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Simultaneous Inhibition of Key Growth Pathways in Melanoma Cells and Tumor Regression by a Designed Bidentate Constrained Helical Peptide

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Abstract

Protein-protein interactions are part of a large number of signaling networks and potential targets for drug development. However, discovering molecules that can specifically inhibit such interactions is a major challenge. S100B, a calcium-regulated protein, plays a crucial role in the proliferation of melanoma cells through protein-protein interactions. In this article, we report the design and development of a bidentate conformationally constrained peptide against dimeric S100B based on a natural tight binding peptide, TRTK-12. The helical conformation of the peptide was constrained by substitution of α -amino isobutyric acid---an amino acid having high helical propensity---in positions which do not interact with S100B. A branched bidentate version of the peptide, bound to S100B tightly with a dissociation constant of 8 nM. When conjugated to a cell penetrating peptide, it caused growth inhibition and rapid apoptosis in melanoma cells. The molecule exerts anti-proliferative action through simultaneous inhibition of key growth pathways

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including reactivation of wild-type p53 and inhibition of Akt and STAT-3 phosphorylation. The apoptosis induced by the bidentate constrained helix is caused by direct migration of p53 to mitochondria. At moderate intravenous dose, the peptide completely inhibits melanoma growth in a mouse model without any significant observable toxicity. The specificity was shown by lack of ability of a double mutant peptide to cause tumor regression at the same dose level. The methodology described here for direct protein-protein interaction inhibition may be effective for rapid development of inhibitors against relatively weak protein-protein interactions for *de novo* drug development.

Introduction

The cancer genome project has demonstrated that many, if not all, tumors accumulate multiple mutations resulting in several dysregulated pathways favoring uncontrolled proliferation^{1,2}. These combinations of dysregulated pathways may be necessary to overcome the multiple tumor suppressor functions present in differentiated cells²⁻⁴. Thus, specific targeting of multiple dysregulated pathways, either through a single agent or through multiple agents may provide useful therapeutic advantage. Although small molecules are sometimes known to be protein-protein interaction inhibitors, rarely do they exhibit low off-target effects. Peptides may offer significant advantages here. Therapeutic peptides may be entering a promising phase with significant developments in overcoming the delivery problem⁵⁻⁷. Thus, developing peptide-based molecules targeting protein-protein interactions may be an important strategy for new class of protein-protein interaction inhibitors.

Secondary structure mimetics have been proposed as effective protein-protein interaction inhibitors⁸⁻¹⁰. Due to resemblance of the secondary structure mimetic to an extant protein, it may be superior to small molecules in causing less undesirable off-target effects. However, many protein-protein interactions, that are drug targets, are weak. Attaining high enough affinity for a secondary structure mimetic, where the parent protein-protein interaction is weak, remains a major challenge. In many situations, a low nanomolar dissociation constant of the receptor-drug complex is desirable or even mandatory¹¹. Since, many proteins are oligomeric in nature we propose that properly designed bidentate secondary structure mimetics (more than one secondary structure mimetics connected by a designed tether) may be a simple way to enhance affinity in such cases.

The S100 family of proteins has been implicated in growth of wide variety of tumors and other cellular processes¹², although their precise role is still unclear in many situations. Increased levels of S100B are observed in several tumors¹³ and have been suggested to contribute to tumor progression by interacting with and down-regulating p53, thus inhibiting its function as a tumor suppressor¹⁴⁻¹⁸. Recent work suggests that other pro-survival pathways may also be regulated by S100B¹⁹. Thus, inhibition of S100B may simultaneously regulate several key growth regulatory pathways and exert broad anti-tumor effect. Classes of melanomas and gliomas are prime examples of cancers where over-expression of S100B plays a crucial role in cancer development and progression²⁰. In this article, we have designed and developed a bidentate helically constrained peptide that

specifically and effectively blocked S100B. This results in altered regulation of more than one key growth pathways and causing anti-proliferative effects and rapid apoptosis. We also report *in vivo* efficacy of the peptide in a mouse model of melanoma.

Results and Discussion

Tumor suppressor protein p53 acts as a barrier to several steps in oncogenic transformation²¹. In several types of tumor cells disabling p53 function through lowering of the p53 protein level breaches this barrier. Reactivation of p53 in such cancer cells is generally acknowledged to be an important anti-cancer strategy²². Most effort towards reactivation has been directed towards inhibition of Mdm2, an ubiquitin ligase that plays a crucial role in p53 degradation²³. Much less effort has gone into inhibition of other proteins that are involved in p53 degradation. S100B, through its interaction with the C-terminal part of the p53, is involved in p53 degradation in certain types of cancer such as, melanoma and glioma. Knockdown experiments²⁴ and inhibition of this interaction by a small molecule, pentamidine²⁵, have demonstrated that S100B is an important drug target in these types of tumors²⁵. However, pentamidine is thought to inhibit other targets as well and is associated with serious toxicity²⁶. Peptides are generally more biocompatible and thus, we have designed a highly specific, tight binding peptide based inhibitor of S100B.

Synthesis and characterization of constrained peptides

S100B binds to the C-terminal regulatory region of p53 and help down-regulate its level^{27,28}. S100B has emerged as a drug target in these types of tumors²⁹. In an attempt to make a peptide based inhibitor of S100B-p53 interaction, a conformationally constrained helix was designed based on a S100B tight-binding peptide from CapZ, called TRTK-12 (TRTKIDWNKILS)³⁰. p53 and CapZ binding regions on S100B overlap partially. Thus, it is expected that a peptide modeled on CapZ should compete with p53 for S100B as well^{31,32}. As such TRTK-12 may not be suitable for *in vivo* applications as unstructured peptides are generally unstable *in vivo*. Constrained peptides are superior to unmodified peptides as they are generally more stable and longer lasting *in vivo*. In addition, small peptides generally do not have definite structures in solution and hence binding to the receptor involves sacrifice of entropy as the structure becomes more ordered upon binding. A constrained helix, thus, may have higher affinity when compared to its unconstrained counterpart and enhanced *in vivo* stability as was shown previously^{10,33}.

