigment granules (by dry weight); 1.0 ml of a 2.5 µmole of compound to be tested in an unspecified solvent; 4.0 ml water; and 1.0 ml buffer or acid. The contents were mixed for 15 min, centrifuged and an appropriate aliquot of the supernatant removed for spectrophotometric determination of the compound's concentration. The difference between the amount added and the amount remaining in the supernatant yielded the amount of compound that is bound to the pigment.

Since the experimental data of Potts includes zero (0) amounts bound, a factor of one was added to all the values in order to make a logarithmic transformation of the amount bound to melanin.

Calculation of binding energy. Binding energy was calculated using Macromodel™, a molecular modelling program developed by Prof. C. Still of Columbia University. A minimized structure of a tetramer of 5,6-dioxoindole (melanin monomer) was obtained using the multiconformer mode and the MM2 forcefield. Figure 1 illustrates the lowest energy conformation calculated for the tetramer.

Binding energies were then calculated by minimizations on the complexes, as well as the isolated molecules.

All structures were minimized by varying suitable torsion angles in a batch process using a modified MM2 forcefield that is used in Macromodel™ [8]. First, each of the structures was allowed to interact with the tetramer (which was kept fixed), at a distance of 3-7 Å. A search for a low energy, or favored, orientation was carried out by allowing the reactant all degrees of freedom to move in the X, Y and Z axes, yielding an approximate minimum energy conformation for the compound-melanin

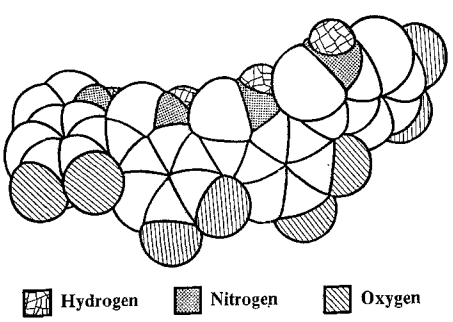

Figure 1. Computer-drawn plot of the low-energy conformation of the melanin tetramer.

complex. Convergence was judged to have been reached when the changes in energy after successive minimization steps were less than 0.25 kJoules. This first step gives the approximate energy associated with the interaction. The energy associated with this favored orientation was then further minimized using the MM 2 forcefield. Each compound was then moved to a distance of 15 Å, where the interaction with the tetramer would be negligible; this process yields the conformational energy associated with the two isolated structures. The binding energy is defined as the energy of the compound-melanin complex minus the sum of the melanin tetramer and chemical energies in isolation.

Results and discussion

The binding energies presented in the table represent the affinities of the 16 test compounds for the melanin tetramer, in the absence of other competitive binding molecules or biological processes, as calculated by Macromodel™. Figure 2 presents these data graphically relative to experimentally-derived data of Potts[6] on melanin-binding of these compounds in vitro, and includes a computer-fit of the data by a simple linear equation ($R^2 = 0.811$; $p \leq 0.000002$). The goodness of fit, as evidenced by the F statistic, $F_{(1,14)} = 60.09$, for the expression indicates that this molecular modelling technique can be considered a significant predictor of the interaction of these compounds with melanin in vitro.

The curve shape for the fitted function is similar to that observed in studies of the binding of drugs to receptors. The primary assumption in studies of the combination of drug with receptor is that the fraction of maximum effect elicited is proportional to the number of receptor sites occupied, at a given concentration of drug, and that the

The calculated binding energies for selected compounds whose percentage bound to melanin in vitro is known

Compound	Binding energy calculated from molecular modelling[a]	Percentage bound to melanin granules in vitro[b]
Phenol	–10.0	0.0
Pyridine	–10.0	0.0
Glucose	–11.0	0.0
Amitriptyline	–16.0	11.0
SKF-6270 A[c]	–24.0	25.0
Aniline	–16.8	26.0
Quinine	–27.0	31.0
KS-24-S[c]	–20.1	33.0
BP-400-S[c]	–20.4	34.0
Tetramethyl-p-phenylenediamine	–19.0	39.0
Bromophenol Blue	–21.0	40.0
TZ-11-S[c]	–24.0	43.0
Prochlorperazine	–22.0	50.0
Chlorpromazine	–21.5	52.0
Chloroquine	–28.0	82.0
Methylene Blue	–30.0	87.0

[a] Binding energy is the difference between the energy associated with interaction of melanin and compound indicated and the energy of the individual compound in isolation. [b] Percentage bound to melanin in vitro as determined by Potts[6]. [c] Structures are of substituted phenothiazines[6].

