## SUPPORTING INFORMATION

## MATERIALS AND METHODS

All chemicals and reagents were ACS-grade or higher and typically purchased from Sigma-Aldrich unless otherwise indicated. $^{15}NH_4Cl$ and $D_6$-DMSO were purchased from Cambridge Isotope Laboratories (Andover, MA). All buffers were passed through Chelex-100 resin to remove trace metals prior to use.

### MD simulations

Calculations were performed with the programs CHARMM [1] and NAMD [2]. Molecular dynamics (MD) simulations of S100B-Pentamidine complex were initiated from the co-crystal structure (PDB 3CR4) of S100B in complex with pentamidine. The initiator methionine (Met0) was removed as it would be cleaved in cells and the two terminal residues (His90, Glu91), which were unstructured in the crystal, were not included. The simulated system consisted of the S100B dimer in complex with four pentamidine molecules and four calcium ions. The waters observed in the crystal structure were retained and 14,331 water molecules and 10 randomly placed sodium ions were added to make a neutral solvated system with dimensions 79 Å x 79 Å x 79 Å. The CHARMM protein force field [3] with CMAP backbone correction [4] was used to model the protein, and the TIP3P model was used for water [5]. The CHARMM general force field [6] was used for pentamidine and its modifications. Following a short energy minimization, heating and equilibration, a 16-ns production MD simulation in the NPT ensemble was performed using the NAMD simulation program [2] with snapshots output every 5 ps. Water geometries and bonds involving hydrogen atoms were constrained using the SHAKE algorithm [7] and a 2-fs integration time step was used for dynamics. Long-range electrostatic interactions were handled with the particle-mesh Ewald method [8] with a real space cutoff of 12 Å and a switching function was applied to the Lennard-Jones interactions in the range of 10 to 12 Å. Production simulations in the NPT ensemble maintained 298 K temperature and 1 atm pressure using Langevin thermostat [9] and Langevin piston barostat, respectively. The first 2 ns from the first production trajectory were considered equilibration and were not included in the analysis. In order to increase sampling, nine other trajectories were initiated from snapshots randomly chosen from the initial trajectory. The cumulative sampling time was 98 ns. From the simulations, probability distributions of selected atoms were calculated on a 3D grid composed of 1 Å X 1 Å X 1 Å volume elements (voxels) and normalized with respect to number of MD snapshots. Accordingly, a voxel occupancy of 1% indicates that the voxel is occupied in one out of every 100 snapshots. When calculating the occupancies, in order to capture interactions that involve direct interactions with the protein, only those atoms that lie within 5 Å of the protein were included in the probability map calculation.

In order to obtain the conformational distributions of pentamidine, hexamidine, and heptamidine in solution, the compounds were simulated in the absence of protein with the aqueous environment modeled using the GBMV implicit solvent model [10] with the solvent dielectric constant set to 80. Three 20-ns simulations were performed on each molecule with snapshots saved every 5 ps. Distance probability distributions between the terminal amidine hydrogens were calculated with a bin width of 1 Å.

### Compound Synthesis

The following synthetic protocol was adapted from a previously published protocol [11]. Cesium Carbonate (10.93 g/31.69 mmoles/4 equivalents) and 1,7-dibromoheptane (**compound-2**) (1.025 g/3.97 mmoles/ 0.5 equivalents) was added to 4-cyano phenol (**compound-1**) (1 g/ 8.5 mmoles/1 equivalent) in 50 mL of acetone. The reaction mixture was refluxed for 48 hours at 60 °C, then cooled to room temperature. Acetone was removed under reduced pressure to give a solid residue. Water (75 mL) was added to the residue and extracted with dichloromethane (25 mL x 3). The organic layer was separated and washed with 10% sodium hydroxide in water (10 mL x 3) and washed with water until neutral to pH paper. The organic layer was dried with sodium sulfate, filtered, and evaporated to give pure solid **AC-1** (2 g, 70%yield) (Supplementary Scheme 1, Step 1).

