Chapter 24

Inhibiting S100B in Malignant Melanoma

Kira G. Hartman, Paul T. Wilder, Kristen Varney,
Alexander D. Jr. MacKerell, Andrew Coop,
Danna Zimmer, Rena Lapidus and David J. Weber

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55176

1. Introduction

The development of new therapies for patients diagnosed with malignant melanoma is in high need. In this chapter, the design and testing of inhibitors are discussed for S100B, a calcium-binding protein that down-regulates the tumor suppressor p53. Because p53 is wild type in many malignant melanoma patients, the restoration of p53 with S100B inhibitors (SBiXs) represents a new and potentially effective strategy for sensitizing melanoma cells to p53-dependent apoptosis pathways and for targeting this deadly cancer. Such a strategy requires blocking of the S100B-p53 protein-protein interaction (PPI) and involves methods including computer aided drug design (CADD), screening technologies, nuclear magnetic resonance (NMR), X-ray crystallography, and medicinal chemistry approaches. The ultimate goal is to design a highly specific and potent inhibitor of S100B that has clinical value.

2. The S100 protein family

The S100 family of EF-hand calcium-binding proteins has more than 20 members, with the genes encoding these proteins present only in vertebrates [7]. S100 proteins (S100s) are expressed in both a cell type and tissue-specific manner to provide diverse functional roles including calcium homeostasis, cell-cell communication, cell proliferation, differentiation, cytoskeletal dynamics, and cell morphology [7-10]. On the other hand, dysregulation of S100 expression is observed in several types of cancers, including malignant melanoma [7-9]. They are also problematic when elevated in several cognitive disorders including those arising from traumatic brain injuries [12-16]. While S100 proteins themselves have no inherent enzymatic

activity, they regulate important biological processes via specific Ca^{2+}-dependent protein-protein interactions [17,18].

The first members of the S100 family were discovered in 1965 in a subcellular fraction from bovine brain tissue and were named based on their solubility in 100% saturated ammonium sulfate. When this protein fraction was examined in detail, two similar, but distinct proteins were discovered and designated S100*α* and S100β that are now referred to as S100A1 and S100B, respectively [17,19]. As with S100A1 and S100B, other S100s have a similar molecular weight (9-12 kDa), have homologous amino acid sequences (>40%), and typically exist as symmetric homodimers, or as heterodimers, held together by noncovalent interactions as pairs of four-helix bundles [20,21]. Two EF-hand helix-loop-helix calcium-binding structural motifs, first defined using the "E" and "F" helices from the X-ray crystal structure of parvalbumin, are present in each S100 subunit [22]. The N-terminal "*S100"* or "*pseudo*" EF-hand (EF1) is comprised of 14 rather than the original 12 residues and this 14 amino acid sequence readily distinguishes S100s from other EF-hand calcium signaling proteins. The canonical EF-hand (EF2) is found at the C-terminus of each subunit and typically binds Ca^{2+} with a higher affinity than EF1. The two EF-hand domains are connected by a stretch of amino acid residues (<25 residues) termed the "hinge region". This "hinge" together with the C-terminal loop of the S100 protein contains the least amount of sequence homology and, therefore, represents the two regions that give each family member their individual target-binding specificity [7,8,23]. In addition to binding Ca^{2+}, several S100s bind Zn^{2+} at a separate site from the EF-hand calcium-binding domains. The Zn^{2+} site can also bind other metals (i.e. Mn^{2+}, Cu^{2+}, and others) and has two ligating residues contributed from each subunit at the dimer interface to provide tetrahedral coordination that is typical for Zn^{2+} [24]. However, for S100B, Zn^{2+}-binding is not sufficient to induce target binding on its own, but rather functions by increasing the affinity S100B has for Ca^{2+} and its target proteins [25,26].

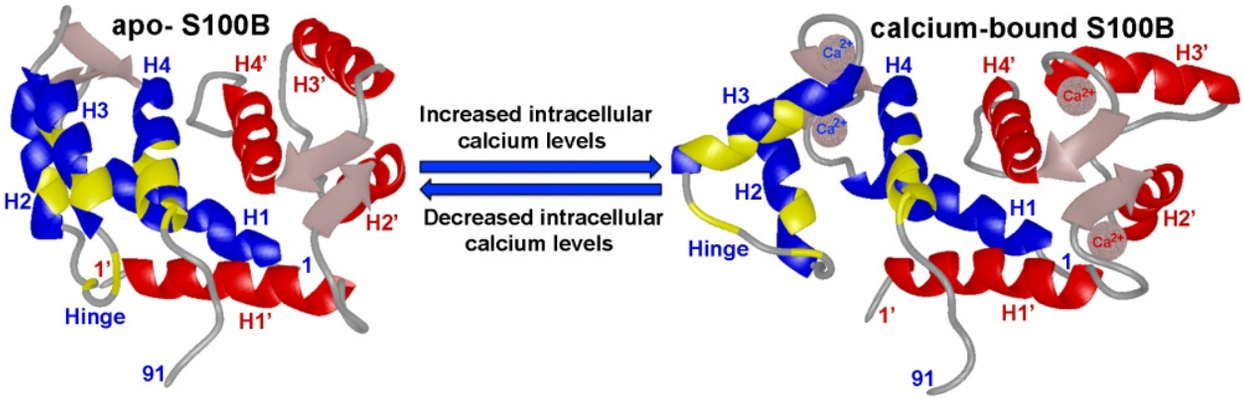

Figure 1. Structures of apo- versus holo-S100B. Ribbon diagrams comparing the NMR solution structures of calcium-free (left) and calcium-bound S100B (right), illustrating the 90° reorientation of helices 3 and 3' in each subunit of S100B. Shown in yellow on one subunit is the S100B target binding site that is exposed after the Ca^{2+}-dependent conformational change.

http://dx.doi.org/10.5772/55176

3. S100B structure & interactions with ions

As with most S100 proteins, each 91 amino acid subunit of S100B has four alpha helices arranged into two helix-loop-helix (HLH) calcium-binding motifs connected by the flexible "hinge" region. Helix 1 and 2 make up the S100 EF-hand, while helix 3 and 4 form the canonical EF-hand (Figure 1). Each S100B subunit, therefore, binds two molecules of calcium, though with significantly different affinities [27]. While the canonical EF-hand binds Ca^{2+} with moderate affinity (K_D = 20 – 50 μM), the weaker S100 EF-hand binds Ca^{2+} with a much lower affinity (K_D = 200 – 500 μM) [27-30]. Calcium binding induces a dramatic conformational change in S100B where helix 3 rotates by 90º to become perpendicular to helix 4 (Figure 1). This change in conformation exposes a unique binding pocket, which in turn, binds to targets specific for each S100 protein generating a biological response [23]. However, it is clear that the dissociation constants of calcium from most S100s *in vitro*, including S100B, are too weak to compete on their own for free Ca^{2+} typically present in the cytosol (100 to 500 nM). Interestingly, it is now understood that the affinity of S100s for Ca^{2+} is increased by as much as 300-fold when bound to biologically relevant target proteins (i.e. at 100 nM free Ca^{2+}) suggesting that S100s typically only sequester free Ca^{2+} when their biologically relevant targets are present at optimal levels within the cell [31-34]. While the mechanism for this allosteric tightening of Ca^{2+} is not completely understood, it is known that when a target peptide derived from CapZ (termed TRTK-12) is bound to S100B, a loss in μs-ms motions occurs throughout the protein including in the side chain of a Ca^{2+}-coordinating residues. These results were consistent with the hypotheses that stabilizing motions, particularly for Ca^{2+}-coordinating residue(s) in EF2, could be responsible for the significant increase in Ca^{2+}-ion binding affinity observed when a target is bound to Ca^{2+}-S100B [35]. Likewise, the binding of S100B to Zn^{2+} (K_D = 90 ± 20 nM) stabilizes residues in the C-terminus of the protein, resulting in an increase in both Ca^{2+}- and target-binding affinities [28,36].