Previously, we have chosen substitution of a helix-promoting amino acid, α -amino-isobutyric acid (aib), for helix stabilization due to its straightforward incorporation into solid-phase peptide synthesis protocol and possibly better scaling up potential³⁴. The structure of the S100B-TRTK-12 complex indicates that the C-terminal half of the bound peptide is helical, while the N-terminal part is in an extended conformation (Figure 1A). In the helical section of the bound peptide, N8 and S12 residues (peptide nomenclature) are pointing away from the protein and not interacting with it. We, thus, decided to substitute these two residues with aib in the TRTK-12 sequence to derive the mono-aib-TRTK peptide (Table I)¹⁰.

Conformational preference of the mono-aib-TRTK was judged by NMR (2D-NOESY). 2D NOESY spectra show many NH-NH cross peaks suggesting presence of helical segments (Figure S1). The distance constraints obtained from 2D NOESY spectra along with dihedral angle constraints derived from J-coupling constants were used to derive the structural preference of the free peptide (Table SI). The derived structure from NOE and dihedral angle constraints shows the helical nature of the peptide (Figure 1B). The binding of mono-aib-TRTK to S100B was measured by a fluorescence anisotropy assay. In this assay, a fluorescein end-labeled peptide was titrated with increasing concentrations of S100B and the fluorescence anisotropy at each point was determined. The data were fitted to a binding equation assuming two identical binding sites on a dimer, thus yielding an average macroscopic dissociation constant³⁵. The mono-aib-TRTK binds to S100B with a K_d of $300 \pm 50 \times 10^{-9}$ M (Figure 1C).

In order to enhance the binding affinity of the peptide towards the dimeric S100B, a bidentate peptide was created in which two helically constrained monomeric peptides are linked (Figure 1D), either through two symmetry related cysteines or through α and ϵ amino groups of a lysine residue (Figure S2). In the NMR structure of the TRTK-12-S100B dimer complex (pdb1MWN), the distance between two symmetry related Ser12 residues of two TRTK-12 molecules is approximately 30 Å. A deep groove separates these two residues where a cross-linker can be sterically accommodated (Figure 1A). In order to span this distance the aib substituted TRTK-12 peptide was extended in the C-terminal direction by GGGCG sequence for cross-linking through the cysteine residues. Modeling shows that the distance between the two cysteines in the bound mono-aib-TRTK should be around 12–15 Å range. Thus, a cross-linker of suitable length was selected (bis-maleimido hexane) based on the above-mentioned modeled distance. This strategy has already been reported for a synthetic transcription factor³⁶. The cross-linked peptide was synthesized and purified as described in the methods. The bis-maleimido hexane cross-linked dimeric peptide (Cl-aib-TRTK) showed approximately an order of magnitude tighter binding than the monomeric counterpart (Table 1). However, the yield of cysteine cross-linked reaction is generally low and hence an attempt was made to create a bidentate peptide that can be synthesized entirely on solid phase. The branching sequence (K-Ahx-K-Ahx-K, between the two C-terminal aib residues) was chosen such that it should span a distance of about 30 Å. The synthesis of the branched peptide (Br-aib-TRTK; Figure S3) in solid phase gave much superior yield, thus making scale-up possible. A non-aib-substituted version of Br-aib-TRTK was also prepared (called Br-TRTK; which contains the parent TRTK-12 sequence TRTKIDWNKILS instead of the aib substituted sequence TRTKIDWBKILB in Br-aib-TRTK; see Table I; Table S2). Both Cl-aib-TRTK and Br-aib-TRTK have similar affinity towards S100B and were used. Full atomic structures of Cl-aib-TRTK and Br-aib-TRTK are given in Figure S2.

Helix stabilizing effect of aib substitution was judged by far UV circular dichroism spectroscopy (Figure 1E). Mean residue ellipticity value at 222 nm is a good indicator of helix content of proteins and peptides³⁷. Br-aib-TRTK shows enhanced mean residue ellipticity value (negative) at 222 nm when compared to that of the Br-TRTK, indicating enhancement of helix content upon aib substitution. On the other hand, Br-aib-TRTK shows decreased mean residue ellipticity value (negative) at 200 nm indicating a lower random coil component. Increase in helix content is expected to reduce the susceptibility of the peptides

for protease cleavage. This expectation is borne out as Br-aib-TRTK shows enhanced resistance against protease cleavage (Figure 1F).

Binding affinity and specificity of the bidentate constructs were judged by the fluorescence anisotropy assay described above. Binding isotherm of Br-aib-TRTK with S100B is shown in Figure 2A; when fitted to a single site binding equation, the binding isotherm yielded a dissociation constant of 8 ± 3 nM. The stoichiometry of the complex was determined by repeating the titration monitored by fluorescence anisotropy at peptide concentrations well above the dissociation constant. Under these conditions, the complex formation is linear until saturation of all the peptide. From that point onwards there is no further increase in anisotropy. The stoichiometry can be determined from the point of singularity. This yielded a stoichiometry of S100B dimer: Br-aib-TRTK as 1:1.1 (inset, Figure 2B) indicating that the bidentate ligand is binding to a single dimer. The selectivity of the bivalent peptide, Br-aib-TRTK was demonstrated by approximately 7-fold weaker binding of Br-aib-TRTK to S100P, the closest paralog of S100B (Figure S4)³⁸. We have also synthesized a mutant branched peptide (Br-aib-TRTK-AA) bearing two substitutions in the interacting residues (Table I). This peptide binds to S100B with a K_d of 135 ± 18 nM (Figure 2B). Br-TRTK peptide was also tested for binding. It binds to S100B with an approximately 2.4 fold higher K_d (2.4 fold weaker affinity) (Table I).