1.0 g of **AC-1** (2.99 mmoles) in 25 mL of anhydrous dioxane and 20 mL of dry methanol was saturated with hydrochloric acid gas at 0-5 °C under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 36 hours, after which the solvent was reduced by 50%. 30 mL of anhydrous ether was added and the resulting precipitate was filtered, washed with anhydrous ether (10 mL x 5) and dried under vacuum to yield **AC-2** (0.99 g, 75% yield) (Supplementary Scheme 1, Step 2).

Dry solid **AC-2** (0.9 g, 2.26 mmoles) was dissolved in 50 mL of dry ethanol and 50 mL of 2 M ethanolic ammonia, to which ammonium chloride (0.025 g, 0.5 mmoles) was added. This mixture was refluxed at 80 °C for 32 hours under nitrogen atmosphere, after which it was cooled and concentrated. The solid obtained was filtered and washed with cold ethanol (5 mL x 3) and ether (10 mL x 3) to give 0.7 g (85% yield) of diamidine product **SBi4211**/heptamidine (Supplementary Scheme 1, Step 3). The compound was then dissolved in $D_6$-DMSO to a concentration of 50 mM and aliquoted for use in all further experiments.

### Cell Culture Conditions

The human melanoma cell line MALME-3M was purchased through American Type Culture Collection (Manassas, VA). Cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 20% fetal bovine serum and 1% penicillin/streptomycin and cultured at 37 °C at 5% $CO_2$ and sub-cultivated twice a week.

### siRNAs and Cloning of Hairpin Loops

The 21-nt double-stranded anti-S100B siRNA (Ambion, catalog number AM16704) and the 23-nt double-stranded scrambled negative control siRNA (Ambion, catalog number 4611) were synthesized and purified using standard methods. The siRNA duplex sequences are listed in Supplementary Table 1. The sequences of both the anti-S100B siRNA and the scrambled negative control siRNA duplexes were then cloned into

pSuppressorNeo (pSupNeo) vector (Imgenex). The hairpin RNAs produced by the pSupNeo vector were designed to be identical to the siRNA duplex oligonucleotides. The sequences inserted into the pSupNeo vector are listed in Supplementary Table 2.

## Establishment of Stable RNAi MALME-3M Cell Lines

MALME-3M cells were cultured in T-25 flasks at 37 °C to 60-70% confluency. The cells were then transfected with the S100B or scrambled siRNA hairpin-generating vector using Mirus TransIT-LT1 reagent. For each, 6 μL of TransIT-LT1 was added to 60 μL of serum-free RPMI media in a 5 mL polystyrene round-bottom tube and incubated at room temperature for 15 minutes. Next, 2 μg of the vector-based hairpin siRNA was added to the mixture and again incubated at room temperature for 15 minutes. The complete mixture was then added drop-wise to the cells in complete growth medium. The transfected cells were incubated for 48 hours at 37 °C, after which they were transferred to IMDM medium containing 20% fetal bovine serum and 1% penicillin/streptomycin and neomycin (0.5 mg/mL) and single-cell diluted into 96-well plates. Wells were screened for neomycin-resistant clones, expanded, and then the levels of S100B protein were determined. A "high S100B" clone (SCR) containing the scrambled shRNA and a "low S100B" clone (#7) containing the anti-S100B shRNA were selected, and thereafter maintained in neomycin-containing medium. Western blot showing protein levels of S100B and control GapDH are shown in Supplementary Figure 1.

