A Search for Inhibitors of S100B, a Member of the S100 Family of Calcium-Binding Proteins

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Abstract: Typically, malignant melanoma has wild-type p53, and yet this cancer proliferates. S100B, which binds p53 and is up-regulated in melanoma, down-regulates wild-type p53 tumor suppressor function. Inhibitors of the S100B-p53 interaction were identified using computer aided drug design (CADD) combined with NMR methodologies and represent potentially new chemotherapeutics for melanoma.

Key Words: S100B, p53, calcium, inhibitors, drug design, cancer.

INTRODUCTION

The S100 Protein Family and Cancer

There are now several members of the S100 family of EF-hand Ca^{2+}-binding proteins (Fig. **1**) that are found in mammals [1-3]. S100 proteins were named because they are soluble in 100 percent saturated ammonium sulfate [4]. S100B is a 21.5 kDa symmetric highly conserved (> 97% identity) homodimer among mammals [1, 4]. Low levels of S100B generally have trophic effects, while uncontrolled cell growth results from higher levels [5-8]. Problematic is that elevated levels of S100B are found in malignant melanoma [9-12], renal cell tumors [13] and malignant mature T-cells (such as doubly negative $CD4^-$ /$CD8^-$ adult T-cells in leukemia patients) [14]. Furthermore, S100B is up-regulated by cytokines that stimulate gliosis such as interleukin-1β and the basic fibroblast growth factor [15]. As is the case for S100B, a number of other S100 proteins are regulated in a tissue-specific manner [16]. S100A1, calcyclin (S100A6), and S100B are elevated significantly in metastatic human mammary epithelial cells [17], and increased levels of mts1 in transgenic mice induce metastatic mammary tumors [18]. For mts1 (S100A4), protein levels are controlled in benign cell lines *via* a *cis*-acting element 1300 base pairs upstream of the rat mts1 start site [18], and expression of antisense RNA to mts1 suppresses metastatic potential for a high-metastatic Lewis lung carcinoma [19]. Protein levels of S100B correlate with malignant melanoma, so it is used as markers for this cancer [20-23]. In general, S100 antibodies are used clinically to identify and classify cancer in several tissues and cell types including brain, bladder, breast, cervix, head and neck, intestine, kidney, larynx, lung, lymph, mouth, skin, and testes among others [13, 14, 17, 24-38]. More recently, S100B was shown to not only be a prognostic marker, but rather that it contributes to cancer progression in malignant melanoma by interacting with the transcription activating protein, p53, and inhibiting its function as a tumor suppressor [39-41].

The Tumor Suppressor Protein, p53

p53 is a transcription activator that signals for the up-regulation of genes involved in cell cycle arrest and apoptosis [42, 43] and plays a pivotal role in the maintenance and regulation of normal cellular functions. Its inactivation affects cell cycle checkpoints, apoptosis, gene amplification, centrosome duplication and ploidy [43-50]. If p53 is mutated, as found in over 50% of human cancers, the cell cycle proceeds unregulated and cell growth accelerates. In such conditions, apoptosis pathways are also not induced, and proliferating cells transform into cancerous ones [44, 51]. However, if p53 levels are too high, then phenotypes associated with aging occur such as problems with skin and bone [52]. As one might expect, p53 is highly regulated by post-translational modifications including those involving interactions with other proteins (i.e. S100B, mdm2, etc.) to regulate its protein level and activity [46, 53-56].

The DNA-binding domain of p53 has a Zn^{2+}-binding site and contains two antiparallel β-sheets that serve as a scaffold for a loop-sheet-helix DNA binding domain [57]. Upon binding specific DNA sequences, the p53 tumor suppressor activates the transcription of numerous downstream targets including a cyclin-dependent kinase inhibitor ($p21^{WAF/CIP1}$), cell cycle control proteins (cyclin G, GADD45), genes involved in apoptosis (i.e. Bax), and a protein, mdm2 (or hdm2 for humans), which in turn negatively regulates p53 protein levels inside the cell as part of a feedback loop to keep p53 protein levels in check [42, 43, 58]. Mdm2 down-regulates p53 by an ubiquitin-mediated pathway and is dependent on the phosphorylation state of p53 in the N-terminal transactivation domain. The 3D structure of the N-terminal domain of mdm2 somewhat resembles EF-hand proteins, despite the fact that it does not bind calcium [59]. In its most active form, p53 is a tetramer held in the C-terminal region as a dimer of dimers to form an X-type four-helix bundle [60-62]. It is the tetramer domain of p53 that interacts most tightly with S100B (K_D=24±10 nM), and it's the binding of S100B