In order to determine the binding site of this bidentate peptide, HSQC of ^{15}N labeled S100B was carried out in the presence and absence of Br-aib-TRTK. Residues that show significant backbone chemical shift changes (greater than 150 Hz for a sum of ^1H and ^{15}N) were mapped on to the structure of the S100B-TRTK-12 structure (Figure 2C). Significant chemical shift changes occur in the region where TRTK-12 binds and in some cases also in residues that line the deep groove that separates the two TRTK-12 binding sites. These changes are consistent with binding of TRTK parts of Br-aib-TRTK in the TRTK-12 binding sites and the linker in the deep groove separating the two TRTK binding sites.

Effect of TRTK based peptides on cell proliferation and viability—Knockdown of S100B in many melanoma and other cancer cells have been studied before^{24,39,40}. Based on these studies, S100B has been established as a legitimate drug target. The specificity of Br-aib-TRTK in cellular assays was determined first and SK-Mel5 cell line (bearing a wild-type p53 allele) was chosen as a model for melanoma cells. For this purpose six D-arginine residues were added to N-termini of each peptide (Arg-Br-aib-TRTK and Arg-Br-aib-TRTK-AA); these peptides were added to SK-Mel-5 melanoma cells, bearing the wild-type p53 allele. Both L- and D-Arginine residues transduce peptides efficiently inside the cell⁴¹. D-Arginine residues were deemed more desirable as they are likely to be more protease resistant. The hexa-D-arginine conjugated version (Arg-Br-aib-TRTK) shows similar binding affinity to S100B as that of Br-aib-TRTK (Figure 2A; Table I). Confocal microscopic images with a fluorescein end-labeled Arg-Br-aib-TRTK showed that Arg-Br-aib-TRTK enters SK-Mel-5 cell within a short period of time after addition (Figure S5). Treatment of SK-Mel5 cells with Arg-Br-aib-TRTK causes it to quickly become round (in about 15 minutes). Visible signs of apoptosis, such as blebbing, were observable within 2 hrs (Figure 3A). Phalloidin staining indicated that reorganization of actin fibers was the underlying reason for the shape change (Figure S6) In order to find out if other melanoma

cells are also affected by the peptide, another human melanoma cell line bearing wild-type p53 (Malme-3M) was treated with Arg-Br-aib-TRTK. Figures S7 A and B show that this cell line also showed significant apoptotic effects upon addition of Arg-Br-aib-TRTK. Similarly, strong growth inhibition was seen with another human melanoma cell line C-81-46A (data not shown) suggesting that S100B is an important drug target in many types of melanomas.

Apoptotic nature of Arg-Br-aib-TRTK induced cell death was also confirmed by generation of DNA ladder, which is often characteristic of apoptotic cell death (Figure 3B). The apoptosis of SK-Mel-5 cells was quantified using flow cytometry. 49 % of cells were determined to be apoptotic and 11 % necrotic at 2 hrs using annexin V stain. Corresponding figures for 6 hrs were 57 % and 14 %, respectively (Figure 3C). Cell cycle assay also suggests a substantial fraction of apoptotic cells upon treatment with Arg-Br-aib-TRTK (Figure S8). A dose dependent change of percentage of apoptotic cells was observed in the flow cytometry assay (Figure S9). That the apoptosis induction was not due to the hexa-arginine sequence was demonstrated by using TAT (48–60) as a substitute cell penetrating peptide (CPP). The TAT conjugated Br-aib-TRTK was found to be equally effective in inducing apoptosis (Figure S7C). On the other hand, a peptide with an unrelated sequence with a hexa-D-arginine tag shows no effect (Arg-KYKETDL) (Figure S7D), demonstrating that the nature of the cell penetration tag has no effect on the biological efficacy. The S100B protein is secreted in many cell types and influences cell functions by binding to extra-cellular receptors such as RAGE⁴². To test whether the inhibition of the extra-cellular form of S100B plays any role in observed effects of Arg-Br-aib-TRTK, we have treated SK-Mel-5 cells with Br-aib-TRTK, which lacks the cell penetration tag. This molecule shows no apoptotic effect on SK-Mel-5 cell line (Figure S7E), suggesting that the effect of Arg-Br-aib-TRTK is due to specific inhibition of intracellular targets, likely S100B.

Whether the peptide predominantly binds S100B inside the cell was tested by a pull-down assay using biotin conjugated Br-aib-TRTK as a bait and trapping on an avidin column from SK-Mel-5 cell lysate. Coomassie blue stained SDS-PAGE gels of the pulled down fraction with biotin labeled Br-aib-TRTK showed pre-dominant presence of a band having molecular weight of S100B (data not shown). When western blot was performed on the same gel, the same band was pre-dominantly seen, indicating that S100B is the major cellular target of Arg-Br-aib-TRTK. A scrambled control peptide yielded no S100B band (Figure S10). MTT assay indicates that Arg-Br-aib-TRTK induces cell death at low micromolar concentrations with an IC₅₀ of about 5 μ M. Both the double mutant and the non-aib substituted peptides show a much reduced effect in this concentration range, suggesting that high affinity as well as helix stabilization to be important for its function (Figure 3D).