## Western Blot Analysis

Total cell lysates were prepared in RIPA lysis buffer (Upstate Biologicals) supplemented with 1X protease inhibitor cocktail EDTA-free (Roche) and 1X phosphatase inhibitor cocktail II (Calbiochem). Lysates were cleared by centrifugation at 16,000 x g for 15 minutes at 4 °C. Protein concentration was determined by a Bradford assay (Bio-Rad). Twenty-five micrograms of each lysate were electrophoresed on a 12% Bis-Tris NuPage gel (Invitrogen) and transferred onto a PVDF membrane (Invitrogen) according to manufacturer's recommendations, then blocked with 5% nonfat dry milk in TBS with 0.5% Tween-20 (USB Corporation) and incubated with the indicated antibody. In this study, the following antibodies and dilutions were used: S100B mouse monoclonal antibody (BD Transduction Laboratories) at 1:1,000 and GapDH mouse monoclonal antibody (CalBiochem) at 1:10,000 as a loading control. The blots were then incubated with secondary antibodies conjugated to horseradish peroxidase. Protein-antibody complexes were detected using Amersham ECL Western Blot Detection Reagents following manufacturer's recommendations (GE Healthcare) Signal was quantitated with an EpiChemi3 Imaging System (UVP) with an attached Hamamatsu CCD camera (Hamamatsu Photonics) and analyzed with UVP Labworks Image Acquisition and Analysis Software v. 4.6. The density of the S100B bands were normalized to their respective GapDH control band, and the average normalizations and standard deviation were calculated for each of the cell lines (n=5). Statistical analysis was performed using a two-tailed Student's t-test calculated with Excel (Microsoft) with a p value $< 0.05$ was considered statistically significant.

## Growth inhibition assay

Compounds were tested for their ability to inhibit the growth of MALME-3M melanoma cells that express relatively high levels of S100B (SCR) as compared to the same cell line with a reduction of S100B protein (#7) using a modification of the high-throughput screening assay performed by Bachman et al [1]. Using a Biomek FX Laboratory Automation Workstation (Beckman-Coulter) equipped with a 96-channel pipetting head, 20 μl of IMDM (Invitrogen) supplemented with 20% fetal bovine serum and 1% Pen/Strep, were added to each well of a 384-well tissue culture plate (Corning) containing enough cells such that growing uninhibited they reach ~80% confluence in 5 days. The cells are propagated in the presence of 0.5 mg/ml neomycin to maintain the vector expressing S100B siRNA to knockdown the protein or the same vector containing a scrambled control siRNA; however, the neomycin is not added to the media for the growth inhibition assay. After 24 hours of growth at 37°C in a 5% $CO_2$ humidity-controlled incubator the cells were treated with 20 μl of compound in the same culture media, while in the control cultures only a equivalent amount of DMSO added with the compound is added. After four additional days of incubation, the cells were lysed by the adding 40 μl of lysis buffer consisting of 1.2% Igepal with 1:10,000 dilution of SYBR Green I (Invitrogen). The covered plates were incubated for another 24 hours at 37 °C in a 5% $CO_2$ humidity-controlled incubator. At every incubation step the 384-well culture plates were wrapped in plastic film. This did not appear to affect the growth of the cells but was very effective in preventing edge affects due to evaporation of the media from the outer wells. The fluorescence intensity was then read through the bottom of the plate using a FLOUstar Optima (BMG) fluorescent plate reader using 485 nm excitation and 520 nm emission filter. The SYBR-green fluorescence is used to measure total DNA that in turn correlates with cell number. The $IC_{50}$ of the compounds were determined using serial dilutions and done in quadruplicate, as above, with no more than 1.0% DMSO or 200 μM compound.

## Bacterial Expression and Purification of Wild-Type S100B

Recombinant S100B protein (rat and bovine) was expressed in *Escherichia coli* [HMS174(DE3) strain] and purified as previously described [12]. Yields of S100B protein were typically 20–30 mg of purified protein per liter of bacterial culture. For NMR experiments, S100B protein was prepared using defined medium that included $^{15}$N-labeled $NH_4Cl$ as the only nitrogen source.

## Differential Scanning Calorimetry

Measurements were performed using a VP-DSC microcalorimeter (MicroCal, Inc., Northampton, MA). Protein solutions for the calorimetric experiments were prepared by dialysis against the buffer 5 mM HEPES at pH 7.2, 15 mM NaCl, 10 mM $CaCl_2$, and 0.5 mM TCEP. To avoid a protein concentration dependence of S100B denaturation temperature, we carried out all the DSC experiments at comparatively low protein concentration (40-50 μM per monomer). The compound solutions were prepared in the same dialysis buffer and diluted in the dialyzed sample solution (200 μM final concentration). Reference (buffer) scans were subtracted from the observed traces, and the data were normalized for protein concentration using Origin for DSC software supplied by MicroCal, Inc.. The apparent $T_m$ was determined from the resultant peak maxima. A second scan of the samples showed approximately 70% reversibility for S100B without any ligand, as opposed to scans observed for S100B-

Heptamidine or S100B-Pentamidine, which revealed no reversibility. The differential scanning calorimetry transitions show that heptamidine significantly decreases the apparent unfolding temperature of S100B ($\Delta T_m$=18.3 °C) followed by aggregation versus pentamidine-S100B ($\Delta T_m$=1.0 °C) relative to S100B dimer ($T_m$=112.2 °C) without any ligand ($T_m$ determined from the peak maxima of the transitions).