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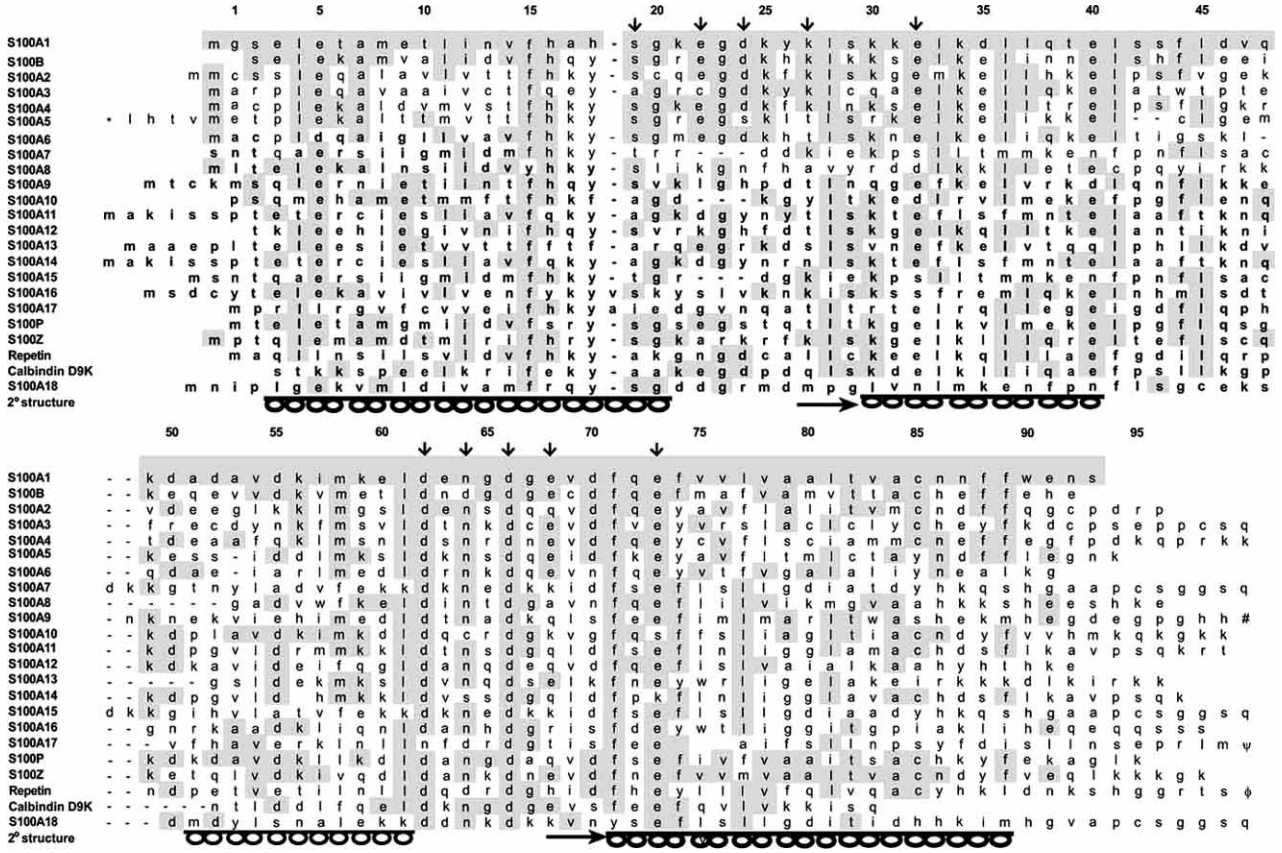

Fig. (1). Alignment of the amino acid sequence for members of the S100 family of calcium binding proteins. Human S100 proteins are aligned based on the sequence of S100A1. Residues involved in coordinating calcium in the S100-type EF-hand at the N-terminus and the typical EF-hand in the C-terminus of the proteins are indicated by downward arrows. Shaded squares indicate amino acid homology, and the secondary structure of one member, S100A1, is indicated at the bottom. The protein labeled S100A18 is newly discovered and under the gene accession code CAI19501. Republished in black and white with permission of Elsevier Publishers from "The Three-dimensional Solution Structure of Ca^{2+}-bound S100A1 as Determined by NMR Spectroscopy", Wright *et al*., 2005, 353, 410-426.

to this domain that is responsible for dissociating the p53 tetramer [63-65]. Directly C-terminal to the tetramer domain is a basic region termed the "extreme C-terminus" or the "C-terminal negative regulatory domain" of p53, which is unstructured when free in solution, but is helical when bound to the calcium-binding protein S100B [66] (Fig. **2**). If the negative regulatory domain is unmodified, p53 has lowered transcription activation activity (i.e. latent state). In fact, deletion of the last 30 residues, covalent modification, and/or binding of antibodies to this domain activates p53 by preventing non-specific DNA binding and/or perhaps by inhibiting S100B binding [67, 68]. Although, it is really the tetramer domain of p53 that is most important for the S100B-p53 interaction since this domain and full-length wild-type p53 bind to S100B more tightly than the extreme C-terminus [64, 69].