Reactivation of p53 is crucial for the anti-proliferative effect—Since, S100B plays a crucial role in down-regulation of wild-type p53 in many melanomas, it is expected that treatment with Arg-Br-aib-TRTK and consequent inhibition of p53 degradation will result in increase of p53 levels. Hence, the level of p53 was checked by western blotting after treatment of SK-Mel-5 cells with Arg-Br-aib-TRTK. The concentration of p53 reaches significantly higher levels within 2 hrs of treatment in SK-Mel-5 cells (Figure 4A). If this increased level of p53 plays a critical role in apoptosis induction, then a p53^{-/-} melanoma

cell line would be resistant to the effect of the peptide. We used a p53^{-/-} melanoma cell line UIISO-Mel-6 (Figure 4B) and found that Arg-Br-aib-TRTK has little apoptotic effect in the concentration range tested, suggesting a critical role played by the p53 in growth regulation and apoptosis induction upon treatment with Arg-Br-aib-TRTK. Arg-Br-aib-TRTK also shows reduced cell death in the SK-Mel-28 cell line bearing a mutant p53 allele compared to Sk-Mel-5 cells bearing wild-type p53 allele (Figure 4C), suggesting that wild-type p53 is essential for efficacy of the peptide.

Apoptosis induction by increased levels of p53 occurs by two major mechanisms: The slower canonical transcription dependent pathway⁴³ and the faster newly discovered transcription independent mitochondrial translocation dependent pathway. Given the rapidity of apoptosis initiation, we have examined whether the apoptosis was occurring through direct mitochondrial translocation pathway⁴⁴. The direct translocation of p53 to mitochondria was judged by co-incidence of fluorescence of tagged p53 antibody (green) and a mitochondria specific dye (red) using fluorescence confocal microscopy (Figure 4D). Extensive merging of red and green fluorescence upon Arg-Br-aib-TRTK treatment (causing yellow fluorescence) is indicative of p53 migration to mitochondria. In order to explore whether translocation of p53 to mitochondria is the principal cause of apoptosis, we have used a specific blocker of the direct mitochondrial pathway of apoptosis. It has been reported that a small molecule pifithrin- μ blocks this pathway without affecting the canonical transcription-dependent pathway of p53 initiated apoptosis while a close analog, pifithrin- α blocks the canonical transcription-dependent pathway without affecting the direct mitochondrial pathway⁴⁵. Figures 5A and B show the effect of pifithrin- μ on the apoptosis induced by Arg-Br-aib-TRTK. Interestingly in the presence of pifithrin- μ the cells change shape very quickly, but blebbings are not seen, whereas in its absence both shape change and blebbings are seen, indicating initiation of apoptosis. This was verified by flow cytometry (Figure 5B) in which virtually no apoptosis was seen upon treatment of Arg-Br-aib-TRTK in presence pifithrin- μ . Effect of pifithrin- α was measured by its ability to antagonize anti-proliferation activity of Arg-Br-aib-TRTK. No significant effect was observed, consistent with a lack of transcription dependent effects of p53 upon Arg-Br-aib-TRTK treatment (Figures 5C and S11). We have also measured upregulation of a p53 dependent gene GADD45, PUMA and p21 upon addition of Arg-Br-aib-TRTK (Figure 5D). At 2 and 6 hrs post-treatment, no significant upregulation of the genes were observed. In fact, there is some reduction in GADD45 RNA, the reason for this remains unclear. Clearly, the apoptotic effect caused by Arg-Br-aib-TRTK is independent of levels of expression of p53 dependent genes.

Inhibition of a second non-p53 dependent pathway of cell growth

A hint that another growth pathway is stimulated by over-expression of S100B in these tumor cells came from measurement of cell proliferation and mobility at concentrations of Arg-Br-aib-TRTK well below that of IC₅₀ of cell death as measured by the MTT assay. The effect of Arg-Br-aib-TRTK on proliferation of SK-Mel-5 cells was measured by [³H] thymidine incorporation (Figure 6A), which directly measures DNA synthesis, rather than the metabolic activity measured by the MTT assay. The measured IC₅₀ by this method was approximately 1.5 μ M (lower than that of cell death) indicating possibility of inhibition of a

growth pathway other than stabilization of p53. Specificity of this effect was tested by comparing the effect of a non-specific peptide and Arg-mono-aib-TRTK with that of the Arg-Br-aib-TRTK. The monomeric counterpart is clearly less efficacious than the branched counterpart and the non-specific peptide is completely ineffective (Figure 6B), suggesting that the increase of affinity of the bidentate peptide plays an important role in growth inhibition.

Cancer cells are not only characterized by uncontrolled proliferation but also increased cell motility and invasion. Recent work suggests that p53 controls these important aspects of tumor progression as well ⁴⁶. We have thus attempted to determine the effect of Arg-Br-aib-TRTK on cell motility, a vital component of tissue invasion by cancer cells. At concentrations in which Arg-Br-aib-TRTK does not cause rapid apoptosis in large number of cells, it causes remarkable inhibition of motility as shown in Figure 6C. This suggests that inhibition of S100B not only cause inhibition of cell proliferation and apoptosis, but also inhibition of cell motility and possibly consequent tissue invasion as well.

