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Binding of an inhibitor of the p53/MDM2 Interaction to MDM2

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The mode of action of the secondary metabolite chlorofusin, which antagonises the interaction between p53 and MDM2, involves direct binding to the *N*-terminal domain of MDM2.

The 53 KDa phospho-protein p53 plays a critical role in the regulation of cell proliferation, and controls cell growth and development of genetic abnormalities by inducing G1 arrest or apoptosis in response to DNA damage.[1] Dysfunction of p53, for example by mutation of one of the well-known mutational 'hot spots' of p53, is frequently associated with the development of cancers. Indeed, 50% of all human tumours are found to contain a mutated form of its gene.[2] The protein forms a stable complex with MDM2.[3] In this complex, the DNA-binding domain of p53 is concealed by MDM2 so that it is unable to activate transcription of the genes needed for induction of G1 arrest.[4] Therefore, when over-expressed, MDM2 acquires tumorigenic potential. The crystal structure of the MDM2-p53 complex has been determined, revealing three hydrophobic and aromatic amino acids of p53 that make contacts within a hydrophobic cleft of MDM2.[5] The fact that only a small peptide region of p53 is necessary for the complex formation, suggests that the MDM2 pocket may be a suitable binding site for small molecules that would consequently free wild-type p53. It therefore seems possible that tumors expressing abnormally high levels of MDM2 could be treated by means of small molecules that are able to bind to MDM2 and restore normal function to p53.

Chlorofusin is a secondary metabolite from the *Microdochium caespitosum* species that antagonises the interaction between p53 and MDM2. This activity was originally discovered during a screening program for inhibitors of the interaction, carried out by a DELFIA-modified ELISA assay.[6] The primary structure of chlorofusin (Fig. 1) has previously been reported.[6] Here, the interaction between chlorofusin and the N-terminal domain of MDM2 is investigated by surface plasmon resonance.

The N-terminal region of MDM2 [MDM2(1–126)] was over-expressed in *E. coli* BL21 (DE3) cells, and purified using an N-terminal His-Tag. In the purification of the protein using His-Bind Quick 900 cartridges containing Ni^{2+} cations, the MDM2 fragment was eluted with the supplied Elute buffer, containing 1 M imidazole. It was then dialysed into a phosphate buffer containing a surfactant (10 mM sodium phosphate, pH 7.0, 100 mM NaCl, 0.1% Tween 20), and concentrated by centrifugal filtration in preparation for the binding assay with chlorofusin.

Surface plasmon resonance (SPR) is a sensitive technique for the detection of molecular binding interactions.[7–9] One of the interactants is immobilised on the surface of the sensor chip, while the other is injected in continuous flow over the surface. Interactions are detected by the change in refractive index at the detector surface, which reflects a change in surface concentration as molecules bind or dissociate. Proteins are immobilised by covalently attaching them to the surface of the sensor chip, for example, by amine coupling. SPR was used to investigate the interaction between MDM2 and chlorofusin, using amine coupling to immobilise the MDM2 and a control protein.

The concentration of the dialysed and concentrated MDM2 sample was determined by the Bradford method[10] as 120 μM. In the study of binding by SPR, a carboxymethylated dextran surface of a sensor chip was used in cell 1. In cell 3, the MDM2 was attached to the carboxymethylated dextran surface of a sensor chip by amine coupling. Ubiquitin was similarly coupled to provide a control surface in cell 2. Chlorofusin was then passed over these surfaces at various concentrations. Fig. 2 shows the response when 62.5 μM chlorofusin was injected over the three channels.

The binding curves for each concentration of chlorofusin were simultaneously fitted to a simple bimolecular association model, but the data did not fit well. The data were also globally

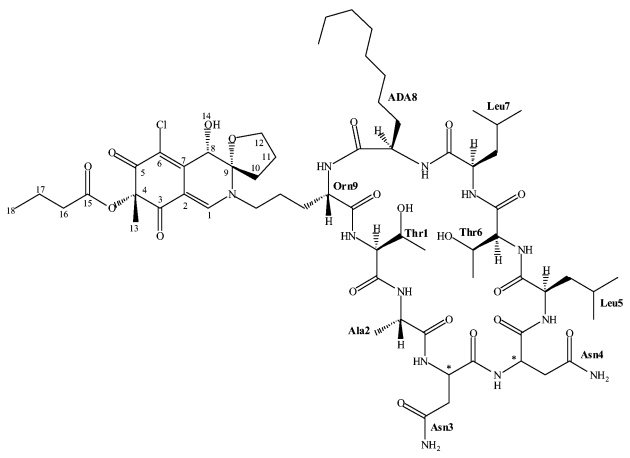

Fig. 1 Primary structure of chlorofusin. The absolute stereochemistries for C-4, C-8 and C-9 may be as shown, or with all three inverted. The asparagine α carbons marked with an asterisk have opposite stereochemistry; one is *R* and one is *S*.