The S100B-p53 Interaction

The first evidence of an S100-p53 interaction *in vivo* was provided by co-immunoprecipitation experiments with S100B done in human primary malignant melanoma cancer cells [70]. Similar co-IPs experiments were also demonstrated for the S100A4-p53 interaction [39]. While most proteins that bind or modify the C-terminus of p53 activate the tumor suppressor, the opposite effect was observed for both S100A4 and S100B; in both cases, DNA binding to p53 in gel shift assays is decreased (Fig. **3**), and correspondingly, p53 function as a transcription activator is decreased when bound to S100 protein [39, 40]. In the case of S100A4 (mts1), the S100-dependent effect on p53 transcription activation varied for some genes and showed a time and cell-density dependence [39]. For S100B, p53 protein levels and downstream effector genes, hdm2 and p21 were also measured after human large-cell lung carcinoma cells (H1299; p53 -/-) were transiently co-transfected with p53 and S100B expression vectors [40, 71]. As expected, expression of p53 triggered expression of mdm2 and p21, but co-expression with the S100B protein markedly reduced the accumulation of p53 (>100-fold), mdm2, and p21 protein levels [40]. Similarly, endogenous wild-type p53 in human breast cancer cell line MCF-7 (p53 +/+) [72] was inhibited when transiently transfected with S100B [40]. These data indicate that the basal levels of p53, mdm2 and p21 can be induced by exposure to bleomycin, while over-expression of S100B protein reduces p53 levels, and blocked mdm2 and p21 accumulation again showing that S100B lowers the endogenous expression of p53 downstream effector genes.

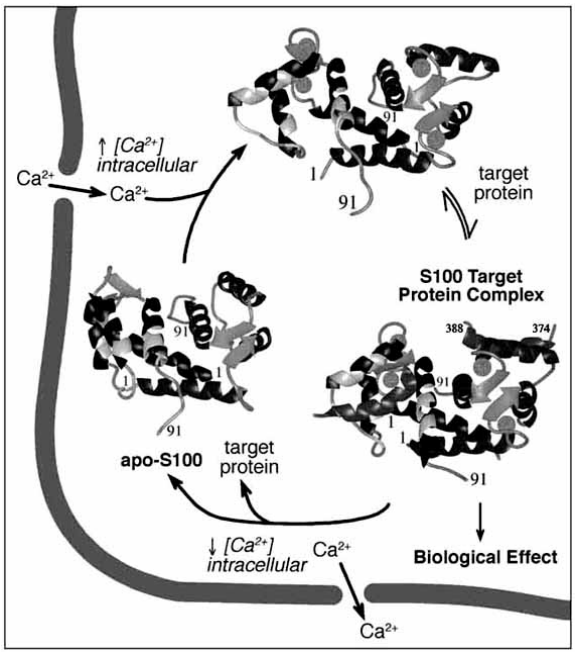

Fig. (2). The Ca^{2+}-dependent interaction of S100B with the tumor suppressor, p53. Dimeric S100B with regions shaded (light gray) for residues that interact with the negative regulatory domain (residues 367-388) of the tumor suppressor protein, p53. Republished in black and white with permission of The American Chemical Society from "Identification and characterization of small molecule inhibitors of the calcium-dependent S100B-p53 tumor suppressor interaction", Markowitz *et al*., 2004, 47, 5085-5093.