A recent article has suggested that S100B directly activates non-receptor Src kinase and consequently the PI-3-Kinase pathway ⁴⁷. In order to examine whether such a pathway is involved in growth inhibition observed here, we have examined some of the known phosphorylation targets of this and other related growth pathways, namely Akt, and ERK (Figure 6D). These targets are strongly inhibited within 0.5–2 hrs, suggesting profound inhibition of Src kinase and down regulation of several Src-kinase regulated pathways. Another key downstream target of the Src-kinase pathway that plays a central role in motility and proliferation is STAT-3. Phosphorylation of STAT-3 is also quickly suppressed upon addition of Arg-Br-aib-TRTK. The kinetics of inhibition of phosphorylation of STAT-3 and AKT in comparison with the kinetics of enhancement of p53 levels suggests two different pathways are influenced by S100B. Thus, inhibition of S100B likely resulted in inhibition of Src-kinase and key growth regulators, e.g. STAT-3, ERK, PI3 kinase pathway and importantly with simultaneous activation of p53. Each of these is already a legitimate anti-cancer targets with molecules in different phases of clinical trials ²³ (<http://clinicaltrials.gov/ct2/show/NCT00955812>, <http://clinicaltrials.gov/ct2/show/NCT00876109>).

To compare how inhibition of Mdm2 which also leads to reactivation of p53 compares with S100B inhibition that affects multiple pathways, we have measured the effect of nutlin, a known Mdm2 inhibitor, on SK-Mel-5 cells. Cell viability was assayed using the MTT assay. Nutlin caused much slower apoptotic cell death and at higher concentrations (Figures S12). We have also compared GADD45 transcript levels in nutlin and Arg-Br-aib-TRTK treated SK-Mel5 cells. The slower acting nutlin increases the levels of GADD45 transcript levels at 24 and 72 hours, whereas the Arg-Br-aib-TRTK shows no increase (time window within which apoptosis happens; Figure S13). Even though inhibition of both targets leads to stabilization of p53, the cell death kinetics and gene expression pattern are completely different, suggesting a different mechanism of the anti-proliferative effect. One possible mechanism is that nutlin acts through the nuclear pool of p53 and transcription dependent pathway, whereas Arg-Br-aib-TRTK acts through mitochondrial translocation. Thus, targeting of S100B may be a superior strategy for therapy in melanomas.

The Branched aib substituted TRTK peptide strongly inhibits tumor growth in mouse model—

The effect of Arg-Br-aib-TRTK was tested in a syngenic mouse model of melanoma. Initially, the peptide was tested for immunogenicity in the mouse model. Figure S14 shows the anti-IGg response after secondary immunization. Clearly, the peptide does not elicit much antibody response and thus should be suitable for repeated injections. B16F10 mouse melanoma cells were implanted in syngenic host. B16F10 cells were efficiently killed in cell culture by Arg-Br-aib-TRTK (Figure S15). Arg-Br-aib-TRTK was found to be non-lethal and show no demonstrable toxicity upto a tested concentration of 50 mg/Kg. Histopathology of some key target organs after 8 days of treatment was normal, indicating no significant toxicity (Figure S16). At two dose levels (50 mg/kg and 25 mg/kg) the tumor growth was completely inhibited up to 8 days (maximum tested) (Figure 7A). In contrast, at the higher dose level, the double mutant peptide Arg-Br-aib-TRTK-AA shows very little tumor growth inhibition (Figure 7B). Histopathology of tumor sections, when compared to untreated tumors, show large areas devoid of live cells (Figures 7C). The tissue sections treated with Arg-Br-aib-TRTK-AA look very similar to the untreated ones (Figure 7D). Immuno-histochemistry of growth marker PCNA indicates almost complete loss of this antigen in the residual tumor mass upon treatment, indicating almost complete loss of proliferating cells (Figure 7E). The tumor sections after only one treatment of Arg-Br-aib-TRTK was stained for p53, which shows significant enhancement in contrast to a control tumor section (Figure 7F). In order to test the final tumor tissue dose, the tissue distribution of the peptide upon tail vein injection was measured with ^{99m}Tc labeled Arg-Br-aib-TRTK. Tumor received less than 5% of the injected dose (Figure S17). Although, this dose may be sufficient to see biological efficacy, further reduction of dose may be obtained with a better delivery system.

Two central issues in development of new chemical entity against cancer today are that of specificity and potential of resistance development. A combination of dysregulated pathways is the cause of uncontrolled proliferation; specific inhibition of one or more of these pathways could be important for new generation of cancer therapy. Imatinib is a classical example of anti-cancer action through specific inhibition of a dysregulated pathway. There appears to be many targets involving protein-protein interactions in such kind of dysregulated pathways. However, protein-protein interactions are generally less prone to small molecule inhibition due to exposed and polar nature of the interface. Nature mimetic peptides may have distinct advantage over small molecules in this aspects, provided stability, delivery and scalability of synthesis can be addressed.

One of the mechanisms of resistance development in cancer chemotherapy involves acquiring mutations which weakens drug-receptor interaction without altering the native function⁴⁸. T315I mutation in Bcr-Abl kinase is an example of this. Nature mimetic peptides may also offer significant advantage in this respect. Protein-protein interactions have evolved by optimizing specific and non-specific interactions. Thus, a protein/peptide mimic having minimal changes from the parent interacting proteins and which closely resembles a natural protein or a part of a protein, has better chance of attaining native-like interactions than a small molecule. In case of peptide mimetics, which have very similar interactions as that of the natural partner, we hypothesize that mutations having detrimental

effect on the drug-target interaction without affecting the native function will likely be rare. Translation of designed peptides to practical use requires addressing other hurdles including easy scalability of synthesis. The total solid phase synthesis of branched bidentate molecules offers potential scalability. Previous reports with stapled α -helix mimetics and broader nature of targets reported here, make a strong case that this class of molecules may occupy an important niche in the future drug discovery field in the area of cancer therapeutics.