Thus, as with many EF-hand proteins, S100 signaling proteins do not bind Ca^{2+} with high affinity unless they are bound to their biologically relevant protein target(s) [32-34,37,38]. In other words, in the absence of a bound target, the Ca^{2+}-binding affinity for most S100 proteins is relatively low (i.e. in the μM range [1,17,27,39], but when bound to peptides (i.e. TRTK-12) or full-length targets, the Ca^{2+}-binding affinity can be increased by 5- to 300-fold, respectively [32-34,37,38,40]. This property is physiologically necessary because while there are over 600 EF-hand Ca^{2+}-binding domains within any given cell, Ca^{2+} homeostasis must be maintained with sufficient free Ca^{2+} ion concentrations for proper signaling (i.e. 100 to 500 nM). Thus, as a physiological control mechanism, S100s and many other EF-hand proteins do not sequester significant amounts of free Ca^{2+} unless their functionally relevant molecular target is available [29,34,38]. It is especially important for drug design that we continue to investigate and understand this phenomenon at the molecular level because S100 inhibitor binding must mimic the EF-hand-target complex and allosterically tighten Ca^{2+} ion binding affinity upon complex formation to be effective inside the cell [35,37]. For S100B, this includes targets such as p53, hdm2, hdm4, Rsk1 and RAGE, among others, which subsequently contributes to a Ca^{2+}-mediated growth response in a cell-specific manner, including those in skin and brain (Table 1).

4. S100B pathology

The protein S100B is found in melanocytes, glial cells, chondrocytes, and adipocytes, exhibiting both intra- and extracellular functionality. The cellular responses elicited by S100B can vary depending on several factors, including concentration (nM or μM), cell type, and cellular location [8,9]. Of particular concern is the role of elevated S100B in melanoma (Figure 2), the most deadly of all skin cancers, notorious for its resistence to chemotherapy and radiation. Clinical studies have established S100B as an effective biomarker for melanoma; however, this is only the case when highly specific S100B antibodies are used [12]. For example, in one study, samples from 412 melanoma patients at varying stages were compared to those diagnosed with non-melanoma skin cancers and inflammatory cutaneous diseases. Using a cutoff value of 0.2 μg/l serum S100B, a positive correlation was observed for patients having S100B levels above the cutoff level and advancement of tumor stage, indicative of a contribution by S100B to micro- and/or macro-metastases [41-43]. Though elevated S100B cannot be used to identify tumor thickness or lympth node status, it is predictive of poor patient prognosis, increased tumor recurrence, and low overall survival [9,41-44]. Subsequent studies reinforce these findings and consistently show elevated levels of S100B to be a sensitive and specific marker of melanoma progression with the ability to detect metastases or relapse at much earlier timepoints. S100B levels can also be used to monitor treatment strategies for rapid identification of whether a particular therapy is promising or for deciding to take an alternative approach [9]. While S100B is a useful prognostic indicator for melanoma, its use as a biomarker for several other cancers with elevated S100B is still under investigation; including colorectal cancer [45-47], several gliomas [48,49], mengiomas [50], non-small cell lung cancer (NSCLC) [51], renal cell carcinoma (RCC) [52], and thyroid carcinoma [53]. In addition, these clinical observations underscore the need to fully understand the role of elevated S100B in cancer, which is ongoing [2-4,54].

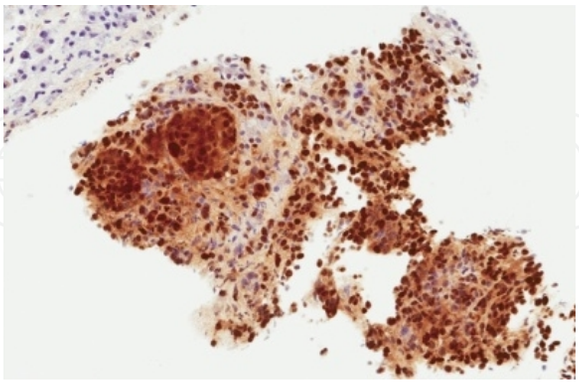

Figure 2. Staining for elevated S100B in a human malignant melanoma biopsy. Elevated S100B is stained brown in this human biopsy recorded for a patient before entering a S100B inhibitor clinical trial (SBi1). Patients are also tested for their p53 status and S100B:p53 ratio as recommended by Lin et al [2-4].

Although not considered in detail here, S100B also plays an important role in the brain, and as with cancer, several cognitive disorders show over-expression of S100B in brain tissue and

are associated with pathological states including Alzheimer's disease (AD), Down's syndrome (DS), and schizophrenia [55-59]. One mechanism for this pathology is that elevated intracellular levels of S100B present in glial cells are excreted and regulate neighboring neuronal cell activity. At low levels, the presence of this extracellular S100B is sufficient to promote neurite extension and growth, while elevated S100B levels are toxic and lead to neuronal cell apoptosis [9,60]. As with skin cancer, the clinical utility of S100B as a marker to identify and characterize neurological diseases and traumas is complicated by overlapping expression of S100B and other S100s in several cell types, its multiple mechanisms of secretion, and its association with more than one neurodegenerative disorder [14]. However, as found for melanoma, lowering S100B levels upon drug delivery is one means used to evaluate drug efficacy for treating schizophrenia [16,61]. Furthermore, the development of S100B inhibitors themselves may be useful for the treatment of these neuropathies, making the identification of such compounds important for advancing efforts towards understanding and treating cancers and cognitive disorders in which S100B levels are at pathologically high levels [62].