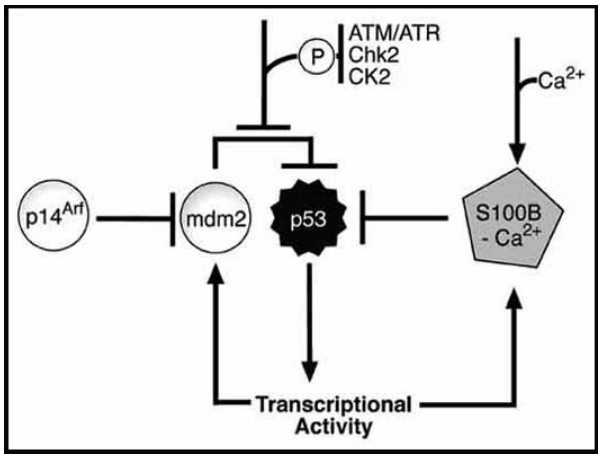

Fig. (3). Scheme for the down-regulation of wild-type p53. p53 is activated upon DNA damage or under stress and up regulates the transcription of genes involved in apoptosis (i.e. ↑Bax etc.) and cell cycle-dependent growth arrest (i.e. ↑p21 etc.). As part of a feedback control mechanism, p53 also up regulates the transcription of genes involved in its own inactivation (i.e. hdm2 and S100B). As part of a cell growth response (↑Ca^{2+}), the Ca^{2+}-dependent interaction between S100B and p53 induces a conformational change in p53 and tetramer dissociation of the tumor suppressor [63], which likely contributes to its degradation (i.e. perhaps involving hdm2/ubiquitin- and/or protease-dependent pathways). Thus, down-regulation of p53 by S100B and hdm2 ultimately facilitates cell growth. Too much S100B, as found in melanoma, leads to cell proliferation. Republished with permission of The American Society for Biochemistry and Molecular Biology, from "Inhibiting S100B restores p53 levels in primary malignant melanoma", Lin *et al*., (2004) 279, 34071-34077; permission was conveyed through The Copyright Clearance Center, Inc.

As a proof of principle for rational drug design directed towards S100B, small interfering S100B antisense RNA ($siRNA^{S100B}$) was found to restore wild-type p53 levels and function in primary malignant melanoma [70]. In this experiment, $siRNA^{S100B}$ inhibited S100B production in melanoma (3-fold) and wild-type p53 levels were restored by nearly the same factor (2.3-fold). Importantly, the restored p53 protein is functional because downstream transcription activation targets of p53, namely p21, hdm2, and bcl2 were all elevated when $siRNA^{S100B}$ was introduced. Therefore, small molecule inhibitors designed to inhibit S100B function may positively promote the activity of wild type p53 in an analogous manner to $siRNA^{S100B}$. Inhibiting elevated levels S100B represents a new therapeutic strategy for the treatment of malignant melanoma and other cancers with elevated S100B.

Feedback Regulation of p53

It is well-established that p53 activates the transcription of mdm2, a protein that is involved in ubiquitin-dependent degradation of p53 itself as part of a feedback loop [48, 58, 73]. In an analogous situation to mdm2, p53 also activates the transcription of S100 proteins, including S100B [70, 74]. The S100B promoter has six relatively equally spaced sequences corresponding to the consensus sequence for p53 binding (≥16/20 nucleotide match), and one region within the promoter region that matches the p53-binding consensus sequence perfectly (20/20 nucleotide match). Using DNA band shift binding assays, it was demonstrated that the S100B promoter constructs containing the 20/20 matching sequence have the highest DNA binding affinity to p53 [40]. Furthermore, p53 activates S100B transcription in reporter gene assays (i.e. CAT assays) containing various constructs of the S100B promoter [70].

It is now clear that the p53-dependent activation of the S100B promoter is also itself negatively regulated. This conclusion was first suspected from a comparison of the transcription activity of the full-length S100B promoter to promoter constructs from a gene involved in cell-cycle control such as GADD45. While none of the GADD45 sites have a 20/20 matching sequence, this promoter is more highly activated by p53 than the S100B promoter. Furthermore, removing portions of the S100B promoter increases its p53-dependent transcription activation, which is consistent with the S100B promoter being negatively regulated when the full-length promoter is intact. However, if the 20/20 p53 consensus region of the S100B promoter is removed, then, not surprisingly, the p53-dependent transcription activation activity was diminished significantly [70]. Negative regulation of the p53 region of the S100B promoter is logical because genes involved in p53 function ought to be activated prior to genes such as S100B and hdm2, which ultimately negatively regulate p53. In summary, S100B is now the sec-

ond protein that is both (i) activated at the transcriptional level by p53 and (ii) then subsequently inhibits p53 function *via* feedback control (Fig. **3**). The important distinction between these proteins (mdm2 *vs*. S100B) is that the S100B interaction with the p53 is Ca^{2+}-dependent and links p53 biology to extracellular growth responses (Fig. **2**); whereas, mdm2 binds the N-terminus of p53 and does not depend on calcium for binding p53 [75].