Methods

Synthesis of branched dimers and purification

Details of the monomeric peptide synthesis is given in Mazumder *et al*³⁶. The two peptides, bound to two subunits, are oriented in approximately head-to-head fashion. The distance between two C-termini is approximately 30 Å. The branching was designed in a manner to keep this distance between the two peptides. The wild type branched molecules and its hexa-arginine tagged variety were synthesized on a PS3 Protein Technologies peptide synthesizer or Prelude peptide synthesizer (protein Technologies International) at 0.3 mmol scale by using a solid-phase peptide synthesis strategy using 9-fluorenylmethoxy carbonyl chemistry and Rink amide PEGA resin. The branching of the peptide starts from α -NH₂ as well as ϵ -NH₂ group of (fmoc)Lys(Fmoc) and the linker 6 -Aminohexanoic acid to maintain the distance 30 Å. All the protected amino acids were purchased from Novabiochem. Aspartic acid and threonine had tert-butyl, tryptophan had tert-butyl carbamate and Arginine had pentamethyl-dihydrobenzofuran as the side-chain protecting group. N-terminal Fmoc-protected amino acids were coupled in the COOH form using O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium tetrafluoroborate (TBTU) as the activating agent with N-hydroxybenzotriazole (HOBT) and N,N-diisopropyl ethylamine (DIPEA) as a base. For each coupling the ratio of amino acid, TBTU, HOBT and DIPEA is maintained 8:1:1:2 (in molar equivalents) with respect to the resin bound amino acid and coupling is continued for 45 minutes. After each coupling, the residual uncoupled amino acids in the growing chain were capped by acetylation using acetic anhydride, Lutidine in dry DMF in the ratio 2:3:50 followed by repeated washing in dry DMF. Peptides were cleaved from the resin, and side-chain protecting groups were removed by incubating in reagent K (trifluoroacetic acid: phenol: thioanisole: H₂O: ethanedithiol, 82.5:5:5:5:2.5) for 3 h at room temperature. The peptides were purified by reverse phase HPLC on micro-bondapac C-18 (Water Associates) column using 0–30% acetonitrile (in 0.1 TFA) gradient over 30 minute. The synthesized peptides were characterized by NMR and ESI mass spectrometry or MALDI-Mass spectrometry in positive ion mode.

Labeling of Peptides—All peptides were labeled with 5, 6-carboxy fluorescein at the N-termini in solid phase. Dry resin bound N-terminal deprotected peptide was reacted with 10 times molar excess of 5(6)-carboxy fluorescein and HBTU (1:1) in dry DMF for 4 hours in dark at 25°C. The resin was washed thoroughly with 20% piperidine in DMF, DMF and diethyl ether and dried under nitrogen atmosphere. For biotin labeling, a linker (6-aminohexanoic acid) was first coupled and then labeled with EZ-Link NHS-LC-LC-Biotin (Thermo Scientific) in solid phase.

Spectroscopy—For direct titration, 50–100 nM fluorescein-labeled dimeric peptidomimetics were titrated with S100B, and the anisotropy was determined at each point. The excitation wavelength was 490 nm, and the emission wavelength was 520 nm. The excitation and emission band passes were 10 and 10 nm, respectively. Anisotropies were fitted to the following equation:

$$A_3 + (A_1 - A_3) * ((A_2 + X + A_4) - \text{SQRT}((A_2 + X + A_4) * (A_2 + X + A_4) - 4 * X * A_4)) / (2 * A_4)$$

Where A_1 is the final anisotropy, A_3 is the initial anisotropy, A_2 is the dissociation constant and A_4 is the peptide concentration. Reactions were carried out in 50 mM Tris-buffer, pH 7.4 containing 10 mM CaCl_2 , 50 mM NaCl and 1 mM DTT.

NMR spectroscopy: NMR spectra for mono-aib-TRTK were done on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe at 500 μM . Structure calculation using DYANA was based on NOESY mixing times of 200 and 400 ms. J values were obtained directly from the resolved 1 dimensional spectra. For HSQC NMR spectra were collected at 37°C with a Bruker Avance III950 MHz NMR spectrometer (950.24 MHz for protons) equipped with four frequency channels and a triple-resonance z-axis gradient 5 mm TCI cryoprobe. All proton chemical shifts are reported with respect to the H_2O or HDO signal taken to be 4.658 ppm relative to external TSP (0.0 ppm) at 37°C. The ^{15}N chemical shifts were indirectly referenced using the following ratios of the zero-point frequencies at 37°C: 0.10132905 for $^{15}\text{N}^{49-51}$. Uniformly ^{15}N -labeled rat S100B was used to collect the 2D ^1H , ^{15}N -fast HSQC 52 during the titration of peptides into Ca^{2+} -S100B and changes in S100B backbone ^{15}N and ^1H were monitored and readily assigned. The sequence specific assignment of ^1H , ^{13}C , and ^{15}N resonances in Ca^{2+} -bound S100B was determined previously using standard heteronuclear multidimensional NMR experiments. For calcium-bound S100B, the 3D ^{15}N -edited clean-HOHAHA-HSQC, the 3D ^{13}C , ^{15}N -edited HNCA, HN(CO)CA, CBCA(CO)NH and HNCACB, and the 3D HCCH-TOCSY data for through-bond correlations along the backbone and sidechains. These assignments were confirmed by the observation of characteristic through-space correlations in the NOESY experiments, particularly in regions of regular secondary structure, as previously described 53 . Upon binding the peptide, correlations in the HSQC experiment and subsequent chemical shift perturbations were followed after subsequent additions of the peptide, and the ^1H and ^{15}N assignments in the S100B-peptide complex were confirmed using ^{15}N -edited NOESY-HSQC and ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC data, as done previously with a peptide derived from the C-terminus of p53 54 . NMR samples contained 0.25 mM S100B (monomer), 10 mM HEPES, pH 7.2, 15 mM NaCl, 10 mM CaCl_2 , 0.34 mM NaN_3 , 0.2 mM TPEN, and 10% D_2O either with or without 0.5 mM branch TRTK peptide. The combined ^1H and ^{15}N chemical shift perturbations is the change in chemical shifts that occurs upon peptide binding for ^1H and ^{15}N in ppm multiplied by 950.24 and 96.287, respectively, and then adding absolute values for ^1H and ^{15}N together.