Cellular Activity	Protein	References
Ca^{2+} Homeostasis	AHNAK*	[26]
Cell Cycle Regulation	Hdm2, Hdm4	[63]
	NDR	[64,65]
Cytoskeletal Regulation	Caldesmon*	[66]
	Calponin	[67]
	CapZα	[68]
	GFAP	[69]
	IQGAP1	[70]
	MARCKS*	[71]
	Src kinase	[26]
	τ-protein*	[72]
	Tubulin	[73]
Energy Metabolism	Fructose 1,6 bisphosphate aldolase	[74]
	Phosphoglucomutase	[75]
Growth & Survival	p53*	[1,28,76,77]

*PKC-mediated phosphorylation target proteins

Table 1. Targets of S100B

5. S100B targets

The ability of S100B to bind a diverse array of protein and enzyme targets is attributable to its broad consensus target-binding sequence [63]. S100B targets include proteins involved in calcium homeostasis, cell-cycle regulation, cytoskeletal regulation, energy metabolism, and

growth/survival (Table 1). One common theme among several S100B-target interactions is that they regulate protein phosphorylation [78]. For example, S100B associates with nuclear Dbf2-related (NDR) protein by binding a region distinct from the active site and inducing a conformational change, which stimulates autophosphorylation, and ultimately activates the protein [64]. S100B also regulates phosphorylation by binding to kinase substrates such as those of protein kinase C (PKC) and sterically blocking phosphorylation [76,77,79] (Table 1). This includes the myristoylated alanine-rich C-kinase substrate (MARCKS), τ-protein, and caldesmon to name a few [66,80,81]. One noteable S100B target is the PKC substrate, p53, which is activated by phosphorylation in the C-terminal negative regulatory domain (NRD). In addition to blocking PKC-dependent phosphorylation, the S100B-p53 complex formation shifts the p53 tetramer to dimer to monomer equilibrium towards oligomer dissociation [76,78]. Thus, for p53, when S100B levels are too high, PKC-mediated activation of p53 is inhibited and p53 tetramers are dissociated. Consequently, p53 cannot bind DNA, which affects its transcriptional activity [2,28,76,77,82,83] and inhibits its ability to control cell cycle progression and apoptosis [2-4]. Other S100B targets include the E3 that designates p53 for ubiquitination, Hdm2, and the Hdm2 regulator, Hdm4 [63]. Thus, studies are underway to understand how S100B complexes involving Hdm2/Hdm4 contribute to lowering p53 levels in melanoma. Complicating this is the fact that both of these negative regulators of p53, Hdm2 and S100B, are themselves transcriptionally regulated by p53 [4,63]. This feedback regulatory mechanism results in time-dependent regulation of p53 that depends on having correct levels of all four proteins for proper regulation of cell cycle growth arrest and apoptosis [63]. Since elevated S100B disrupts the maintenance of p53 levels and promotes a cancerous phenotype, the development of small molecule inhibitors designed to target Ca^{2+}-bound S100B has become a high priority. Specifically, investigations are focused on the identification of compounds capable of blocking the Ca^{2+}-dependent S100B-p53 interaction in malignant melanoma (Figure 3). Ideally, administration of such compounds would reactivate p53 in malignant melanoma, as found for $siRNA^{S100B}$, to induce normal apoptosis pathways and reduce proliferation/survival of the cancer cells [2-4].

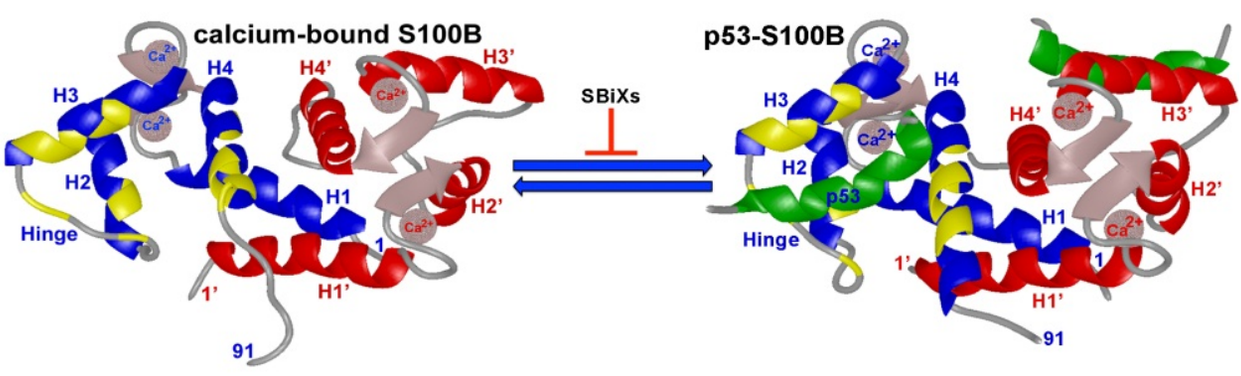

Figure 3. Illustration of the p53-binding site on S100B. Shown are ribbon diagrams Ca^{2+}-bound S100B (NMR), $p53^{367-388}$-bound Ca^{2+}-bound S100B (NMR, PDB entry 1DT7). The helices of S100B are colored in blue, while the $p53^{367-388}$ peptide is shown in red. Gray spheres represent the two calcium molecules per subunit. S100B inhibitors (SBiXs) are being developed to inhibit the Ca^{2+}-dependent formation of the S100B-p53 complex.

http://dx.doi.org/10.5772/55176

6. Targeting the S100B-p53 interaction

Binding of S100B to p53 blocks PKC-dependent phosphorylation, p53 tetramerization, and p53-dependent transcription activation [28,63,76,82,83]. Therefore, efforts to restore wild-type p53 activities in malignant melanoma are underway as part of a drug design strategy [28]. A combination of approaches is being used, including those involving target validation and screening, computer aided drug design, structural biology, medicinal chemistry, and *in vivo* biology and drug testing methods (Figure 4). In one case, a previously FDA approved drug was discovered to block the S100B-p53 interaction. The wealth of available data associated with this compound, including its use in animals and human clinical trials made repurposing it for use in malignant melanoma a fairly quick transition.

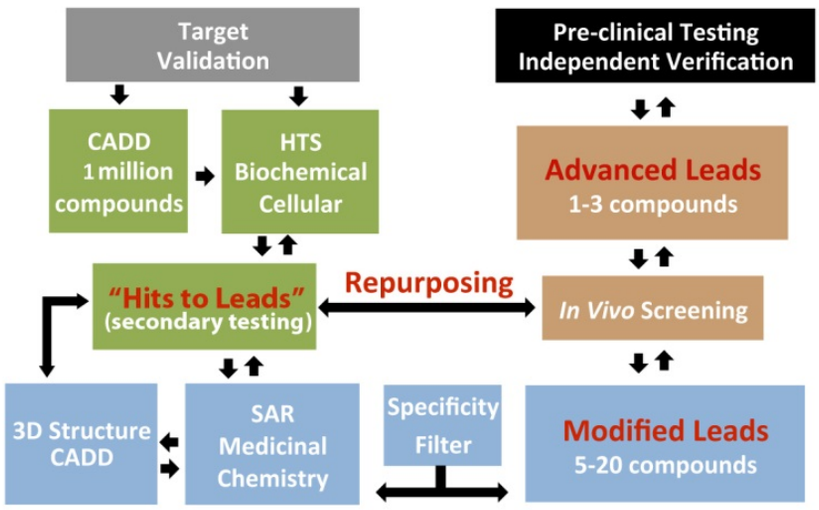

Figure 4. Summary of strategies for advancing hits from screening studies to leads, modified leads, and then advanced lead compounds. This schematic is a general guideline for the early stages of drug development. The data from these approaches is compiled and used to choose "candidate" compounds that are studied more extensively in humans for their toxicology and effectiveness in treating cancer. It is ofter advantageous to consider repurposing compounds that are already known to be safe in humans as a means to advance this process more quickly.

