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Anti-viral cyclam macrocycles : rapid zinc uptake at physiological pH†

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NMR and UV-vis spectroscopic studies show that Zn(II) binds to cyclam rapidly at micromolar concentrations, an observation relevant to the anti-viral activity and co-receptor binding of anti-HIV cyclams.

Cyclam (1,4,8,11-tetraazacyclotetradecane) macrocycles exhibit anti-HIV activity. Particularly potent is the xylyl-bicyclam 1-1′-[1,4-phenylenebis(methylene)]-bis(1,4,8,11-tetraazacyclotetradecane) (AMD3100) which has recently been on clinical trials for treatment of AIDS, and also has a remarkable ability to mobilize stem cells.[1,2] Cyclams inhibit the entry of the virus into white cells by binding to the co-receptor protein CXCR4 in the outer membrane. Antiviral activity correlates with the strength of CXCR4 binding. Complexation of AMD3100 to Zn(II) enhances co-receptor binding strength and anti-HIV activity, whereas Pd(II) binding inactivates the drug, the activity order being:[3]

$$Zn(II)_2 > AMD3100 > Ni(II)_2 > Cu(II)_2 \gg Co(III)_2 \gg Pd(II)_2.$$

Cyclam has a high affinity for Zn(II) (log K = 15.5)[4] and at physiologically-attainable concentrations of AMD3100 in blood[5] (micromolar) where the Zn(II) concentration is *ca.* 19 μM, it can be calculated that nearly all the drug would exist as a Zn(II) complex at physiological pH of 7.4. Metal cyclam complexes commonly adopt one of the configurations shown in Fig. 1, which differ in the chirality of the bound N atoms.

The orientation of the N–H bonds is important for co-receptor recognition. Carboxylate oxygens of specific glutamate and aspartate side-chains of CXCR4 can coordinate directly to Zn(II) cyclam, and also form strong H-bonds to cyclam N–H groups.[6] Such combined coordination and H-bonding on either side of the cyclam ring can stabilise the *cis*-V configuration. Although the thermodynamics of Zn(II) binding is favourable, there appear to be no reported data on the kinetics of Zn(II) binding to cyclams at pH 7. We show here by 800 MHz ^{1}H NMR spectroscopy that Zn(II) uptake by cyclam under physiologically-relevant conditions is relatively rapid. Also UV-vis spectroscopic studies show that Zn(II) binds even more rapidly than Cu(II).

First we studied the reaction of $ZnCl_2$ (15 μM) with cyclam (15 μM) at 310 K (body temperature), pH 7.4, by 800 MHz ^{1}H NMR spectroscopy. The disappearance of peaks for free cyclam was relatively rapid (initial rate 7.2 × 10^{-9} M s^{-1}) and several sets of new peaks appeared and changed in intensity over a period of *ca.* 2 h (Fig. 2). At equilibrium *ca.* 66% of cyclam is bound to Zn(II), and the remainder is presumably bound to the cacodylate buffer. At concentrations of cyclam used in later UV-vis experiments (745 μM, *vide infra*) the initial rate was much faster (> 1.8 × 10^{-6} M s^{-1}).

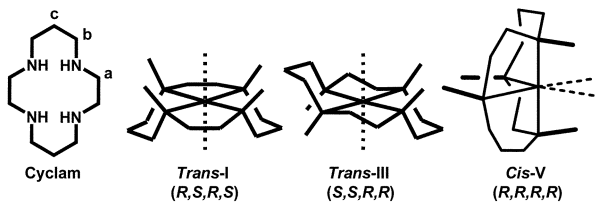

Fig. 1 Cyclam and some configurations of metal cyclam complexes.

Based on our recent ^{1}H, ^{13}C and ^{15}N studies of complexes such as $[Zn(cyclam)(H_2O)_2]Ac_2$, which has a *trans*-III configuration in the crystalline state, including Kaplus-type analyses of vicinal couplings[7] (and studies of analogous ^{111}Cd(II) complexes),[8] it is possible to conclude from Fig. 2 that Zn(II) is taken up rapidly by cyclam and that this gives rise to the unusual *trans*-I/*cis*-V configurations of Zn(II) cyclam which slowly equilibrate with the *trans*-III configuration, usually the most stable configuration in the solid state.