## NMR Spectroscopy

Purified $^{15}$N-labeled was dialyzed against 0.25 mM Tris (pH 7.5) and 0.25 mM DTT, concentrated to 10-15 mM using Amicon Ultra centrifugal filter units with a 10 kDa MWCO, concentration was determined using Bradford reagent (BioRad) and protein was then aliquoted and stored at −20 °C. For 1D NMR experiments to determine binding, control samples were made with 1 μM compound, 0.34 mM $NaN_3$, 15 mM NaCl, 10 mM $CaCl_2$, and 10 mM Tris-D11 in 100% $D_2O$. Protein was lyophilized and resuspended in $D_2O$ twice, and samples were made with the same conditions as the control, but with the addition of 10-30 μM $^{15}$N S100B. This sample was then titrated into the control sample, and 1D experiments with water suppression using WATERGATE[14; 15] were collected at various concentrations of protein. Change in height of the compound peaks was monitored and fit using MicroCal Origin software. Although it was determined that pentamidine binds with a stoichiometry of 2, each site was fit separately since the site-dependent dissociation constants were significantly different (>100-fold), non-cooperative, and required a separate set of conditions to analyze properly. The resulting binding constants for each titrated peak were then averaged; two experiments were performed for each compound and the average of both experiments is reported, along with the standard deviation. Some peaks were omitted from final analysis due to noise from protein signals at higher concentrations of S100B. The $Ca^{2+}$-loaded S100B-SBi4211 HSQC sample was prepared in a manner similar to that previously described [16] and contained 0.1 mM S100B subunit, 0.25 mM SBi4211, 0.34 mM $NaN_3$, 15 mM NaCl, 5% DMSO-$d_6$, 10 mM $CaCl_2$, 10% $D_2O$, and 10 mM TES, adjusted to pH 7.2 with HCl. Heteronuclear single-quantum coherence (HSQC) NMR data were collected at 37 °C with a Bruker Avance 800 US2 (800.27 MHz for protons) instrument equipped with pulsed-field gradients, four frequency channels, and triple-resonance, z-axis gradient cryogenic probes. Data were processed with NMRPipe [17], and proton chemical shifts were reported with respect to the $H_2O$ or HDO signal taken as 4.658 ppm relative to external TSP (0.0 ppm). The $^{15}$N chemical shifts were indirectly referenced as previously described using the following ratio of the zero-point frequency: 0.10132905 for $^{15}$N to $^{1}$H. A labeled HSQC of $^{15}$N-S100B at pH 7.2, which has not previously been reported, is given in Supplementary Figure 2.

## Protein Crystallization

Bovine S100B protein was dialyzed into buffer [0.25 mM Tris (pH 7.2) and 0.25 mM DTT], concentrated to 80–100 mg/mL (~8–10 mM subunit concentration using Amicon Ultra centrifugal filter units with a 10 kDa MWCO, concentration was determined using Bradford reagent (BioRad) and protein was then aliquoted and stored at−20 °C. We obtained diffraction quality crystals for the SBi4211 –$Ca^{2+}$-S100B complex by sitting drop vapor diffusion at 22 °C by mixing 1 $\mu$L of S100B protein and SBi4211 compound [40 mg/mL S100B, 4.0 mM SBi4211, 7.5 mM $CaCl_2$, and 10 mM sodium cacodylate buffer (pH 7.21)] with 1 $\mu$L of reservoir solution [7.5 mM $CaCl_2$, 100 mM sodium cacodylate (pH 7.21), 25% PEG3350] and equilibrating for 2–3 days. After crystals formed, they were cryoprotected in a harvest solution [4.0 mM SBi4211, 7.5 mM $CaCl_2$, 10 mM sodium cacodylate buffer (pH 7.21), 27% PEG3350, and 5% glycerol] for 30–60 s and then flash-cooled in liquid nitrogen. Space groups and unit cell parameters are given in Supplementary Table 3. The crystal had one S100B subunit in the asymmetric unit.