Screening for S100B inhibitors was initiated using computer-aided drug design methods (CADD) [28,84], and in all steps of identifying and prioritizing "hits" during these and other screens, the pharmacological activity of compounds was evaluated semi-quantitatively, providing an unbiased means of eliminating compounds that do not fulfill specific "drug-like" criteria [84,85]. Compounds identified in screens are also evaluated regarding their potential for absorption, distribution, and metabolism/excretion (ADME) properties [86]. Among many CADD approaches, a recent structure-based technique termed Site Identification by Ligand Competitive Saturation (SILCS) is now used extensively [87-89]. The simultaneous presence of benzene, propane and water in MD simulations of the target protein (ie. S100B) in this fragment-based computational approach identifies potential binding regions for aliphatic moieties, aromatic moieties and hydrogen bond donors and acceptors, while simultaneously allowing for increased flexibility and conformational changes to occur within the drug-binding

site [87-89]. In addition, SILCS is very useful for strategically modifying "hits" or "lead compounds" to span a larger area of the protein surface [87,88]. CADD methods such as these are particularly important for blocking protein-protein interactions (PPIs) such as that for the S100B-p53 complex since at least three distinct target binding pockets have been identified on S100B (Figure 5) [27,29,30,37,63,68,90,91]. As a result, the drug pentamidine diisethionate (Pnt), which is referred to as SBi1 (designated SBiX, where 'X' is an arbitrary compound number), was identified at a very early stage of the screening process as an effective inhibitor of the S100B-p53 complex [84]. Pnt was approved by the FDA as an antimicrobial agent for the treatment of Pneumocystis carinii pneumonia (PCP), which allowed for repurposing of this drug for *in vivo* testing for efficacy in treating malignant melanoma (Figure 4). To this end, a clinical trial is ongoing at the University of Maryland Medical Center (UMMC) to determine the efficacy of Pnt in melanoma patients *(0794GCC: "Treatment of melanoma with wild-type p53 and detectable S100B using pentamidine (SBi1): a Phase II trial with correlative biomarker end-points"*; CA135624; PI: Dr. Ed Sausville, M.D.; Co-PI: Dr. David J. Weber). Although, there are promising results for the use of Pnt for the treatment of malignant melanoma, efforts have continued with the goal of engineering a compound with higher efficacy and more specificity for targeting S100B (versus other S100 proteins).

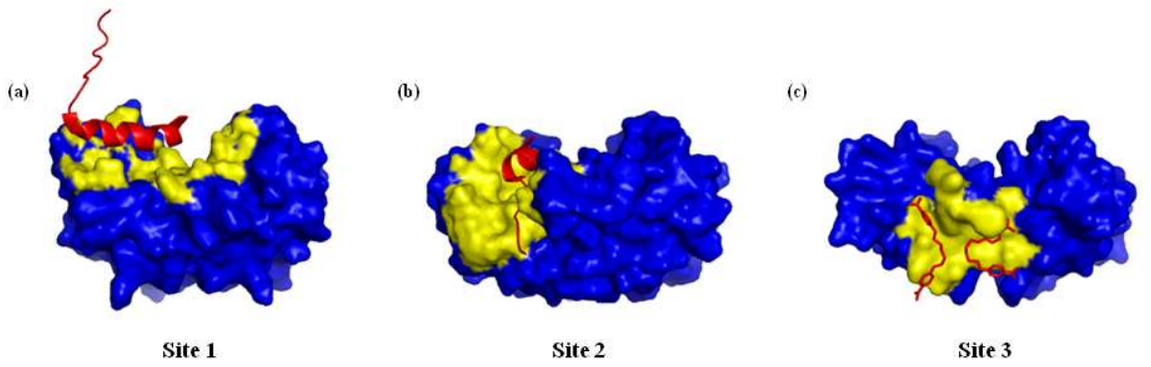

Figure 5. Three binding pockets are targeted on S100B. Surface representation of the structures of Ca^{2+}-S100B bound to (a) the C-terminal negative regulatory domain of p53 (PDB ID: 1DT7)(1), (b) TRTK-12 (PDB ID: 1MWN) [5], and (c) pentamidine, also referred to as SBi1 (PDB ID: 3CR4) [6]. Sites 1, 2, and 3 are labeled. The protein is depicted as a blue surface and the regions within 3 Å of the bound peptide or small molecule are colored yellow. TRTK-12, p53 peptide, and pentamidine are shown in red.

In addition to CADD, biochemical and cellular screening methods are continuously ongoing to identify "hits" that can be considered further as scaffolds for drug development (Figure 4). One sensitive method done *in vitro* is a fluorescence polarization competition assay (FPCA), which can identify PPI inhibitors in a high-throughput manner. This competition assay takes advantage of a small molecule inhibitor causing the dissociation of a small peptide-bound fluorophore from a larger protein-peptide complex. Specifically, SBiX inhibitors can readily be identified since the smaller, faster rotating free fluorophore-bound peptide will exhibit lower polarization when competed away by the SBiX and compared to the larger S100B-peptide complex. Importantly, the labeled-peptide must differ in molecular weight from the S100B-peptide complex to provide a reasonable dynamic range for the assay [92].

While it is important to show that an S100B-compound complex forms *in vitro*, it is also important to demonstrate that the SBiX has anti-cancer activity in cellular assays (i.e. growth inhibition, reduction in survival, increase apoptosis activity etc). In addition to showing compound efficacy in cells, these assays are important for providing information about other properties of the compound(s) including its membrane permeability and overall toxicity. One reliable method for identifying inhibitors of S100B-dependent pathways is via the creation of matched cell lines that only differ in S100B expression level. For example, MALME-3M melanoma cells were selected because they express elevated S100B protein levels and retain wild-type p53. Small interfering RNA (siRNA) was then stably transfected into the MALME-3M cells to give a scrambled siRNA control with high S100B levels and an isogenetically matched $siRNA^{S100B}$ construct resulting in low S100B (unpublished data). These matched cell lines provided a means for a large-scale screen of compound libraries to identify SBiXs with potency and specificity towards pathways involving S100B. The "hits" in this cellular assay were then routinely tested for direct binding and secondary cellular assays were completed by comparing the effect of these hits on primary malignant melanoma cells side-by-side with normal melanocytes [84]. Compounds that result in indiscriminate cell death were considered toxic rather than from specific inhibition of S100B or an S100B-dependent pathway and highlighted the importance of including normal melanocytes in every screen. Preferential growth inhibition of melanoma with high S100B as compared to little or no effect on the melanocytes or cells containing $siRNA^{S100B}$ was considered to be an early indication that the compound may have promising therapeutic value. This is exemplified with pentamidine, since treatment of C8146A primary melanoma cells resulted in significant cell growth inhibition, but little or no effect was noticed from this drug on normal melanocytes [84].

