**Publication**


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A Constrained Helical Peptide against S100A4 Inhibits Cell Motility in Tumor Cells.

Abstract

S100A4, a member of a calcium-regulated protein family, is involved in various cellular signaling pathways. From many studies over the last decade or so, it has become clear that it is involved in tumor metastasis, probably playing a determinative role. However, except the phenothiazine group of drugs, no significant inhibition of S100A4 has been reported. Even the phenothiazines are very weak inhibitors of S100A4 action. In this study, we report design and development of a conformationally constrained helical peptide modeled on the non-muscle myosin-2 peptide that binds to S100A4. This conformationally constrained peptide binds to S100A4 with a dissociation constant in the nanomolar range. We also synthesized a peptide for experimental control that bears several alanine mutations in the peptide-protein interface. We demonstrate that the former peptide specifically inhibits motility of H1299 and MCF-7 cells in a wound healing assay. Structures of several S100A4-ligand complexes suggest that it may be possible to develop a smaller peptide-ligand molar correlate having high affinity for S100A4. Peptide-ligand conjugates of this kind may play an important role in developing drug leads against this anti-metastasis target. S100A4 is an important member of the large S100 protein family (1). S100A4 forms homodimers and interacts with many target proteins in a calcium-dependent manner, in both extra-cellular and intra-cellular spaces (2). It strongly promotes cell motility and is known to play an important role, perhaps a determinative role, in tumor metastasis (3). S100A4 knock-out mice resist growth of transplanted tumors (4). In spite of being such a potentially important drug target against metastasis, very few inhibitors of S100A4 partner protein interactions are known. To our knowledge, phenothiazines are only known class of inhibitors that inhibit S100A4 action, presumably through an aggregation-inhibition mechanism. However, IC50 of this effect is in the range of 50-100 μM. It is doubtful whether such large levels are pharmacologically achievable in vivo (5). A number of recent studies have identified an existing anti-hepatic drug niclosamide to act as a metastasis inhibitor (6, 7). Probably, through regulation of expression of S100A4 and other genes via inhibitor WNT pathway (6, 9). Whether its down-regulates S100A4 specifically, is not clear. Being a relatively small protein (a molecular mass of about 21 kDa for the homodimer), the available binding surfaces for a potential inhibitor are not very extensive. Thus, we attempted to develop a peptide-based high affinity inhibitor and demonstrate its potential in cellular milieu. If successful, such a peptide can be conjugated to small molecules, such as, pertussisome (a known binder of S100B, a paralog of S100A4) or its variants or phenothiazines to generate a very high affinity inhibitor. This article is protected by copyright. All rights reserved.
A Constrained Helical Peptide Against S100A4 Inhibits Cell Motility in Tumor Cells

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S100A4, a member of the calcium-regulated protein family, is involved in various cellular signaling pathways. From many studies over the last decade or so, it has become clear that it is involved in tumor metastasis, probably playing a determinative role. However, except the phenothiazine group of drugs, no significant inhibitor of S100A4 has been reported. Even the phenothiazines are very weak inhibitors of S100A4 action. In this study, we report design and development of a conformationally constrained helical peptide modeled on the non-muscle myosin peptide that binds to S100A4. This conformationally constrained peptide binds to S100A4 with a dissociation constant in the nanomolar range. We also synthesized a peptide for experimental control that bears several alanine mutations in the peptide–protein interface. We demonstrate that the former peptide specifically inhibits motility of H1299 and MCF-7 cells in a wound-healing assay. Structures of several S100A4–ligand complexes suggest that it may be possible to develop a smaller peptide–small molecule conjugate having high affinity for S100A4. Peptide–drug conjugates of this kind may play an important role in developing drug leads against this antimitastatic target.