At first, the implications of this feedback loop seem to be troublesome to a drug-design program to restore p53 activity since elevated levels of p53 does, in turn, up-regulate S100B in any surviving primary malignant melanoma cells [76] (<5%). Nonetheless, the S100B promoter is relatively weakly activated by p53 as compared to other p53-activated promoters such as GADD45. Thus, as with many drug regimens, a single treatment with an S100B inhibitor may not always be sufficient to fully abolish all the cancer cells, but a strategy will require two or more treatments and/or a combination of chemotherapeutic agents. Furthermore, as with all drug discovery programs, it is therefore the goal to obtain a higher affinity binding drug, so lower concentrations of compound will have to be administered per dose.

Summary of the Interrelationship Between S100B and the p53 Tumor Suppressor

A scheme for the interrelationship between S100B and p53 is illustrated (Fig. **3**). In cells, p53 is activated upon DNA damage or under stress and up regulates the transcription of genes involved in apoptosis (i.e. ↑Bax etc.) and cell cycle-dependent growth arrest (i.e. ↑p21 etc.). As part of a feedback control mechanism, p53 also up regulates the transcription of genes involved in its own inactivation (i.e. hdm2 and S100B). In a cell growth response, there is an increase in intracellular calcium (↑Ca^{2+}), and S100B binds calcium and undergoes a conformational change as is necessary to interact with the tumor suppressor protein, p53. This Ca^{2+}-dependent interaction between S100B and p53 induces a conformational change in p53 and tetramer dissociation of the tumor suppressor [63], which likely contributes to its degradation (i.e. perhaps involving hdm2/ubiquitin- and/or protease-dependent pathways). Thus, down-regulation of p53 by S100B and hdm2 ultimately facilitates cell growth. Too much S100B, as found in melanoma and several other cancers, leads to cell proliferation. Therefore, it is necessary to inhibit the S100B-dependent effect in such cancers. A proof in principle for restoring functional p53 in malignant melanoma is in place whereby siRNA designed to inhibit S100B protein was found to restore functional p53 levels [70]. Such an effort to restore wild-type p53 tumor suppressor function, as is typically found in melanoma, is now underway using a rational drug design approach to find small molecule inhibitors of S100B [76]. Furthermore, the role of other S100 proteins as inhibitors of p53 and/or regulators of S100B *via* heterodimer formation will require further characterization.

SMALL MOLECULE INHIBITORS DESIGNED TO BIND CALCIUM-LOADED S100B

Here, we will briefly review an ongoing search for lead compounds that bind S100B and inhibit the S100B-p53 interaction. Such molecules have potential for becoming new and useful cancer therapeutic agents. A flow diagram of the iterative approach used is illustrated that includes (i) determining high-resolution three dimensional structural data for S100B, (ii) screening large databases of small molecules using a computer aided drug design approach (CADD), (iii) testing such compounds in biochemical and biological screens, (iv) further characterizing the promising leads *via* structural biology, and (v) modifying the compounds using synthetic chemistry together with structure-based drug design approaches (Fig. **4**).

Solution NMR Structures of S100B

Before small molecule inhibitors could be identified and designed *via* computer aided drug design (CADD), it was first necessary to characterize the Ca^{2+}-dependence of the S100B interaction with p53 at atomic resolution (Fig. **2**). The Ca^{2+}-dependence of the S100B-p53 interaction can be observed by comparing the structures of three S100B complexes (apo-, Ca^{2+}-bound, and p53-bound S100B; Fig. **2**). Most of the residues that interact with the C-terminal negative regulatory domain of p53 (18 of 21) are buried in the apo-S100B structure. When Ca^{2+} binds to S100B, however, these same residues are exposed due to a large change in the position of helix 3; this conformational change is required for the interaction with the target proteins such as p53. In general, S100B targets have varying sequences with most of its targets binding a consensus sequence including the residues [K/R]-[L/I]-[P/S/N/D]-[W/L/I]-[S/D/L]-x-[L/I]-[L/F]. For p53, specific and important interactions involve residues Leu-385 of the p53 peptide and residues Met-79, Val-80,