## X-ray Data Collection, Model Building, and Refinement

X-ray data for the SBi4211–$Ca^{2+}$-S100B crystals were collected at 100 K using an in-house X-ray generator (MSC Micromax 7; Rigaku Texas, USA) and a Raxis-4++ image plate detector (Rigaku Texas). The reflection intensities were integrated and scaled with the HKL2000 suite of computer programs[18]. The crystal of the SBi4211–$Ca^{2+}$-S100B complex diffracted to 1.65 Å resolution. Preliminary phases were obtained via molecular replacement techniques using the structure of $Ca^{2+}$-bound S100B [PDB entry 1MHO] as a search model and the computer program Phaser from the CCP4 program suite [19; 20]. Model building and refinement of S100B were performed using COOT and REFMAC5[21; 22]. The locations of the SBi4211 molecule and several water molecules were determined by visual inspection of electron density maps calculated with $2mF_o–DF_c$ and $mF_o–DF_c$ coefficients with COOT. The occupancy was set to 1.0 for SBi4211 except for the C9 and C10 atoms in the linker region, which were set to 0.5 occupancy because these atoms reside on a crystallographic 2-fold axis. The stereochemistry and validity of the final structure were checked with MOLPROBITY[23] and PROCHECK[24]. Refinement statistics are given in Supplementary Table 3. The coordinates were deposited in the Protein Data Bank and assigned the accession number 4FQO. Figures were generated with PyMol ("http://www.pymol.org").

## SUPPORTING FIGURES

**Supplementary Scheme 1. Synthesis of SBi4211**

**Supplementary Table 1. Sequences of siRNA duplexes**

| siRNA duplex | Sense strand (5'→3') | Antisense strand (5'→3') |
|---|---|---|
| S100B | GGAAUUCAUGGCCUUUGUU | AACAAAGGCCAUGAAUUCC |
| SCR | AGUACUGCUUACGAUACGGdTdT | CCGUAUCGUAAGCAGUACUdTdT |

**Supplementary Table 2. Sequences for hairpin RNAs**

| Hairpin RNA | Sense strand (5'→3') |
|---|---|
| S100B | TCGACCCGGAATTCATGGCCTTTGTTTTCAAGAGAAACAAAGGCCATGAATTCCTTTTTTGGAAT |
| SCR | TCGACCCAGTACTGCTTACGATACGGTTCAAGAGACCGTATCGTAAGCAGTACTTTTTTTGGAAT |

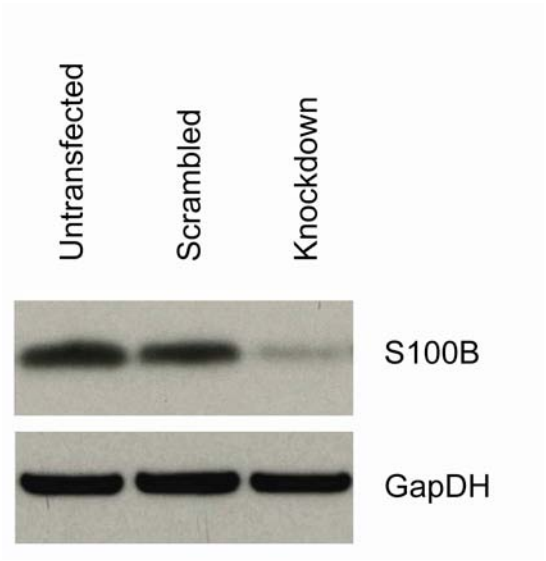
**Supplementary Table 3. Differential Scanning Calorimetry Results**

| **Sample** | **$T_m$ (°C)** | **$\Delta T_m$ (°C)** |
|---|---|---|
| S100B | 112.2 | --- |
| S100B-Pentamidine | 111.2 | 1.0 |
| S100B-Heptamidine | 93.9 | 18.3 |