Key words: chemical biology, peptide, protein–protein interaction

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S100A4 is an important member of the large S100 protein family (1). S100A4 forms homodimers and interacts with many target proteins in a calcium-dependent manner, in both extra-cellular and intracellular spaces (2). It strongly promotes cell motility and is known to play a very important role, perhaps a determinative role, in tumor metastasis (3). S100A4 knock-out mice resist growth of transplanted tumors (4). In spite of being such a potentially important drug target against metastasis, very few inhibitors of S100A4–partner protein interactions are known. To our knowledge, phenothiazines are only known class of inhibitors that inhibit S100A4 action, presumably through an aggregation-promotion mechanism. However, IC50 of this effect is in the range of 50–100 μM. It is doubtful whether such tissue levels are pharmacologically achievable in vivo (5). A number of recent studies have identified an existing antihelmintic drug niclosamide to act as a metastasis inhibitor (6,7), probably through the regulation of expression of S100A4 and other genes via inhibition WNT pathway (8,9). Whether it downregulates S100A4 specifically is not clear.

Methods and Materials

Materials
All Fmoc-amino acids, Rink amide MBHA resin, TBTU, HATU, and HOBT were purchased from Novabiochem (Mumbai, India), DMF, DPEA, DIPC, HPLC water, acetonitrile, TFA were purchased from Spectrochem (Mumbai, India), 5(6)-carboxyfluorescein was purchased from Molecular Probes, Invitrogen (Bangalore, India). Reverse-phase C-18 HPLC column was from Thermo Corporation Limited (Mumbai, India). Ni-NTA Sepharose was purchased from GE Healthcare Bio-Sciences AB (India). Acrylamide, bis-acrylamide, TEMED, PMSF, ethidium bromide, bromophenol blue, commassie brilliant blue, Ampicillin, and IPTG were purchased from Sigma Chemical Company (St. Louis, MO, USA). Luria Broth and agar were purchased from Hi-Media (Mumbai, India). Triton-X 100 was purchased from MP Biomedicals (Mumbai, India). Anhydrous glycerol, NaCl, imidazole, Tris–HCl, sodium carbonate and Sodium bicarbonate, EDTA were purchased from J.T.
Baker (Faridabad, India). Streptomycin sulfate was purchased from Sigma-Aldrich (India). RPMI media and FBS were purchased from Invitrogen (Mumbai, India).

Peptide synthesis

The peptide was synthesized on Rink Amide MBHA resin using Fmoc chemistry in a protein technologies PS3 peptide synthesizer. A capping step with 5% acetic anhydride and 5% 2,6 dimethylpyridine in DMF was incorporated in the protocol after each coupling. Fmoc-amino acids were activated with O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium tetrafluoroborate (TBTU) in the presence of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and/or Hydroxybenzotriazole (HOBt) and Diisopropyl ethyl amine (DIPEA). Peptides were cleaved from the resin, and side-chain protecting groups were removed by incubating with 81% trifluoroacetic acid (TFA), 5% phenol, 5% thioanisole, 3% water, 2.5% 1,2-ethanedithiol (EDT), 2% dimethyl sulfoxide, 1.5% (by weight) ammonium iodide at 25 °C for 3 h followed by precipitation with cold diethyl ether. The precipitate was collected by centrifugation, dried and dissolved in 0.1% TFA and purified by reverse-phase HPLC on C18 column. The peptides were purified by HPLC on a reverse-phase μBondapak C-18 column using 0–80% acetonitrile/water with 0.1% TFA and characterized by MALDI-TOF mass spectrometry. The following program or some minor variation of it was used for all the HPLC gradient runs.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water (0.1% TFA) (mL)</th>
<th>Acetonitrile (0.1% TFA) (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>40</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>45</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The peaks were monitored at 215 and 230 nm. In case of fluorescein-labeled peptide, peaks were monitored at 215 and 480 nm. In all the cases, only major peaks were collected and characterized through mass spectrometry. The peptides were characterized by MALDI-TOF mass spectrometry.