There are few previous studies of the interconversion of zinc cyclam configurations in solution.[7,9] Conversion of *trans*-I or *cis*-V to *trans*-III requires two N inversions as well as rearrangement of the carbon skeleton. The *trans*-I/*cis*-V configurations may be further stabilised by binding to carboxylate side-chains of the CXCR4 co-receptor.[6]

Then we investigated the competitive binding of Zn(II) and Cu(II) by cyclam. This is of interest not only because Cu(II) may be a competitor *in vivo*, but also because Cu(II) cyclam is purple (ε_{511} = 100 M^{-1} cm^{-1})[10] which allows Cu(II) uptake to be followed by UV-vis spectroscopy, whereas Zn(II) uptake cannot be followed directly by this method. Cu(II) is reported to bind to cyclam 5 × 10^{11} times more strongly than Zn(II) (log K = 27.2)[11] suggesting that biologically less active Cu(II) cyclam complexes are more likely to be formed *in vivo*. Also we expected that Cu(II) uptake

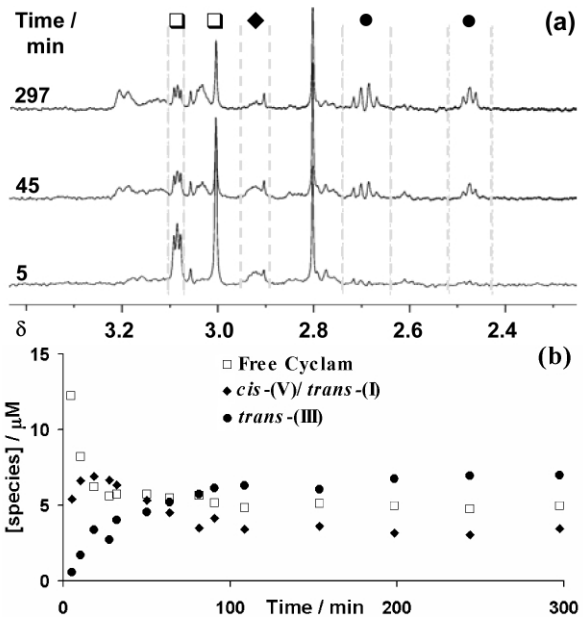

Fig. 2 Cyclam binds Zn(II) rapidly at micromolar concentrations. (a) 800 MHz ^{1}H NMR spectra showing the uptake of Zn(II) (15 μM) by cyclam (15 μM) at 310 K, in 3.3 mM cacodylate buffer pH 7.4 (10% D_2O/90% H_2O), and formation of *trans*-I, *trans*-III and *cis*-I configurations. Each spectrum was acquired in 4.5 min. Assignments (δ/ppm): free cyclam 3.09 H_b, 3.00 H_a; *trans*-III 2.69 H_b, 2.47 H_a; *trans*-I/*cis*-V (overlap) 2.92 H_a; 2.80 impurity. For Zn cyclam, geminal protons are non-equivalent but H_a′ and H_b′ protons are not labelled in the spectrum. (b) Dependence on time of the concentrations of the species detected in (a).

† Electronic supplementary information (ESI) available: UV-vis spectra. See http://www.rsc.org/suppdata/cc/b3/b312752b/

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would be favoured kinetically over Zn(II) uptake since previous studies have shown[12] that the rates of complexation of transition metal ions by cyclam follow the order Cu(II) > Zn(II) > Co(II) > Ni(II), which parallels the rates of water exchange. However, the reported kinetic studies were confined to low pH ranges (*ca.* 2.5–4.5 for Cu(II), and 4–6 for Zn(II)) to avoid precipitation. After a wide search for suitable buffers, we discovered that both Cu(II) and Zn(II) chlorides remain soluble at concentrations as high as 15 mM at pH 7.4 in cacodylate buffer. Although Cu(II) is taken up by cyclam too rapidly at low pH to be followed by conventional UV-vis spectroscopy, the reaction occurs over the course of an hour at pH 7.4, with loss of the peak for free Cu(II) at 750 nm and the growth of the peak for Cu(II) cyclam at 511 nm. The identity of the product was confirmed by ESI-mass spectrometry (peak for $[CuCyclamCl]^+$ = 299 Da/e).

We studied the binding of Cu(II) and Zn(II) either alone or in competition by adding a solution of cyclam to a stirred solution containing $CuCl_2$ and/or $ZnCl_2$ (3.5 ml 1-cm cuvette, 1:1:1 mol ratio, 0.75 mM – determined by limit of detection).