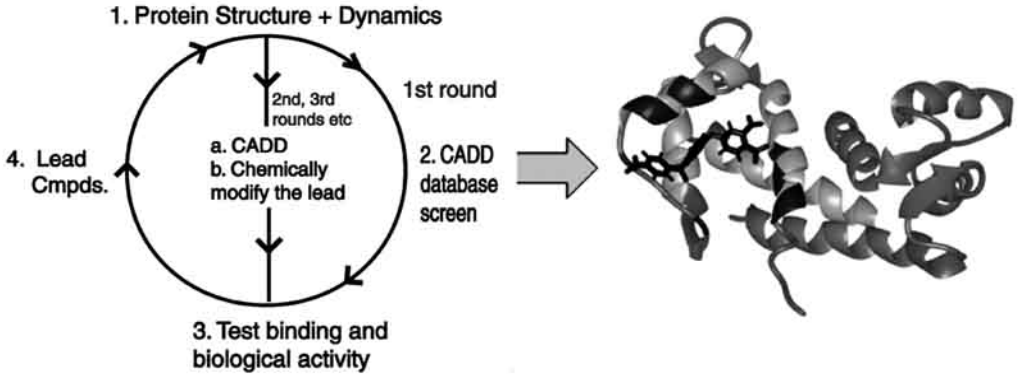

Fig. (4). Diagram of an iterative approach for rational drug design. On the left is a flow diagram of an iterative approach for obtaining molecules that bind S100B and inhibit S100B-p53 complex formation. On the right is a molecule docked into the p53 binding site of S100B.

Leu-44, and Val-56 of S100B are shown (Fig. **5**). This interaction is part of a mini-hydrophobic core at the peptide-protein interface; Phe-385 is located adjacent to this hydrophobic patch, but it is positioned to interact with Phe-87 on S100B. Salt bridges between residues Arg-379 and Lys-386 from p53 to residues Glu-45 on the "hinge" and Glu-86 on helix 4 of S100B are also likely based on the NMR structure. Furthermore, a similar hydrophobic interaction (analogous to Leu-385 of p53) is observed between S100B and Trp-7 from a tight S100B-binding peptide derived from a phage library, the TRTK peptide [77], so the structure of TRTK-S100B can also be used in CADD approaches. The availability of this extensive structural data on S100B combined with its role in down-regulating p53 makes S100B an ideal target for rational drug design.

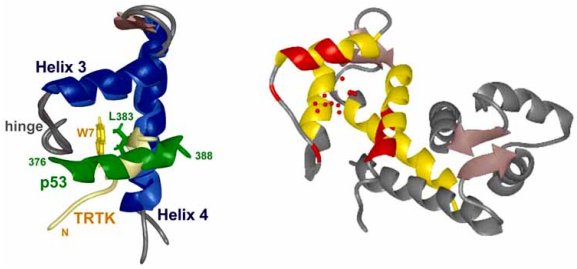

Fig. (5). Close-up view of the p53 binding site on S100B illustrating the p53 binding pocket targeted using DOCK. (A) Helices 3 and 4 of S100B (blue) are superimposed for the S100B-p53 (green) and S100B-TRTK (gray) peptide complexes illustrating the hydrophobic group (Leu-383 of $p53^{367-388}$; Trp-7 of TRTK) protruding into the binding pocket on S100B. (B) The program SPHGEN was used to define the binding pocket on S100B based on these two structures and the Ca^{2+}-S100B structure. The sphere set used by the program DOCK that defines the hydrophobic pocket is illustrated with red spheres, and residues that interact directly with $p53^{367-388}$ are also highlighted (colored red). The tetramer domain of p53 (residues 321-346) also interacts with this same site on S100B.

However, it is clear from thermodynamic and biological data that it is the tetramer domain together with the C-terminal negative regulatory domain of p53 (residues 293 – 393) that interacts most tightly with S100B [40, 64]. As with other S100B target peptides, the interaction of full-length p53 and the p53 tetramer domain peptide (residues 321-346) is also fully calcium-dependent. Likewise, it binds to the same hydrophobic cleft in calcium-bound S100B as TRTK-12 and the C-terminal negative regulatory domain peptides as judged from chemical shift perturbations observed in HSQC spectra [78]. These data together with competition studies between the tetramer domain peptide and TRTK-12 and the peptide derived from the C-terminal negative regulatory domain confirmed that the tetramer domain binds to the same site, which was targeted using CADD. Although, for S100B binding to the p53 tetramer, it is found that the structure of the p53 tetramer is significantly changed and perhaps not any longer folded in the presence of S100B; whereas, the structure of S100B is relatively unchanged when different protein targets are bound when its 3-D structure is compared in various peptide complexes (i.e. p53 peptides, TRTK-12, etc).