One of the most important requirements of any drug development program is to obtain physiological data at an early stage in the process to help determine whether a lead compound is effective and/or shows unanticipated toxicities *in vivo [93]*. This is particularly important for S100 inhibitors since there are over 20 structurally similar proteins in the S100 protein family, and they each regulate specific cellular pathway(s) [94,95]. To address these issues as quickly as possible, an *in vivo* screening assay was developed to test potential lead compounds for melanoma at an early stage in the drug development process. Once promising hits are designated as lead compounds via *in vitro* and cellular testing and secondary validation protocols, they need to be tested *in vivo*. Leads to be tested *in vivo* are chosen based on binding affinities (K_Ds) and/or specificity both in biochemical assays and in cellular assays. For the cellular assays, specificity and potential off-target effects are evaluated by comparing IC_{50} values of isogenic cell lines with or without S100B over-expression [96,97]. The vast majority of lead SBiXs are new chemical entities that require extensive optimization prior to entering *in vivo* testing, with the exception of those compounds that have been repurposed from other studies and trials. Animal models play a fundamental role in such *in vivo* testing. For example, a compound may need to be more lipophilic to pass through the cell membrane and reach its target or side groups may need to be added to allow for oral delivery or brain penetration. In the case of accessible tumors such as melanoma, drugs can be delivered directly to the tumor (intratumoral) without optimization for systemic delivery [93]. In addition, intratumoral delivery can achieve significantly higher drug concentrations at the site of action than can be

obtained via systemic delivery. For this purpose a multi-allelic genetically engineered mouse model was chosen to test SBiX compounds since this model mimics spontaneous tumoriogenesis and heterogeneity as well as provide additional information necessary for additional target validation [98]. One such melanoma model is the RAS-induced INK4a/ARF$^{-/-}$ mouse [99], which was chosen for *in vivo* SBiX screening because it has: (i) an intact S100B-p53 signaling pathway (elevated S100B and wild type p53), (ii) an intact immune system, (iii) tumors which are amenable to intratumoral delivery, and (iv) a proven record in developing new melanoma therapies [99,100]. This screen utilizes 2-3 month old experimental Tyr::RAS^{G12V}/INK4a/ARF$^{-/-}$ male mice that develop spontaneous cutaneous melanomas in the pinna of the ears (30%), torso (23%), and tail (20%) without distant metastasis [99] and uses tumor proliferation rate as the primary outcome. Although the screen is not optimized for obtaining tolerability, PK or PD information, the gross/histological pathology, SBiX levels and p53 pathway reactivation in the tumors are monitored as is necessary to select more advanced leads that have potential for proceeding to pre-clinical testing (Table 2).

In the case of modified leads that have ADME properties favorable for systemic administration, concurrent tolerability (MTD) and pharmacokinetic (PK) assays are also conducted. MTD and PK trials are also performed to determine if a lead is suitable for pre-clinical testing or if it requires additional medicinal chemistry optimization and/or further evaluation prior to pre-clinical testing. If the compound is found to be toxic, then it is eliminated from further consideration. Should successful tumor shrinkage be observed in mice treated with the well-tolerated S100B inhibitors, an effort is then put in place to consider phase 1 or 2b human clinical trials.

Parameter	Leads	Modified Leads	Advanced Leads
K_D	<10 μM	<50 nM	<50 nM
IC_{50} in cells	<10 μM	<50 nM	<50 nM
Off target effects	$K_D \approx IC_{50}$	$K_D \approx IC_{50}$	$K_D \approx IC_{50}$
Activity in target (-/-) cells	<50%	<20%	<10%
CYP2D6 Metabolism	Not determined	No	No
P450 CYP induction	Not determined	<50% at 30 mM	<50% at 30 mM
Bioavailability	Not determined	Preferred oral	Preferred oral
Metabolic stability	Not determined	>80% after 1 hour	>80% after 1 hour
BSA Ligand K_D	Not determined	K_D> 10 mM	K_D> 10 mM
Specificity	>5:1	>50:1	>500:1

Table 2. Some criteria for leads, modified leads, and advanced leads

7. SBiX lead optimization

SBiX leads are typically optimized using structure-based drug design and by examining structure/activity relationships (SAR) using traditional medicinal chemistry approaches. Modified leads are also tested using cellular and *in vivo* assays described above to determine whether the modification improved efficacy, specificity, and other criteria listed in Table 2. Although several leads were identified for S100B and are undergoing optimization via a structure-based drug design approach, diverse scaffolds remain essential at this stage of development in case the existing lead compounds become intractable.

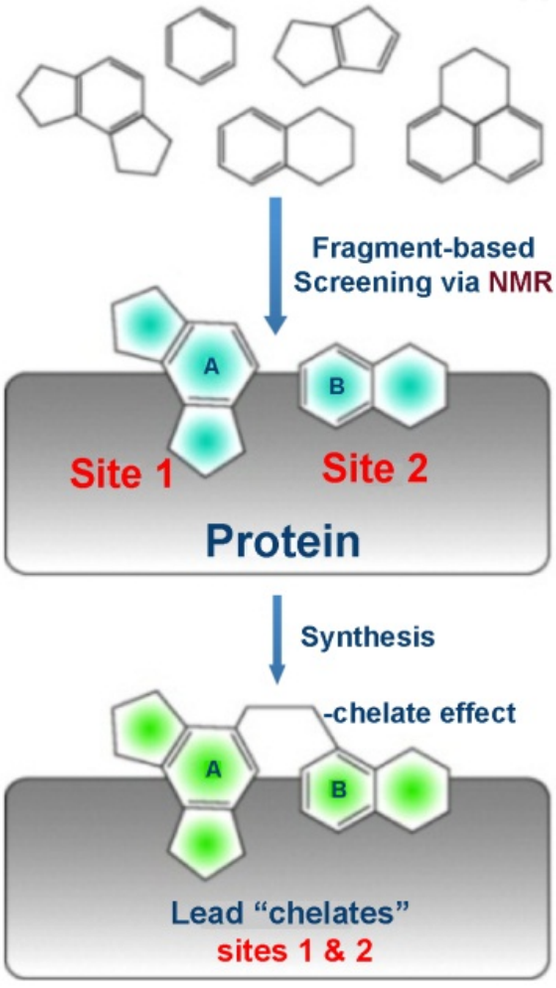

Figure 6. SAR by NMR [11]. NMR screens identify compounds that bind pockets "all over" the protein, which can be linked synthetically to obtain high affinity and specific SBiX inhibitors.

The availability of 3D structures of S100B-SBiX complexes allow for CADD to be used to select compounds from 3D chemical databases with an enhanced potential for binding to S100B [101-105] and/or to engineer compounds de novo via *in silico* methods [106,107]. In an iterative process, new S100B-drug complexes are solved, lead modifications to improve affinity predicted via CADD, predicted compounds synthesized and the resulting compounds

experimentally evaluated. Work is also underway to develop novel inhibitors of S100B via a fragment-based approach that targets multiple binding sites on the protein identified by NMR, X-ray crystallography, and CADD techniques, including the new SILCS technique [87]. The SAR by NMR approach is now a standard method for quickly identifying ^{1}H-^{15}N and/or ^{1}H-^{13}C chemical shift perturbations in HSQC and/or TROSY spectra as a result of an S100B-small molecule interaction; this enables rapid, qualitative identification of binding site(s) on S100B for fragment-based design of new compounds that take advantage of the well-established "chelate effect" involved in linking fragments that bind neighboring sites [108,109] (Figure 6). For fast exchanging inhibitors, saturation transfer differences are collected to identify protons of the lead at the S100B-lead interface [84,110,111]. Thus, progress using a combination of functional-group optimization and the fragment-based approaches offers the potential for improvement in affinities/specificity, and for identifying novel leads. As new lead compounds are identified and structurally characterized (NMR, X-ray crystallography), SBiX affinity is considered as is scaffold diversity due to potential unforeseen problems with compounds that can occur during later-stage preclinical development (e.g. pharmacokinetic limitations, toxicity in humans). Therefore, as many as 3-6 chemical scaffolds are under consideration for development, and this number will be reduced as the project proceeds based on ADME properties, synthetic feasibility, and other pre-clinical/clinical information (Table 2). Such criteria include physiochemical properties related to bioavailability (molecular weight, clogP, TPSA, pKa, nitrogen atoms, carboxylates, H-bond donors, H-bond acceptors, rotatable bonds, H-bonds), and dose-limiting toxicities that may be predictive of the therapeutic index. Importantly, promising leads are routinely tested *in vivo* as early as possible to avoid wasted effort on toxic/ineffective compounds.