For addition of Cu(II) alone, we determined an initial rate of 2.9×10^{-7} M s^{-1} for the disappearance of "free" Cu(II) (Fig. 3). When Cu(II) and Zn(II) were added to cyclam together (1:1:1 mol ratio) the uptake of Cu(II) was markedly slower and proceeded in two phases, the first with an initial rate of 2.1×10^{-7} M s^{-1} and the second at an initial rate of 6.4×10^{-9} M s^{-1}. The reaction with a 10-fold molar excess of Zn(II) over Cu(II) (to mimic ratio in blood plasma) showed only one slow phase (initial rate 0.11×10^{-7} M s^{-1}). One explanation of these results is that Zn(II) binds more rapidly to cyclam than Cu(II) and quickly reaches equilibrium. Thus the two-phase nature of the equimolar reaction could be due to the reaction of Cu(II) with free cyclam followed by displacement of Zn(II) from cyclam by Cu(II) at a slower rate. To test this hypothesis we incubated Zn(II) and cyclam at 310 K for 30 min to allow uptake of Zn(II), and then added Cu(II). The rate of uptake of Cu(II) by cyclam in this solution (two-phase, 2.2×10^{-7} M s^{-1} and 6.6×10^{-9} M s^{-1}) was identical to that observed for simultaneous addition of Zn(II) and Cu(II).

Therefore, under the conditions used here, our results suggest that, surprisingly, Zn(II) binds to cyclam more rapidly than Cu(II) at physiological pH (7.4), thus slowing the uptake of the thermodynamically-favoured metal ion. At pH 7.4, cyclam is largely in the form $cyclamH_2^{2+}$ (pK_a values 11.5, 10.3, 1.6 and 0.9)[13] and so complexation will involve two deprotonations, stepwise N coordination, and N inversions. Such mechanisms have been studied in detail for cyclams only under a few carefully chosen conditions.[14–16]

The studies reported here were carried out because Zn(II) complexes of cyclams can exhibit as high, or higher, anti-HIV activity as metal-free cyclams,[3] and Zn(II) cyclam readily undergoes configurational changes,[7] but there had been no previous work on Zn(II) uptake by cyclams under physiological conditions. We have now shown that uptake of Zn(II) by cyclam is relatively rapid at pH 7.4 and can be accompanied by configurational changes. Thus we conclude that it is likely to be both thermodynamically and kinetically favourable for cyclam anti-HIV drugs to form Zn(II) complexes in blood plasma. Moreover the specificity of recognition of metal cyclams by membrane co-receptor proteins may be determined by the configuration of the macrocycle. Our studies suggest that metal complexation *in vivo* is a factor which should be considered in attempts to understand the mechanism of action of (apo) cyclam drugs, both in terms of their antiviral activity and their side-effects. Direct studies of cyclam metallation in body fluids would now be worthwhile. AMD3100 has recently been withdrawn from clinical use because of side-effects. Perhaps the co-administration of zinc supplements and/or use of configurationally-restrained metal cyclam complexes could offer some advantages.

The ability of Zn(II) to exert kinetic control over the uptake of Cu(II) into specific binding sites is a principle which may be of wider significance in biology, *e.g.* in the assembly of the active sites of metalloproteins. Also zinc recruitment by organic drugs *in vivo* is a strategy which can be utilised in drug design.[17] Finally it is notable that the high sensitivity of modern NMR spectrometers now allows reactions of drugs to be studied under physiologically relevant conditions, and especially at micromolar levels. This is particularly important for understanding the mechanisms of action of metal-based drugs for which ligand substitution reactions can often play an important role.

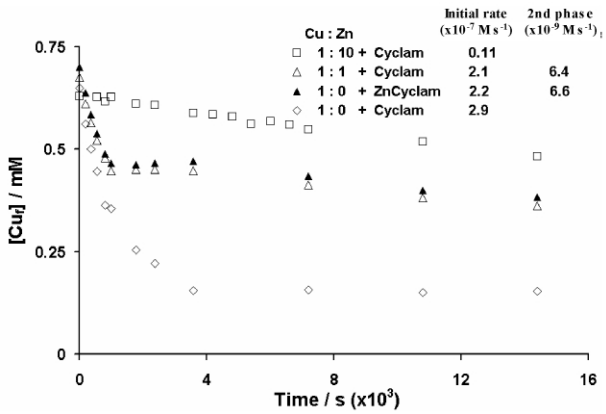

Fig. 3 Zn(II) retards uptake of Cu(II) by cyclam. Plots showing decrease in concentration of Cu_f ("free" Cu(II)) with time for various mol ratios of Cu(II):Zn(II):cyclam. Data taken from UV-vis studies ([Cu(II)] = 0.75 mM, 0.5 M cacodylate buffer, pH 7.4, 310 K).

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