Sequence and nomenclature of peptides derived from NMIIA

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMII</td>
<td>QREILDATADAMNREVSSLKNNKRRGDLPFWYPRAMA</td>
</tr>
<tr>
<td>NMIB</td>
<td>QREILDATADAMNREVSSLKRRGDLPPFWYPRAMA</td>
</tr>
<tr>
<td>NMIB-mut</td>
<td>QREILDATADANPEEASRLLKRRGDLPPFWYPRAMA</td>
</tr>
</tbody>
</table>

Chemical modification of peptides

A small portion of the peptide resin was labeled at the ε-amino group of the N-terminal amino acid with 10-fold molar excess of 5(6)-carboxyfluorescein and HOBt and DIPC at 25 °C for 3 h. Then resin was first washed with 20% piperidine and then with DMF to remove the excess unreacted dye. The labeled peptides were cleaved from the resin and purified as described above.

Expression and purification of S100A4

The S100A4 cDNA/gene was cloned in pKK223-3 vector. Then, it was subcloned in the pCold vector in the HindIII/PstI cloning site. The pCold plasmids containing the S100A4 gene were transformed in BL21 E. coli cells, and the cells were grown in Luria broth containing 100 μg/mL of ampicillin at 37 °C until the A600 reached 0.5. At that point, the cells were incubated at 15 °C for 30 min. Without stirring, then induced with 1 mM IPTG at 15 °C and shaking continued for another 24 h at that temperature. Then cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The harvested cells were suspended in lysis buffer (50 mM Tris–HCl buffer, pH 7.5 containing 300 mM NaCl, 2 mM DTT, 10% glycerol, 20 mM imidazole) and lysed by sonication. The cell debris was removed by centrifugation at 14 000 rpm for 45 min at 4 °C. Affinity chromatographic purification of the protein was performed using Ni-NTA Sepharose column pre-equilibrated with the lysis buffer. After centrifugation, the supernatant was allowed to bind with the pre-equilibrated column material for 1.5 h at 4 °C. Then, the column was washed subsequently with a buffer having the same composition of lysis buffer but in addition contained 300 mM imidazole. The protein was eluted from the column with buffer having the same composition as that of the wash buffer but in addition contained 50 mM imidazole. The protein was characterized by running 15% SDS-PAGE gel. The purified protein was dialyzed against 50 mM Tris–HCl buffer, pH 7.5 containing 150 mM NaCl, 2 mM DTT, and 10% glycerol and then stored at −80 °C. The protein purity was estimated to be 95%.

Fluorometric titrations

Binding of the peptides was determined by direct titration of fluorescein-labeled peptides with unlabeled S100A4. All the anisotropy, measurements were carried out in 50 mM Tris–HCl buffer pH 7.5 containing 150 mM NaCl, 2 mM dithiothreitol (DTT), 10% glycerol, and 0.5 mM CaCl2 at 25 °C. All the binding assays were carried out in a Quantamaster 6 (PTI) T-geometry fluorometer fitted with a circulating water bath to maintain the temperature at 25 °C. Excitation and emission wavelengths used for each titration were 490 and 525 nm, respectively, with a band pass of 5 nm in each channel. The anisotropy data were fitted into a single-site binding equation, and Kd values were obtained by fitting them in Kyplot (Koichi Yoshioka, 2015).

Table: Parameters of fluorescence quenching

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E0</td>
<td>0.65</td>
</tr>
<tr>
<td>Kd</td>
<td>0.6</td>
</tr>
<tr>
<td>R2</td>
<td>100</td>
</tr>
<tr>
<td>n</td>
<td>1</td>
</tr>
</tbody>
</table>

The following program or some minor variation of it was used for all the HPLC gradient runs.
where \( A \) stands for the observed anisotropy, \( A_o \) stands for limiting anisotropy at infinite protein concentration, \( A_i \) stands for the initial anisotropy, that is the anisotropy of the free peptide, \( K_d \) is the dissociation constant, \([S100A4]\) is the total concentration of S100A4 in terms of dimer and [peptide] is the concentration of the peptide used. The equation was obtained from the equilibrium equation. For the competition experiment, 150 \( \mu \)M of S100A4 was mixed with 3 \( \mu \)M carboxy-fluorescein-labeled NMII and back-titrated with NMIIB.