8. Summary

Ongoing collaborative efforts involving biology, structure determination, CADD and synthetic chemistry have lead to the development of a collection of inhibitors of S100B. These efforts include identification of the FDA approved compound pentamidine, which is currently being evaluated in human clinical trials. In addition, the work has identified several novel chemical scaffolds that are undergoing optimization and have laid the foundation for the application of fragment-based approaches to design additional novel scaffolds. Notably, while the goal of this research is to develop a potent inhibitor of S100B for the treatment of malignant melanoma, we anticipate that the knowledge gained to date will be of utility in designing specific inhibitors of other members of the S100 protein family for the treatment of a range of S100 associated disease states.

Acknowledgements

Support from the NIH (CA107331; to DJW), The Center for Biomolecular Therapeutics (CBT), and the University of Maryland Computer-Aided Drug Design Center is appreciated.

Author details

Kira G. Hartman[1], Paul T. Wilder[1,3,4], Kristen Varney[1,2,3], Alexander D. Jr. MacKerell[2,3,4], Andrew Coop[2,4], Danna Zimmer[1,3], Rena Lapidus[3,4] and David J. Weber[1,3,4*]

*Address all correspondence to: dweber@som.umaryland.edu

1 Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD, USA

2 Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD, USA

3 Center for Biomolecular Therapeutics, University of Maryland School of Medicine, Baltimore, MD, USA

4 University of Maryland Marlene and Stewart Greenebaum NCI Cancer Center, Baltimore, MD, USA

References

[1] Rustandi, R. R., Baldisseri, D. M., and Weber, D. J. (2000) *Nat Struct Biol* 7, 570-574

[2] Lin, J., Blake, M., Tang, C., Zimmer, D., Rustandi, R. R., Weber, D. J., and Carrier, F. (2001) *J Biol Chem* 276, 35037-35041

[3] Lin, J., Yang, Q., Wilder, P. T., Carrier, F., and Weber, D. J. (2010) *J Biol Chem* 285, 27487-27498

[4] Lin, J., Yang, Q., Yan, Z., Markowitz, J., Wilder, P. T., Carrier, F., and Weber, D. J. (2004) *J Biol Chem* 279, 34071-34077

[5] Rustandi, R. R., Baldisseri, D. M., Inman, K. G., Nizner, P., Hamilton, S. M., Landar, A., Zimmer, D. B., and Weber, D. J. (2002) *Biochemistry* 41, 788-796

[6] Charpentier, T. H., Wilder, P. T., Liriano, M. A., Varney, K. M., Pozharski, E., MacKerell, A. D., Jr., Coop, A., Toth, E. A., and Weber, D. J. (2008) *J Mol Biol* 382, 56-73

[7] Marenholz, I., Heizmann, C. W., and Fritz, G. (2004) *Biochem Biophys Res Commun* 322, 1111-1122

[8] Donato, R. (2001) *Int J Biochem Cell Biol* 33, 637-668

[9] Harpio, R., and Einarsson, R. (2004) *Clin Biochem* 37, 512-518

[10] Zimmer, D. B., Cornwall, E. H., Landar, A., and Song, W. (1995) *Brain Res Bull* 37, 417-429

[11] Fesik, S. W. (1993) *J Biomol NMR* 3, 261-269

[12] Gaynor, R., Herschman, H. R., Irie, R., Jones, P., Morton, D., and Cochran, A. (1981) *Lancet* 1, 869-871

[13] Van Eldik, L. J., and Griffen, W. S. T. (1994) *Biochimica et Biophysica Acta* 1223, 398-403

[14] Steiner, J., Bogerts, B., Schroeter, M. L., and Bernstein, H. G. (2011) *Clinical chemistry and laboratory medicine : CCLM / FESCC* 49, 409-424

[15] Steiner, J., Marquardt, N., Pauls, I., Schiltz, K., Rahmoune, H., Bahn, S., Bogerts, B., Schmidt, R. E., and Jacobs, R. (2011) *Brain Behav Immun* 25, 1233-1241

[16] Rothermundt, M., Ahn, J. N., and Jorgens, S. (2009) *Gen Physiol Biophys* 28 Spec No Focus, F76-81

[17] Kligman, D., and Hilt, D. C. (1988) *Trends in biochemical sciences* 13, 437-443

[18] Donato, R. (1991) *Cell Calcium* 12, 713-726

[19] Moore, B. W. (1965) *Biochemical and biophysical research communications* 19, 739-744

[20] Isobe, T., Ishioka, N., and Okuyama, T. (1981) *European Journal of Biochemistry* 115, 469-474

[21] Hunter, M. J., and Chazin, W. J. (1998) *J Biol Chem* 273, 12427-12435

[22] Kretsinger, R. H. (1979) *Adv Cyclic Nucleotide Res* 11, 1-26

[23] Drohat, A. C., Baldisseri, D. M., Rustandi, R. R., and Weber, D. J. (1998) *Biochemistry* 37, 2729-2740

[24] Baudier, J., Galsser, N., Haglid, K., and Gerard, D. (1984) *Biochimica et Biophysica Acta* 790, 164-173

[25] Barber, K. R., McClintock, K. A., Jamieson, G. A., Jr., Dimlich, R. V., and Shaw, G. S. (1999) *J Biol Chem* 274, 1502-1508

[26] Gentil, B. J., Delphin, C., Mbele, G. O., Deloulme, J. C., Ferro, M., Garin, J., and Baudier, J. (2001) *J Biol Chem* 276, 23253-23261

[27] Markowitz, J., Carrier, F., Varney, K. M., Mackerell, A. D., Jr., and Weber, D. J. (2005) *AACR Meeting Abstracts* 2005, 1140-a-

[28] Rustandi, R. R., Drohat, A. C., Baldisseri, D. M., Wilder, P. T., and Weber, D. J. (1998) *Biochemistry* 37, 1951-1960

[29] Markowitz, J., Rustandi, R. R., Varney, K. M., Wilder, P. T., Udan, R., Wu, S. L., Horrocks, W. D., and Weber, D. J. (2005) *Biochemistry* 44, 7305-7314

[30] Markowitz, J., Mackerell, A. D., Jr., Carrier, F., Charpentier, T. H., and Weber, D. J. (2005) *Curr Top Med Chem* 5, 1093-1108

[31] Ferreon, J. C., Lee, C. W., Arai, M., Martinez-Yamout, M. A., Dyson, H. J., and Wright, P. E. (2009) *Proc Natl Acad Sci U S A* 106, 6591-6596