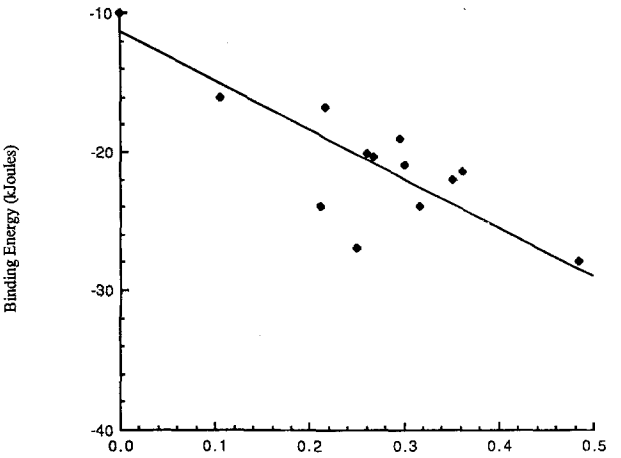

Figure 2. The relationship between percentage binding of 13 different chemicals to melanin in vitro and their binding energies as calculated by molecular modelling:
Binding Energy = $-35.47\,(\pm 4.58)^{*}X + -11.35\,(\pm 1.32)$
$r = 0.901$ $R^2 = 0.811$
$F_{(1,14)} = 60.09$ $p = 0.000002$

percentage occupancy is determined by the affinity of the reactant for the receptor[9]. This occupancy theory of Clarke can be expressed mathematically as follows:

$$\frac{\text{Effect}}{E_{max}} = \frac{[\text{Drug}]}{K_d + [\text{Drug}_{total}]}$$

where E_{max} is the maximum effect or response, and K_d is the dissociation constant for the interaction of the drug with the receptor. When examined graphically, this equation describes a rectangular hyperbolic function. A logarithmic transformation of the x-axis of such a plot yields a sigmoidal function, which has a linear region where changes in drug concentration are related to changes in effect. In the case of melanin, the calculated binding energy serves as a gauge of each chemical's in vitro pharmacodynamic binding capacity, as does the ratio of Effect/E_{max} in the above equation. Thus, it seems reasonable that the analysis of binding energy, as calculated from conformational analysis, versus the Log [1 + Amount Bound] should yield a similar relationship. In fact, the statistical analysis shows that there is a linear relationship between binding energy and the log [1 + Amount Bound], suggesting that the data fall within the linear region of the sigmoidal curve. The true test of the power of this approach would be to increase the number of compounds modelled and to measure their in vitro binding.

Molecular modelling methods do not take into account the solvation factors; and, it is not possible to include solvation factors at the present stage in the program's development. This is one of the biggest limitations on molecular modelling as normally used. Most of the parameters for modelling are obtained from the gas

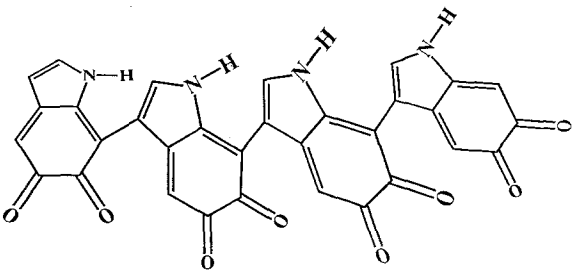

Figure 3. Conventional structure of the low-energy conformation of the melanin tetramer showing the sites for hydrogen bonding.