### Isothermal titration calorimetry

All ITC experiments were performed in a VPITC instrument from Microcal Inc. The titrations were carried out in 50 \( \mu \)M Tris-HCl buffer pH 7.5 containing 150 \( \mu \)M NaCl, 2 \( \mu \)M DTT and 0.5 \( \mu \)M CaCl\( _2 \) at 25 °C. The protein (10 \( \mu \)M in terms of dimer) was taken in the cell (volume 2 mL), and the peptide (100 \( \mu \)M) was added from a syringe with continuous stirring. The data were fitted to a single-site binding isotherm using ORIGIN 7.0 software.

### Cell culture

H1299 (human non-small cell lung carcinoma) and MCF 7 (human breast adenocarcinoma) cells were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA, and were maintained in RPMI-1640 medium and Eagle’s minimum essential medium, respectively, supplemented with 10% fetal bovine serum (FBS) and 2 \( \mu \)M L-glutamine. Eagle’s minimum essential medium was further supplemented with 0.01 mg/mL human recombinant insulin as recommended for MCF-7 maintenance.

### Cell migration assay

The cells (H1299 or MCF 7 cells) were grown to confluent monolayer and ‘wounds’ were created using a sterile pipette tip. The cells were treated with 20 \( \mu \)M NMII, NMIIIB, NMIIIB mut peptides, or 50 \( \mu \)M CTK7A, as indicated. The cell migration was monitored up to 48 h depending on the cell line. Photographs were taken at the indicated time-points.

### Results and Discussion

To develop a peptide-based inhibitor of S100A4, we focus on the structure of the complex of S100A4 with a peptide from non-muscle myosin-IIA (Figure 1) (10). This peptide binds in a predominantly helical conformation. Previous studies have suggested that conformationally stabilized helices are good inhibitors and can be delivered inside the cell (11–13). We decided to use this peptide as the starting point and develop a conformationally stabilized helix by incorporating \( \alpha \)-amino isobutyric acid (aib) residues in non-interacting positions as was outlined previously (14). Aib is known to increase conformational preference of peptides for helical conformations (14,15), and such constrained helices can be efficacious inhibitors in an in vivo setting. Positions of aib substitutions are shown in Figure 1.

The aib-substituted peptide (NMIIIB; denoting non-muscle myosin II derived peptide having aib whose one letter abbreviation is B) was synthesized in solid phase and purified by HPLC. The peptide was characterized by mass spectrometry and showed predominantly one peak of the correct molecular mass. The wild-type peptide, NMII, was also synthesized, purified, and characterized as described.
above. A mutant peptide (NMIIB-mut), which has five alanine residues in the protein–peptide interface instead of the natural amino acids in the wild-type sequence, was also synthesized. All of the mutated residues are located at the peptide–S100A4 interface (Figure 1B). For measuring binding to S100A4, the peptides were labeled with fluorescein at the N-terminal amino group in the solid phase and purified by HPLC. The purity and identity of the peptide was established by mass spectrometry. S100A4 was purified to homogeneity as detailed in the Methods and Materials. To check whether incorporation of aib residues conformationally constrain the peptide to more helical conformations, we have measured CD spectra of NMII and NMIIB (Figure 2A). NMIIB shows distinctly higher intensity at 222 nm and greatly reduced intensity at 200 nm, indicating considerable increase in the helical content and reduction in the random coil component. The purified labeled peptide was titrated with S100A4, and fluorescence anisotropy was monitored (Figure 2B). Titration of NMIIB with S100A4 yielded a hyperbolic plot which when fitted to a single-site binding equation gave a dissociation constant of 49 ± 6 nM. Tight binding of NMIIB was confirmed by isothermal titration calorimetry (ITC) (Figure 2C). A value of 11.5 ± 0.3 nM was obtained for the dissociation constant. The difference in measured $K_d$ values by two different techniques, if significant, may originate from the concentrations of S100A4 used in two experiments. Titration of NMII with S100A4 yields a dissociation constant of 106 ± 16 nM (Figure 2D). This indicates that aib substitution and consequent conformational constraint led to modest gain in binding affinity. The corresponding binding isotherm of NMIIB-mut shows little rise in anisotropy, indicating a very weak binding (Figure 2E).