[32] Wright, N. T., Cannon, B. R., Wilder, P. T., Morgan, M. T., Varney, K. M., Zimmer, D. B., and Weber, D. J. (2009) *Journal of molecular biology* 386, 1265-1277

[33] Wright, N. T., Inman, K. G., Levine, J. A., Cannon, B. R., Varney, K. M., and Weber, D. J. (2008) *J Biomol NMR* 42, 279-286

[34] Wright, N. T., Prosser, B. L., Varney, K. M., Zimmer, D. B., Schneider, M. F., and Weber, D. J. (2008) *J Biol Chem* 283, 26676-26683

[35] Liriano, M. A., Varney, K. M., Wright, N. T., Hoffman, C. L., Toth, E. A., Ishima, R., and Weber, D. J. (2012) *Journal of molecular biology*

[36] Wilder, P. T., Baldisseri, D. M., Udan, R., Vallely, K. M., and Weber, D. J. (2003) *Biochemistry* 42, 13410-13421

[37] Charpentier, T. H., Thompson, L. E., Liriano, M. A., Varney, K. M., Wilder, P. T., Pozharski, E., Toth, E. A., and Weber, D. J. (2010) *J Mol Biol* 396, 1227-1243

[38] Malashkevich, V. N., Varney, K. M., Garrett, S. C., Wilder, P. T., Knight, D., Charpentier, T. H., Ramagopal, U. A., Almo, S. C., Weber, D. J., and Bresnick, A. R. (2008) *Biochemistry* 47, 5111-5126

[39] Baudier, J., and Gerard, D. (1986) *J Biol Chem* 261, 8204-8212

[40] Wright, N. T., Cannon, B. R., Zimmer, D. B., and Weber, D. J. (2009) *Current chemical biology* 3, 138-145

[41] Hauschild, A., Engel, G., Brenner, W., Glaser, R., Monig, H., Henze, E., and Christophers, E. (1999) *The British journal of dermatology* 140, 1065-1071

[42] Hauschild, A., Engel, G., Brenner, W., Glaser, R., Monig, H., Henze, E., and Christophers, E. (1999) *Oncology* 56, 338-344

[43] Hauschild, A., Michaelsen, J., Brenner, W., Rudolph, P., Glaser, R., Henze, E., and Christophers, E. (1999) *Melanoma research* 9, 155-161

[44] Karnell, R., von Schoultz, E., Hansson, L. O., Nilsson, B., Arstrand, K., and Kagedal, B. (1997) *Melanoma Res* 7, 393-399

[45] Huang, M. Y., Wang, H. M., Chang, H. J., Hsiao, C. P., Wang, J. Y., and Lin, S. R. (2012) *DNA Cell Biol* 31, 43-49

[46] Huang, M. Y., Wang, H. M., Tok, T. S., Chang, H. J., Chang, M. S., Cheng, T. L., Wang, J. Y., and Lin, S. R. (2012) *DNA Cell Biol* 31, 625-635

[47] Hwang, C. C., Chai, H. T., Chen, H. W., Tsai, H. L., Lu, C. Y., Yu, F. J., Huang, M. Y., and Wang, J. Y. (2011) *Ann Surg Oncol* 18, 139-145

[48] Preusser, M., Hoischen, A., Novak, K., Czech, T., Prayer, D., Hainfellner, J. A., Baumgartner, C., Woermann, F. G., Tuxhorn, I. E., Pannek, H. W., Bergmann, M., Radl-

wimmer, B., Villagran, R., Weber, R. G., and Hans, V. H. (2007) *Am J Surg Pathol* 31, 1709-1718

[49] Gartner, W., Ilhan, A., Neziri, D., Base, W., Weissel, M., Wohrer, A., Heinzl, H., Waldhor, T., Wagner, L., and Preusser, M. (2010) *Neuro Oncol* 12, 1004-1008

[50] Hancq, S., Salmon, I., Brotchi, J., Gabius, H. J., Heizmann, C. W., Kiss, R., and Decaestecker, C. (2004) *Int J Oncol* 25, 1233-1240

[51] Pang, X., Min, J., Liu, L., Liu, Y., Ma, N., and Zhang, H. (2012) *Med Oncol*

[52] Yang, J. F., Zhang, X. Y., and Qi, F. (2004) *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 29, 301-304

[53] Nagasaka, A., Umekawa, H., Hidaka, H., Iwase, K., Nakai, A., Ariyoshi, Y., Ohyama, T., Aono, T., Nakagawa, H., Ohtani, S., and et al. (1987) *Metabolism* 36, 388-391

[54] American Cancer Society. (2011)

[55] Van Eldik, L. J., and Wainwright, M. S. (2003) *Restor Neurol Neurosci* 21, 97-108

[56] Sorci, G., Bianchi, R., Riuzzi, F., Tubaro, C., Arcuri, C., Giambanco, I., and Donato, R. (2010) *Cardiovascular psychiatry and neurology* 2010

[57] Zimmer, D. B., Chaplin, J., Baldwin, A., and Rast, M. (2005) *Cell Mol Biol (Noisy-le-grand)* 51, 201-214

[58] Mori, T., Asano, T., and Town, T. (2010) *Cardiovascular psychiatry and neurology* 2010

[59] Mrak, R. E., and Griffin, W. S. (2004) *J Neuropathol Exp Neurol* 63, 679-685

[60] Castets, F., Griffin, W. S., Marks, A., and Van Eldik, L. J. (1997) *Brain Res Mol Brain Res* 46, 208-216

[61] Rothermundt, M., Ponath, G., and Arolt, V. (2004) *Int Rev Neurobiol* 59, 445-470

[62] Roltsch, E., Holcomb, L., Young, K. A., Marks, A., and Zimmer, D. B. (2010) *J Neuroinflammation* 7, 78

[63] Wilder, P. T., Lin, J., Bair, C. L., Charpentier, T. H., Yang, D., Liriano, M., Varney, K. M., Lee, A., Oppenheim, A. B., Adhya, S., Carrier, F., and Weber, D. J. (2006) *Biochim Biophys Acta* 1763, 1284-1297

[64] Millward, T. A., Heizmann, C. W., Schafer, B. W., and Hemmings, B. A. (1998) *Embo J* 17, 5913-5922

[65] Bhattacharya, S., Large, E., Heizmann, C. W., Hemmings, B., and Chazin, W. J. (2003) *Biochemistry* 42, 14416-14426

[66] Skripnikova, E. V., and Gusev, N. B. (1989) *FEBS Lett* 257, 380-382

[67] Wills, F. L., McCubbin, W. D., and Kay, C. M. (1993) *Biochemistry* 32, 2321-2328

[68] Inman, K. G., Yang, R., Rustandi, R. R., Miller, K. E., Baldisseri, D. M., and Weber, D. J. (2002) *J Mol Biol* 324, 1003-1014

[69] Bianchi, R., Garbuglia, M., Verzini, M., Giambanco, I., Ivanenkov, V. V., Dimlich, R. V., Jamieson, G. A., Jr., and Donato, R. (1996) *Biochimica et Biophysica Acta* 1313, 258-267

