In the current manuscript, Milanesi et al. characterize the binding mode of the sub-microM binding drug idoxifene to calcium-loaded calmodulin. Interestingly, the relatively high affinity of the 2:1 complex does not preclude extensive mobility of the bound ligands. Since most titrating calmodulin resonances show slow exchange behaviour, the KD was determined using fluorescence of the ligand, whereas the localisation of the binding sites was established by NMR, based on CSPs and NOEs. Critically, variable orientations of idoxifene molecules within each of the two binding sites was implied by NMR-restrained docking to satisfy all observed intermolecular NOEs. While mostly well-established NMR method have been employed here, the obtained experimental data convincingly supports the conclusions drawn about an unusual ligand-protein binding mode, which has significant implications for calmodulin antagonism. I strongly recommend publication in Magnetic Resonance.

The authors may consider the following minor issues:

Apparently, there’s only a single set of resonances for the ligand without (in Fig S1) an indication for significant exchange broadening (Fig. S1). Can the authors say something about the timescale of reorientation in the binding pockets?

As the authors note correctly, the presence of intermolecular NOEs of many ligand resonances to the same protein nuclei, widely distributed across each domain, could be indicative of spin diffusion. The main reason for the choice of 100 ms as mixing time is that it was also used in previous studies. I do no mistrust the interpretation of the observed NOE pattern as a consequence of variable orientations of the ligand in the binding pockets, but additional spectra recorded with shorter mixing time might be of interest (not required though) nevertheless and would settle the point. Also, it is stated that “spin-diffusion effects are not dominant between nuclei within the ligand when it is
bound in the complex”, but no explanation is given.

In the experimental section, recording of a 3D 13C-half filtered-13C-edited NOESY is described, but the experiment is not mentioned or shown in the Results part. If the experiment was in fact used, why was a 15N filter not applied as well? There should be some overlap of aromatic ligand signals with amide protons of calmodulin.

Does w2-13C-half filtered w1-13C-NOESY (line 126) mean 2D w2-13C-half filtered w1-13C-edited-[1H,1H]-NOESY?

The original name of the J-based experiment for methionine e-methyl assignment is Long Range 13C-13C (LRCC) correlation.

Fig. 1: I don’t understand the numbering scheme for idoxifene. Where are positions 1-6? Why are different numbers used for equivalent positions in the rings?

The values used for indirect referencing of 13C and 15N (line 109) according to Wishart et al. refer to internal DSS, but TSP is used here instead.