CADD Database Search Based on Available NMR Structures

Drug design and development is being significantly advanced *via* CADD approaches [79, 80]. While CADD approaches are useful when structural information is only known for ligands of a particular target (i.e. pharmacophore searching [81]), the largest growth of CADD is in systems where the 3D structure of the biological target molecule is known [82]. In such systems CADD can be used to select compounds from 3D chemical databases with an enhanced potential for binding to the target molecule [83-87] or build such compounds *de novo via in silico* methods [88, 89]. In the case of S100B, the availability of multiple 3D structures of the protein allowed for application of database screening methods to this system. In addition, the availability of multiple structures and NMR-derived dynamic data [90, 91] facilitated the inclusion of protein flexibility, which is often ignored during database screening [92]. Accordingly, 3D chemical database searching was accomplished to identify low molecular weight chemical compounds with a high probability for binding S100B, thereby blocking its interactions with p53. Other recent successes of this approach include inhibitors of thymidylate synthase, [93] various proteases [94-98], kinases [99-101] and HIV integrase [102], among others. Notable is the successful identification of small compounds that block protein-protein interactions [103, 104], including studies on Bcl-2 [105], IL-2 [106], mdm2-p53 interactions [107], and p56lck kinase [108]. In these studies, low molecular weight compounds were identified with dissociation constants or IC_{50}s in the low micromolar range.

Screening was performed using the program DOCK [85, 109, 110]. The program SPHGEN was used to identify putative small molecule binding sites on the protein surface with the final binding site selected based on a location adjacent to S100B residues involved directly in S100B-$p53^{367-388}$ and S100B-TRTK peptide complexes. Once the binding site was adequately mapped by SPHGEN, database screening was performed using approximately 200,000 compounds from the non-proprietary portion of the NCI database [111] and nearly 440,000 commercially available compounds from the companies Chembridge and Maybridge Inc. The latter represent a subset of the 3 million small molecular weight compounds virtual database generated in the laboratory of Prof. MacKerell [112]. The screen involved an initial search of the 640,000 compounds from which the 20,000 top compounds were selected based on their interaction energies with the protein. These compounds were then subjected to a more rigorous screen from which the top 500 compounds were selected. Clustering based on chemical fingerprints was then performed to select compounds with maximal chemical diversity for biological assay. From this, a total of 60 compounds were obtained for testing and biological assay.

Biological Assays and *In Vitro* Screening of the Compounds Identified by DOCK

From the CADD screen, 60 potential inhibitors targeting the putative binding site on S100B were tested in fluorescent binding/competition assays. Thirteen of the compounds were found to bind to S100B with a relatively high affinity (1.0

μM < K_D < 120 μM) and inhibit p53 peptide binding [76]. Based on NMR chemical shift perturbations in calcium-bound S100B, it was found that seven of the thirteen small molecules (termed SBi1-SBi7), including one FDA approved drug (pentamidine; SBi1), bind directly in the p53 binding pocket of S100B as predicted by the program DOCK; the remaining 6 compounds were more difficult to evaluate by NMR due to solubility problems. Using saturation transfer difference (STD) NMR methods, it was possible to confirm inhibitor binding and qualitatively identified protons from the small molecule at the small molecule-S100B interface. Mapping regions of the small molecules *via* STD NMR methods is very useful information to start designing more tightly binding inhibitors in the future [76, 113]. With these data, it was possible to calculate a preliminary NMR docked model of the pentamidine-S100B complex using all of the available NMR data including intermolecular NOE correlations between SBi1 and calcium-bound S100B [76]; however, such a NMR docked model requires confirmation in a full-scale 3D structural analysis using NMR and/or X-ray crystallography.

The SBiX inhibitors (X=1-7) that inhibit the S100B-p53 interaction *in vitro* were tested for their ability to enter primary malignant melanoma cells and inhibit cell growth (Fig. **6**). It was discovered that treatment with small molecule inhibitors of S100B restores p53 activity such that S100B is