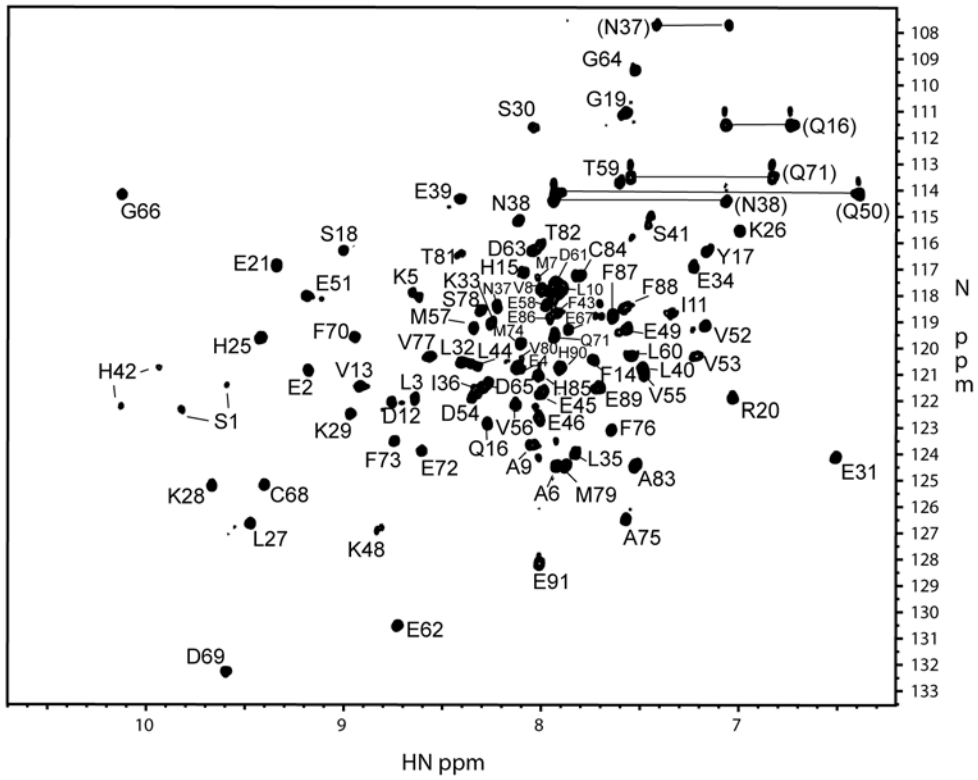
**Supplementary Table 4. $Ca^{2+}$-S100B-Heptamidine Crystal and Refinement Statistics**

| | |
|---|---|
| Diffraction Statistics | |
| Space Group | P41212 |
| Cell dimensions a, b, c (Å) | 63.1, 63.1, 49.0 |
| Cell angles α, β, γ (degrees) | 90.0, 90.0, 90.0 |
| Resolution (Å) | 44.60 – 1.65 (1.65 – 1.68)[a] |
| No. of unique reflections | 11701 (832) |
| Completeness (%) | 99.6 (99.9) |
| $R_{sym}$[b] | 0.068 (0.651) |
| Average (I/σ) | 12.1 (4.4) |
| Multiplicity | 13.5 (13.5) |
| Refinement Statistics | |
| $R_{crys}$[c] (%) | 20.0 (21.3) |
| $R_{free}$[c] (%) | 21.4 (25.0) |
| Protein Atoms | 732 |
| Water Molecules | 89 |
| Non-Hydrogen Atoms | 844 |
| RMSD | |
| Bond Length (Å) | 0.012 |
| Bond Angles (Å) | 1.434 |
| Mean B Values (Å$^2$) | 22.73 |
| Ramachandran Plot (%)[d] | |
| Favored | 98.9 |
| Allowed | 100.0 |
| Outliers | 0.0 |