Cell culture and treatments—Cells were grown in Dulbecco's modified Eagle's media (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37°C in a humidified incubator with 5% CO_2 . Human melanoma UIISO-MEL-6

(p53 $-/-$) were cultured in minimal essential medium with Earle's salts and 10% heat-inactivated FBS. For experimental studies, cells were grown to 80–90% confluence, harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline (PBS) and plated at desired density and allowed to re-equilibrate for 24 h before any treatment. All experiments were conducted in DMEM containing 10% FBS and 1% PSN antibiotic.

Pull down assay—SK-Mel-5 cells were lysed with 1 x cell lysate buffer (Cell Signaling Technology) according to the manufacturer's protocol. 1 mg of lysate was incubated with either biotinylated Arg-Br-aib-TRTK (10 μ M) or biotinylated scrambled peptide (10 μ M) in binding buffer (20 mM Tris, 150 mM NaCl, 5 mM CaCl₂, 0.1% Triton-X100, pH 7.5) at 4°C overnight. The reaction mixtures were then incubated with pre-equilibrated immobilized avidin monomeric agarose beads (Pierce) at 4°C for 2 h. The beads were washed thrice with binding buffer and run on 15% SDS-PAGE gels followed by immunoblotting with polyclonal rabbit anti-S100 antibody (Dako, Denmark).

Laser scanning confocal microscopy—SK-Mel 5 cells (2×10^3) cells were seeded in LAB-TEK chamber microscope slides (Nalge Nunc, International, Naperville, IL) in 400 μ l culture medium. Twenty four hours after seeding, cells were treated with 10 μ M Arg-Br-aib-TRTK for 1 h and 2 hrs and then incubated with 100 nM Mito Tracker Red CMXRos (Molecular Probes) in culture mediums for 30 minutes. After washing thrice with PBS, the cells were fixed with 3.7% (w/v) paraformaldehyde for 15 min, permeabilized with 0.1% (v/v) Triton X-100 for 15 min at room temperature. After blocking with 1.5% BSA in PBS, cells were incubated with the primary antibody against p53 (DO1, Santa Cruz biotechnology, Inc.) for 1h. Cells were then washed with blocking solution 3 times for 1h. Slides were mounted by using DAPI containing Ultra cruz mounting medium (Santa Cruz Biotechnology, Inc.). Images were independently captured by Nikon A1R Confocal Laser Scanning microscope (1.4 NA 60X objective) with the 488 and 543 lines of the krypton/argon laser used for the excitation of FITC and Mitotracker red, respectively and excitation of DAPI was by 404 laser. Fluorescence signals were analyzed by using Nikon NIS elements software.

Animal Studies

Experiments were carried out under protocols approved by the Institutional Animal Ethics Committee and institutional guidelines for the proper use of animals in research were followed. National guidelines had been followed. C57BL/6J female mice (mean initial weight, 20g) were kept in one mice in one cage at 19°C to 23°C with a 12 hour light/12 hour dark cycle. They had free access to water and food. To generate tumor, 1×10^6 B16F10 cells were mixed with Matrigel (BD bioscience) and inoculated S.C. in the right flank of 6 week-old mice. Tumors (typically 2 mm in diameter) were palpable 5 days after subcutaneous injection. Peptide treatments (50mg/kg body weight) were administered by intravenous tail vein injection on the day tumors became apparent (day 0) and then days 1, 2, 3, 4, 5, 6 and 7. Control mice were treated only with saline vehicle. Mice were weighted, and tumor volumes were calculated with the following formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structure of S100B-TRTK peptide complex. The magenta and the yellow chains are the two TRTK peptides, the cyan and the green chains are the two subunits of S100B. (B) NMR derived structure of mono-aib-TRTK. (C) Binding isotherm of mono-aib-TRTK with S100B. (D) A schematic view of the structure of Branched TRTK peptides. (E) Circular Dichroism spectra of Br-aib-TRTK (blue) and Br-TRTK (red). (F) Protease cleavage experiment with Br-aib-TRTK (open circles) and Br-TRTK (solid circles). Details of Protease cleavage experiments are given in Materials and Methods.

Figure 2.

(A) Binding isotherm of Br-aib-TRTK (red circles) and Arg-Br-aib-TRTK (blue triangles) with S-100B. (B) Binding isotherm of Br-aib-TRTK-AA (C) with S100B. The inset shows the stoichiometric titration of Br-aib-TRTK with S100B. The each point is an average of at least three independent experiments. The lines represent best fit to a single site binding equation which is given in the Materials and Methods. (C) Ribbon diagram of Ca²⁺-loaded S100B illustrating residues that exhibit chemical shift differences upon Br-aib-TRTK binding. Amino acid residues of S100B are shown in green and residues exhibiting chemical shift changes upon Br-aib-TRTK binding colored in salmon pink.