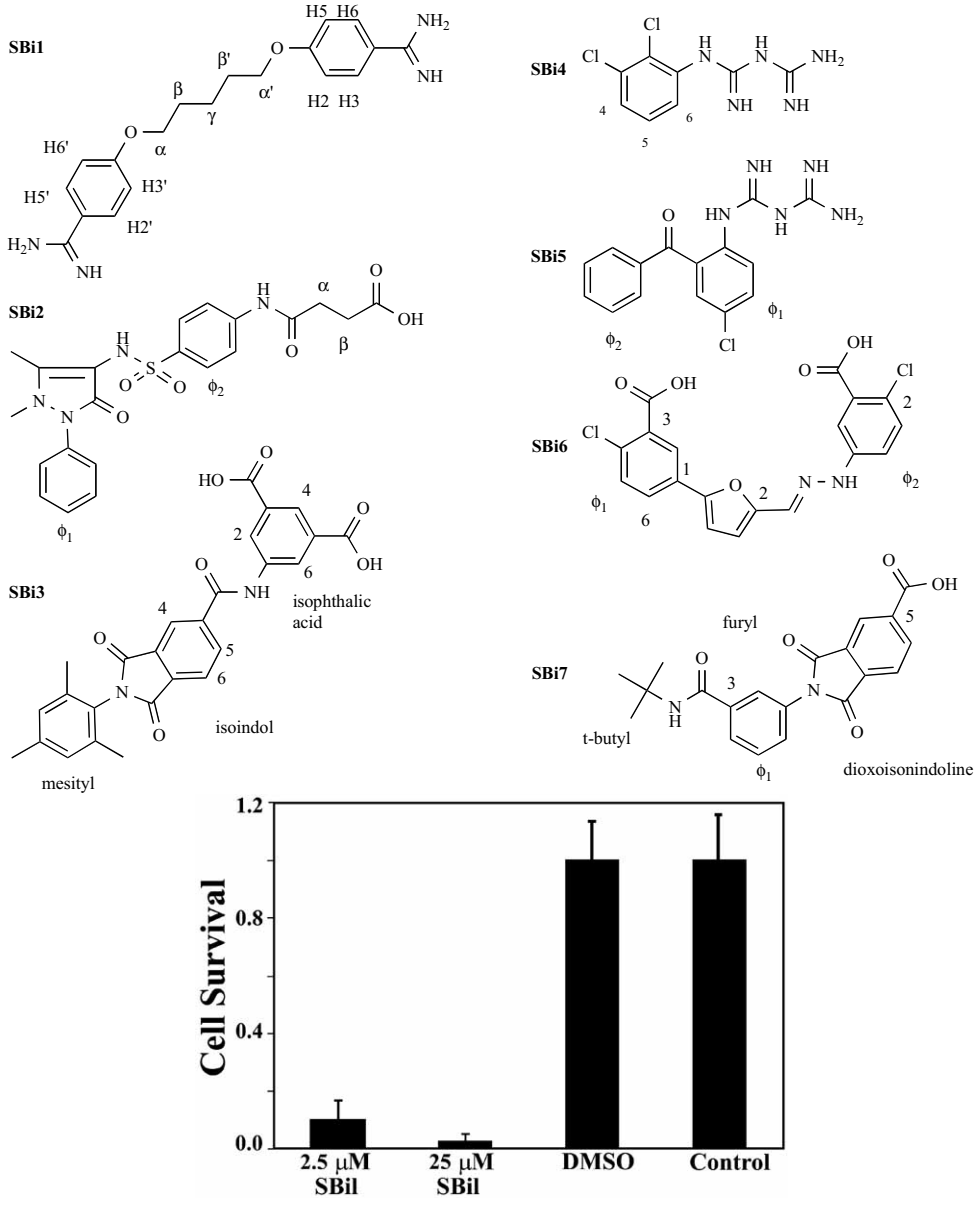

Fig. (6). Chemical structure of the S100B inhibitors (SBi1-SBi7) and cellular data illustrating the ability of pentamidine (SBi1) to inhibit cell growth of primary malignant melanoma cells (C8146A) containing wild-type p53 tumor suppressor. In contrast, a 7.5 fold lesser effect (at 25 μM SBi1) was found in growth in normal neonatal melanocytes [76].

itself then up-regulated (by > 6-fold). This occurs at least partially because S100B is under transcriptional regulation of p53 as part of a feedback loop; although, it cannot be ruled out that the compounds induce a stress response that up-regulates p53 by other pathways. Nonetheless, the relatively high stability of S100B makes this a robust test to detect the restoration of p53 activity and can be used in high throughput screens using 96 well plates. Although slower, other previously published methods for monitoring p53 activity [40] such as inhibiting the S100B-p53 interaction in primary malignant melanomas and the restoration of p53 DNA binding and transcription activation using CAT assays can also be done in the presence of small molecules as described previously for treatments with siRNA directed against S100B [40].

SUMMARY AND FUTURE DIRECTIONS

In summary, a combination of computer aided drug design (CADD) and nuclear magnetic resonance techniques were useful for identifying several lead compounds that bind and inhibit the S100B-p53 interaction. The continuation of this rational drug design approach will include new structure determinations of small molecules bound S100B together with computer guided synthetic strategies to discover/synthesize new lead compounds that interact with S100B with higher binding affinity. This is necessary for the development of a therapeutic drug that can restore wild-type p53 activity in cancers with elevated S100B such as malignant melanoma and astrocytomas.

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