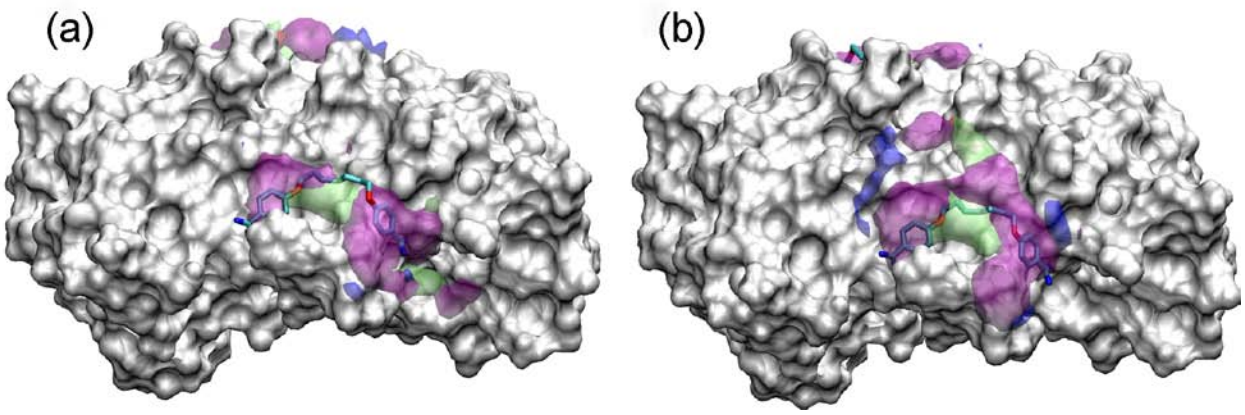
[a]Numbers in parentheses represent the last outer shell. [b]$R_{sym} = \Sigma_h\Sigma_i(|I_i(h)| - |\{I(h)\}|)/\Sigma_h\Sigma_i I_i(h)$, where $I_i(h)$ = observed intensity, and $\{I(h)\}$ = mean intensity obtained from multiple measurements. [c]$R_{crys}$ and $R_{free} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where $|F_o|$ = observed structure factor amplitude and $|F_c|$ = calculated structure factor amplitude for the working and test sets, respectively. [d]According to MolProbity analysis 89 out of 90 residues were in favored (98%) regions and 1 additional residue was in allowed (>99.8%) regions.



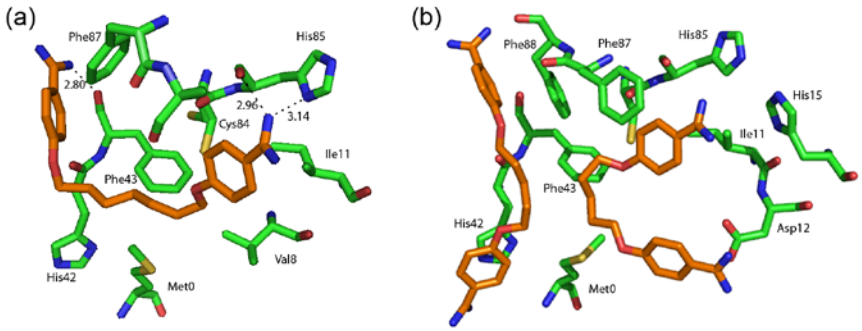

**Supplementary Figure 1**. Western blot of cell lysates from stable RNAi transfections in MALME-3M cells. Lane 1 shows the expression levels of normal MALME-3M cells. Stable MALME-3M scrambled negative control clones and stable MALME-3M anti-S100B clones are shown in lanes 2 and 3, respectively.




**Supplementary Figure 2.** HSQC of $^{15}$N S100B at pH 7.2 with peaks of backbone residues labeled. Sidechain amides are labeled in parentheses.




**Supplementary Figure 3.** Overlay of heptamidine crystal conformation on the protein and probability maps obtained at an occupancy isocontour value of 1.5%.




**Supplementary Figure 4**. Closer view of the compound-binding site for heptamidine (a) and pentamidine (b) on S100B. Protein residues within 3 Å are shown, colored green (carbon), blue (nitrogen), and red (oxygen). Hydrogen bonds are represented with dashed lines, and the distance between the nitrogen atoms is noted. Compounds are colored orange (carbon), blue (nitrogen), and red (oxygen)

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