Figure 3.

(A) Morphology of SK-Mel-5 cells as a function of time upon treatment with Arg-Br-aib-TRTK. (B) Arg-Br-aib-TRTK induced DNA laddering (indication of apoptotic cell death) in SK-Mel-5 cells. SK-Mel-5 cells were treated with Arg-Br-aib-TRTK (10 μ M) for 2 & 6 hours. Isolated genomic DNA samples were separated on a 2% agarose gel. (C) Flow cytometry of SK-Mel-5 cells before and after treatment with 10 μ M Arg-Cl-aib-TRTK, using Annexin V and Propidium Iodide (lower panel) and forward/side scatter (upper panel). (D) MTT assay of cell viability of SK-Mel-5 cells after treatment with Arg-Br-aib-TRTK (black circles) and Arg-Br-aib-TRTK-AA (red Circles) and Arg-Br-TRTK (blue circles).

Figure 4.

(A) Western Blots for p53 in the cell extract of SK-Mel-5 after treatment with 10 μM of Arg-Br-aib-TRTK. (B) MTT assay of cell viability of UIO-Mel-6 ($p53^{-/-}$) cells after treatment with Arg-Br-aib-TRTK (open circles) and Arg-Br-aib-TRTK-AA (solid circles). (C) MTT assay of cell viability of SK-Mel-28 ($p53^{\text{mut}}$) cells after treatment with Arg-Br-aib-TRTK. (D) Confocal microscopic images of merged p53 (green) and mitotracker (red), DAPI (blue), 1 h after treatment with Arg-Br-aib-TRTK.

Figure 5.

(A) Phase contrast microscopy of SK-Mel-5 cells (Upper panel) 10 μ M Arg-Cl-aib-TRTK (Lower panel) 10 μ M Arg-Cl-aib-TRTK, pre-treated with 10 μ M pifithrin- μ , 1 hr after treatment. (B) Flow cytometry of SK-Mel-5 cells after treatment with 10 μ M Arg-Br-aib-TRTK in presence of 10 μ M pifithrin- μ , using Annexin V and Propidium Iodide. (C) Assay of cell viability using incorporation of 3 H-thymidine after treatment with 10 μ M pifithrin- α (green), 20 μ M pifithrin- α (red) and no pifithrin- α (blue). (D) Expression level of some known p53 dependent genes upon treatment with 10 μ M Arg-Br-aib-TRTK.

Figure 6.

(A) Assay of cell viability using incorporation of ^3H -thymidine after treatment with different concentrations of Arg-Br-aib-TRTK for 18 hrs. (B) Assay of cell viability using incorporation of ^3H -thymidine after treatment with different concentrations of (solid circles) Arg-Br-aib-TRTK, (open circles) Arg-mono-aib-TRTK and (open square) a non-specific peptide DR6-KYKETDL for 18 hrs. (C) Wound healing assay with SK-Mel-5 cells. The cells were treated with 2 μ M Arg-Br-aib-TRTK and monitored for 2 h. The pictures show the position after 20 h; left, untreated; right treated with 2 μ M Arg-Br-aib-TRTK. (D) Western Blots of SK-Mel-5 cell extract for phospho-Stat3, phospho-Akt and phospho-ERK after the designated times of treating with 10 μ M of Arg-Br-aib-TRTK.

Figure 7.

(A) Kinetics of implanted tumor (B16F10 melanoma cell) growth in syngenic mouse model when treated with 50 mg/kg i.v. Arg-Br-aib-TRTK (green circles), 25 mg/kg (blue squares) or vehicle control (red circles). (B) Kinetics of implanted tumor (B16F10 melanoma cell) growth in syngenic mouse model when treated with 50 mg/kg i.v. Arg-Br-aib-TRTK-AA (solid circles) or vehicle control (open circles). (C) Histopathology of tumor section after sacrifice of the animals after treatment with Arg-Br-aib-TRTK (upper panel) or vehicle control (lower panel). (D) Histopathology of tumor section after sacrifice of the animals after treatment with Arg-Br-aib-TRTK-AA. (E) Antibody staining for PCNA in tumor sections after treatment with Arg-Br-aib-TRTK (lower panel) or vehicle control (upper panel). (F) p53 staining of the tumor sections after treatment with 1 dose of 50 mg/Kg of Arg-Br-aib-TRTK (left panel) or vehicle control (right panel).

Table I

Peptide Name	Sequence	Relative Affinity	Dissociation Constant (nM)
Mono-aib-TRTK	TRTKIDW <u>B</u> KIL <u>B</u>	1	300±50
Cl-aib-TRTK	TRTKIDW <u>B</u> KIL <u>B</u> GGGCG TRTKIDW <u>B</u> KIL <u>B</u> GGGCG	10	30±9
Br-aib-TRTK	TRTKIDWBKILBKAhx K TRTKIDWBKILBKAhx	37.5	8±3
Br-aib-TRTK-AA	TRTKADABKILBKAhx K TRTKADABKILBKAhx	2.2	135±18
Arg-Br-aib-TRTK	RRRRRRTRTKIDWBKILB KAhx K RRRRRRTRTKIDWBKILB KAhx	27.2	7.7±2.5
Br-TRTK	TRTKIDWNKILSKAhx K TRTKIDWNKILSKAhx	15.6	

Higher numbers indicate tighter binding. The codes used are *mono* for monomer, B for aib, Cl for Cross-linked through cysteine, Br for branched, aib for presence of α-amino isobutyric acid, Ahx is 6-aminohexanoic acid and the last part of the name shows the origin of the sequence.