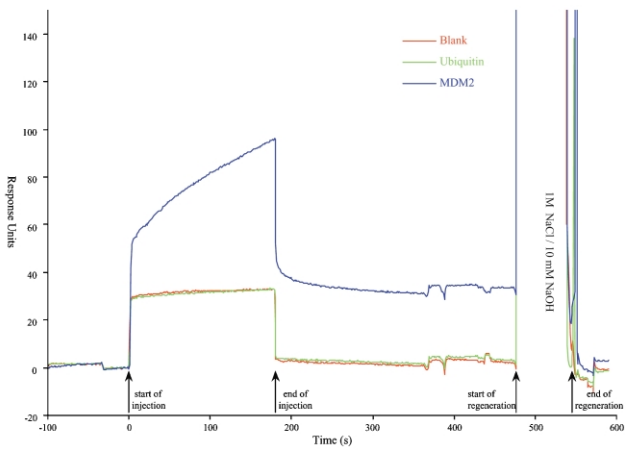

Fig. 2 Chlorofusin (62.5 μM) binding to the blank and ubiquitin-containing cells (lower two traces) and MDM2-containing cell (upper trace). Regeneration was achieved with 1 M NaCl/10 mM NaOH.

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fitted to a biphasic model that has been used in other SPR binding studies, which describes an initial fast binding event followed by a second, slower step. A much better fit (Fig. 3) was obtained for this two-step-binding model.

This could imply a rapid initial binding step which induces a conformational change that increases the affinity between chlorofusin and MDM2, resulting in a second, slower binding step. The standard errors for the calculated kinetic constants gave an overall χ^2 of 26.7 for simultaneous fitting of 13 binding curves. The calculated association and dissociation constants from this kinetic model are summarised in Table 1. Using this fit, the overall affinity constant, K_D, which is defined as the product of K_1 and K_2, was calculated to be 2.11×10^5 M^{-1}, or 4.7 μM. This is in excellent agreement with the IC_{50} value obtained from the original ELISA assay, which was determined as 4.6 μM.[6]

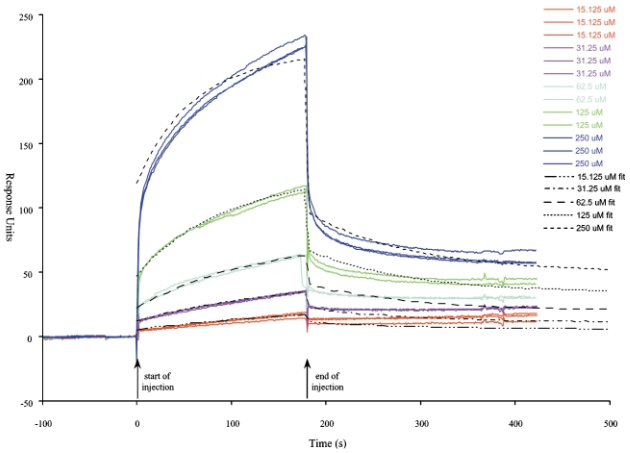

Fig. 3 Chlorofusin binding to MDM2 at low (red lines) to high (dark blue lines) concentrations. The broken lines indicate the fit obtained with the two-step-binding model.

Table 1 Rate and affinity constants for binding of chlorofusin to MDM2, calculated from fitting the data to a biphasic reaction model

k_a	k_d	Affinity
k_{a1} = 49.5 M^{-1} s^{-1}	k_{d1} = 6.59×10^{-3} s^{-1}	K_1[a] = 133 μM
k_{a2} = 2.97×10^{-3} s^{-1}	k_{d2} = 1.06×10^{-4} s^{-1}	K_2 = 0.036
		K_D = 4.7 μM

[a] K_1, which describes the initial binding affinity, is defined as k_{d1}/k_{a1}, and K_2, which describes the equilibrium position between the initial and final bound states, is defined as k_{d2}/k_{a2}.

The work provides further detail of the way in which a small molecule can inhibit a protein–protein interaction. Specifically, the mode of action of chlorofusin, which antagonises the interaction between p53 and MDM2, involves direct binding to the N-terminal domain of MDM2.

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