To demonstrate that NMIIB binds to the same site as that of NMII, we have formed the fluorescein–NMII–S100A4 complex first and then titrated with unlabeled NMIIB, while monitoring the fluorescence anisotropy. The fluorescence anisotropy of NMII decreases progressively until it returns back to the free NMII value (Figure 2F). This indicates that the two peptides compete for the same binding site.

To verify whether such peptides can inhibit cell motility, we have attempted to determine their effect on cell migration in wound-healing assays. MCF 7 cells were treated with NMIIB peptide, and a control was performed in which NMIIB-mut was used. To estimate whether conformational
stabilization is important for in vivo action, we have also used NMII. All the peptides were tagged with a cell penetration tag (6 D-arginine residues) at the N-terminal end to facilitate cell entry. NMIIIB-treated cells had a reduced rate of cell migration compared with the untreated cells, NMII-treated cells and the NMIIIB-mut peptide-treated cells (Figure 3, left panel). The reduced migration was comparable to the migration of the cells treated with CTK7A, a HAT inhibitor, which is known to inhibit cell migration (16). The tumor suppressor p53 is known to be inhibited by S100A4 (17) and thus to eliminate the possibility that the peptide worked through a p53-mediated modulation of cell motility, a p53 null cell line, H1299, was chosen for the next set of wound-healing assays. In the H1299 cells treated with NMIIIB peptide, cell motility was evidently hindered, while the mutant peptide showed no effect on cell migration. Figure 3 (right panels) shows the effect of NMIIIB and NMIIIB-mut on migration of H1299 cells, along with a positive control CTK7A. It is clear that by 12 h NMIIIB inhibits cell migration almost as well as the positive control molecule (Figure 3, right panel). In contrast, cell migration is neither affected in the untreated nor the mutant treated cells, indicating high degree of specificity. This suggests that augmentation of cell motility by S100A4 probably do not depend on the presence of p53. This is consistent with the fact that S100A4 binds to non-muscle myosin directly and regulates its activity and consequently cell motility.

There is now compelling evidence from animal models and patient samples that S100A4 is involved in many steps of metastasis (3). One of the most important aspects is its role in cell motility. However, in spite of its importance in metastasis, probably the single most important cause of cancer death, no suitable inhibitor has been found yet. The only class of small molecules known to inhibit S100A4 function directly belongs to the phenothiazine group (18). However, due to its weak nature of binding, it is unlikely to be successful as a drug. Peptides are drawing increasing attention as possible drug leads and as chemical genetics tool. One possibility of designing an effective inhibitor is to use the 39-mer NMIIA peptide as an inhibitor. However, as is well known, free peptides are often unsuitable for use in vivo and need to be stabilized. The corresponding helically stabilized peptide, NMIIIB has high affinity for the target and when attached to a cell penetration tag, is an effective inhibitor of cell motility. However, the peptide may be too long for further development for in vivo study and probably needs to be shortened significantly. One possibility is to shorten its length and conjugate it with a known ligand-like PCP (5). Figure 4 shows that the PCP-binding...
site is non-overlapping with a large portion of the N-terminal part of the NMIIA peptide. Thus, a conjugate of a shorter version of the stabilized NMIIA peptide a PCP derivative could be suitable for in vivo use. Niclosamide, an inhibitor of the WNT pathway, also indirectly downregulates S100A4 (8). It may be possible to combine the peptide and niclosamide for synergistic antitumostatic action. The peptide, in its fluorophore-labeled version, can also be used for a high-throughput assay to screen for small molecule inhibitors, which may then be used for conjugation.

In conclusion, we have demonstrated that a helically stabilized NMIIA-derived peptide can specifically bind to S100A4 and inhibit cell motility. This opens up the possibility of developing peptide-based therapeutic agents targeting metastasis against this so far undrugged target.

Acknowledgment

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References