phase, whereas the results of Potts[6] are the result of solid-liquid interaction. It is likely that solvation energies play a minor role at the low end of the binding energies, for compounds like aniline and phenol, and also for compounds that are strongly bound, like chloroquine and methylene blue; and, our binding energies bear that out. Nonetheless, the ability to predict strongly bound compounds or those lacking significant binding is important to both the pharmaceutical industry and various drug regulatory agencies when questions arise concerning the safety in humans of a new drug entity. The solvation energies are critical when we are dealing with compounds that bind in the intermediate range and also when they are structurally similar. This is one reason why there is a good deal of variation in the intermediate range. Additionally, the paper by Potts does not detail into what solvents each of the compounds were dissolved. Finally, it must be pointed out that since melanin is a polymer, it will swell to different degrees in different solvents; and, this in turn will effect the binding.

The molecular graphics of the chemical-melanin complex indicates that an important spatial arrangement in the interactions is the ability of a compound to hydrogen-bond with the quinone or with the pyrrole elements in the melanin tetramer shown in figure 3. As a result of these dual structural features, it allows both strongly cationic and anionic compounds to associate with melanin in vitro. However, Lybrand and Kollman[10] have suggested that ligands which interact with biomolecules via specific group-group interactions, such as hydrogen bonds, can be modelled with fewer problems because strong, specific interactions tend to overshadow weaker non-specific interactions such as van der Waals forces. Hence, correlations of modelling results to experimentally-derived results may not be as good in the absence of these strong interactions.

Two general limitations exist in the molecular modelling procedure used:

1) The energy calculations do not take into account solvation and entropy factors involved in binding, and the exclusion of these factors may limit the utility of this method in describing other biologic interactions. However, extension of the method to use of the Amber force-field[11], which accounts for more of the factors involved in the interactions of chemicals with biomolecules, may give a more accurate representation of both in vitro and in vivo situations and, therefore, expand the utility of this approach.

2) The method does not take into account the ability of the compounds to reach melanin-containing structures in vivo. Discrepancies may occur between computed and in vivo results due to lack of access of the compound to melanin-containing structures.

Despite these limitations, however, the present investigation shows that computational conformational analysis can be used to examine properties which may not be amenable to direct experimental observation. It also illustrates the ability to construct useful models of biomolecules from their known precursors when only limited information exists on their actual conformations. There are, at present, limitations to the usefulness of energy calculations as applied to ligand-biomacromolecule interactions. Even with these limitations, however, molecular modelling allows the exploration of the molecular basis for the behavior of complex biologic systems. The ultimate goal of conformational analysis and molecular graphics, such as has been used in the calculation of binding energies here, is to reveal three-dimensional structural elements that might be involved in the interaction of chemicals with the biologic organism.

1 Swan, G. A., Fortschr. Chem. Org. Naturst. *31* (1974) 521.
2 Kinross-Wright, V., Psychiat. Res. Rep. *4* (1956) 89.
3 Hobbs, H. E., Sorsby, A., and Freedman, A., Lancet *2* (1959) 478.
4 Ings, R. M. J., Drug Metab. Rev. (U.S.) *15* (1984) 1183.
5 Larsson, B., and Tjalve, H., Biochem. Pharmac. *28* (1979) 1181.
6 Potts, A. M., Invest. Opthalm. *3* (1964) 405.
7 Tollenaere, J. P., and Janssen, P. A. J., Med. Res. Rev. *8* (1988) 1.
8 Allinger, N. L., J. Am. chem. Soc. *77* (1977) 8127.
9 Clarke, E. J., J. Physiol. *61* (1926) 530.
10 Lybrand, T., and Kollman, P., Biopolymers *24* (1985) 1863.
11 Weiner, S. J., Kollman, P. A., Case, D., Singh, V. C., Alagna, G., Profeta, S., and Weiner, P., J. Am. chem. Soc. *106* (1984) 765.
12 Abraham, Z. H. L., Agbandje, M., Neidle, S., and Acheson, R. M., J. biomolec. Struct. Dyn. *6* (1988) 471.

0014-4754/90/010077-04$1.50 + 0.20/0