[70] Mbele, G. O., Deloulme, J. C., Gentil, B. J., Delphin, C., Ferro, M., Garin, J., Takahashi, M., and Baudier, J. (2002) *J Biol Chem* 277, 49998-50007

[71] Sheu, F. S., Huang, F. L., and Huang, K. P. (1995) *Arch Biochem Biophys* 316, 335-342

[72] Baudier, J., and Cole, R. D. (1988) *J. Biol. Chem.* 263, 5876-5883

[73] Donato, R. (1987) *Cell Calcium* 8, 283-297

[74] Zimmer, D. B., and Van Eldik, L. J. (1986) *J Biol Chem* 261, 11424-11428

[75] Landar, A., Caddell, G., Chessher, J., and Zimmer, D. B. (1996) *Cell Calcium* 20, 279-285

[76] Baudier, J., Delphin, C., Grunwald, D., Khochbin, S., and Lawrence, J. J. (1992) *Proc Natl Acad Sci U S A* 89, 11627-11631

[77] Wilder, P. T., Rustandi, R. R., Drohat, A. C., and Weber, D. J. (1998) *Protein Sci* 7, 794-798

[78] Donato, R., Cannon, B. R., Sorci, G., Riuzzi, F., Hsu, K., Weber, D. J., and Geczy, C. L. (2012) *Curr Mol Med*

[79] Brozzi, F., Arcuri, C., Giambanco, I., and Donato, R. (2009) *J Biol Chem* 284, 8797-8811

[80] Albert, K. A., Wu, W. C., Nairn, A. C., and Greengard, P. (1984) *Proc Natl Acad Sci U S A* 81, 3622-3625

[81] Baudier, J., Mochly-Rosen, D., Newton, A., Lee, S. H., Koshland, D. E., Jr., and Cole, R. D. (1987) *Biochemistry* 26, 2886-2893

[82] Sakaguchi, K., Sakamoto, H., Lewis, M. S., Anderson, C. W., Erickson, J. W., Appela, E., and Xie, D. (1997) *Biochemistry* 36, 10117-10124

[83] Sakaguchi, K., Sakamoto, H., Xie, D., Erickson, J. W., Lewis, M. S., Anderson, C. W., and Appella, E. (1997) *J Protein Chem* 16, 553-556

[84] Markowitz, J., Chen, I., Gitti, R., Baldisseri, D. M., Pan, Y., Udan, R., Carrier, F., MacKerell, A. D., Jr., and Weber, D. J. (2004) *J Med Chem* 47, 5085-5093

[85] Whitlow, J. L., Varughese, J. F., Zhou, Z., Bartolotti, L. J., and Li, Y. (2009) *Journal of molecular graphics & modelling* 27, 969-977

[86] Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001) *Adv Drug Deliv Rev* 46, 3-26

[87] Guvench, O., and MacKerell Jr, A. D. (2009) *PLoS Comp. Biol.* 5, e1000435

[88] Raman, E. P., Yu, W., Guvench, O., and Mackerell, A. D. (2011) *J Chem Inf Model* 51, 877-896

[89] Foster, T. J., Mackerell, A. D., Jr., and Guvench, O. (2012) *J Comput Chem* 33, 1880-1891

[90] Charpentier, T. H., Wilder, P. T., Liriano, M. A., Varney, K. M., Zhong, S., Coop, A., Pozharski, E., MacKerell, A. D., Jr., Toth, E. A., and Weber, D. J. (2009) *Biochemistry* 48, 6202-6212

[91] Markowitz, J., MacKerell, A. D., Jr., and Weber, D. J. (2007) *Mini Rev Med Chem* 7, 609-616

[92] Wilder, P. T., Charpentier, T. H., Liriano, M. A., Gianni, K., Varney, K. M., Pozharski, E., Coop, A., Toth, E. A., Mackerell, A. D., and Weber, D. J. (2010) *International journal of high throughput screening* 2010, 109-126

[93] Zimmer, D. B., Lapidus, R.G., Weber, D.J. (2012) *Methods Mol Biol* in press

[94] Leclerc, E., and Heizmann, C. W. (2011) *Front Biosci (Schol Ed)* 3, 1232-1262

[95] Zimmer, D. B., and Weber, D. J. (2010) *Cardiovascular psychiatry and neurology* 2010

[96] Vitolo, M. I., Weiss, M. B., Szmacinski, M., Tahir, K., Waldman, T., Park, B. H., Martin, S. S., Weber, D. J., and Bachman, K. E. (2009) *Cancer Research* 69, 8275-8283

[97] Weiss, M. B., Vitolo, M. I., Mohseni, M., Rosen, D. M., Denmeade, S. R., Park, B. H., Weber, D. J., and Bachman, K. E. (2010) *Oncogene* 29, 4715-4724

[98] Singh, M., Lima, A., Molina, R., Hamilton, P., Clermont, A. C., Devasthali, V., Thompson, J. D., Cheng, J. H., Bou Reslan, H., Ho, C. C., Cao, T. C., Lee, C. V., Nannini, M. A., Fuh, G., Carano, R. A., Koeppen, H., Yu, R. X., Forrest, W. F., Plowman, G. D., and Johnson, L. (2010) *Nat Biotechnol* 28, 585-593

[99] Chin, L., Pomerantz, J., Polsky, D., Jacobson, M., Cohen, C., Cordon-Cardo, C., Horner, J. W., 2nd, and DePinho, R. A. (1997) *Genes & Development* 11, 2822-2834

[100] Larue, L., and Beermann, F. (2007) *Pigment Cell Res* 20, 485-497

[101] Lawrence, M. C., and Davis, P. C. (1992) *Proteins* 12, 31-41

[102] Martin, Y. C. (1992) *J Med Chem* 35, 2145-2154

[103] Ewing, T. J. A., and Kuntz, I. D. (1997) *J. Comput. Chem.* 18, 1175-1189

[104] Makino, S., and Kuntz, I. D. (1997) *J. Comp. Chem.* 18, 1812-1825

[105] Taylor, R. D., Jewsbury, P. J., and Essex, J. W. (2002) *J. Comp. Aided Mol. Des.* 16, 151-166

[106] Oprea, T. I., Ho, C. M. W., and Marshall, G. R. (1995) De Novo Design: Ligand construction and prediction of affinity, in computer-aided molecular design: Application

in agrochemicals, materials, and pharmaceuticals. (Reynolds, C. H., Holloway, M. K., and Cox, H. K. eds.), American Chem. Soc., Washington, D.C. pp 64-81

[107] Schneider, G., Lee, M. L., Stahl, M., and Schneider, P. (2000) *J Comput Aided Mol Des* 14, 487-494

[108] Hajduk, P. J., Dinges, J., Miknis, G. F., Merlock, M., Middleton, T., Kempf, D. J., Egan, D. A., Walter, K. A., Robins, T. S., Shuker, S. B., Holzman, T. F., and Fesik, S. W. (1997) *J Med Chem* 40, 3144-3150

[109] Hajduk, P. J., Meadows, R. P., and Fesik, S. W. (1997) *Science* 278, 497-499

[110] Mayer, M., and Meyer, B. (2001) *J Am Chem Soc* 123, 6108-6117

[111] Meinecke, R., and Meyer, B. (2001) *J Med Chem* 44, 3059-3065

IntechOpen