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The evolution of S100B inhibitors for the treatment of malignant melanoma

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Abstract

Malignant melanoma continues to be an extremely fatal cancer due to a lack of viable treatment options for patients. The calcium-binding protein S100B has long been used as a clinical biomarker, aiding in malignant melanoma staging and patient prognosis. However, the discovery of p53 as a S100B target and the consequent impact on cell apoptosis redirected research efforts towards the development of inhibitors of this S100B–p53 interaction. Several approaches, including computer-aided drug design, fluorescence polarization competition assays, NMR, x-ray crystallography and cell-based screens have been performed to identify compounds that block the S100B–p53 association, reactivate p53 transcriptional activities and induce cancer cell death. Eight promising compounds, including pentamidine, are presented in this review and the potential for future modifications is discussed. Synthesis of compound derivatives will likely exhibit increased S100B affinity and mimic important S100B–target dynamic properties that will result in high specificity.

Targeting human malignant melanoma

Melanoma of the skin arises when melanocytes experience unregulated cell growth, forming tumors and invading neighboring tissues. Although melanoma is one of the least common skin cancer types, it accounts for 79% of skin cancer-related deaths. According to the American Cancer Society the number of new melanoma cases has been increasing for years, and it is estimated that 76,250 new patients will have been diagnosed with melanoma in 2012 [1–3].

Ideally, an individual only has a 2% risk of developing melanoma of the skin in his or her lifetime [3]. Yet there are several factors that can increase this risk, including excessive exposure to UV light, family history and complexion. Shortly following the diagnosis of melanoma, the disease stage is determined (0–IV) based on the thickness of the melanoma, mitotic rate, presence of ulceration, lymph node involvement and metastasis [1–3]. Staging can also be assessed with the use of clinical biomarkers, which are proteins that are present

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in the blood or other bodily fluids that assess the severity or progression of a disease. Several biomarkers for melanoma have been proposed, including lactate dehydrogenase (LDH), melanoma inhibiting activity protein, and S100B [4]. Although LDH and S100B differ greatly with respect to their biological activities, these two serum markers were shown to be independent prognostic factors in malignant melanoma (MM) patients with distant metastasis [5]. Furthermore, widespread clinical testing for S100B has prompted numerous studies, concluding that elevated S100B levels are indicative of advanced disease stage, poor therapeutic response, increased recurrence and low overall survival [6,7].

In 1980, S100B was found to be over-expressed in cultured human MM cells, and shortly afterwards was also determined to be present at elevated levels in melanoma tumor biopsies but not in normal skin samples and non-melanoma tumors [8–10]. Since then, S100B has proven to be a strong cancer biomarker for melanoma. For example, a study conducted by Hauschild *et al.* with 412 melanoma patients established a threshold value of 0.2 µg/l S100B, where patients expressing levels below this cutoff were considered negative [6]. It was found that S100B serum levels increase with advancing tumor stage and were indicative of micro- or macro-metastases [7,11]. Although S100B cannot be used to identify tumor thickness or lymph node status, it is still of prognostic value. A higher concentration of protein at each stage correlates with increased recurrence and low overall patient survival [6,7, 12]. This suggests that S100B should be used as a means of monitoring the effectiveness of patients' therapy. Rising levels of S100B have consistently proved to be a sensitive and specific marker of cancer progression, with the ability to detect metastases or relapse weeks or even months earlier than alternative methods. Use of S100B as a biomarker can also assist in assigning proper treatment by identifying unsuccessful strategies early on [7].

While the number of available therapies for MM patients is growing, surgery is still almost always the first and best treatment option, often curing early stage melanomas. More advanced cancers, however, require additional treatments including chemotherapy and radiation. Unfortunately, melanoma is notoriously resistant to these conventional treatments and as a result, they are mainly used to relieve painful symptoms, reduce tumor size and extend the life of the patient [1–3].

Immunotherapy of MM has recently received attention following the US FDA approval of a monoclonal antibody targeting CTLA-4 called ipilimumab (Bristol-Myers Squibb) [13]. This treatment functions by blocking CTLA-4 expressed on cytotoxic T lymphocytes, thereby allowing for sustained immune activity and inducing an anti-tumor response [13,14]. Ipilimumab has produced relatively meaningful results in clinical trials; however, only a small percentage of patients respond to the treatment [13]. While continued research on immune-mediated targeting of tumor cells will provide a more complete mechanistic understanding and potentially drive the development of improved monoclonal antibodies, additional treatments must still be pursued.

There are several genes that are frequently mutated in melanoma [15–17] and the development of protein inhibitors capable of targeting these oncogenic signaling pathways are very promising alternative treatments (**Table 1**) [18]. Yet, many of the target inhibitors presented exhibit detrimental off-target effects. For example, the significantly increased activity of the MAPK pathway in melanoma makes its constituents particularly attractive targets [19]. Mutated *BRAF* has been observed in greater than 50% of MMs, with approximately 90% of those cases harboring the *BRAF V600E* substitution mutation [15]. Thus, for the last decade, *BRAF* has been the focus for the targeted therapy of melanoma. Clinical trials began with the pan-kinase inhibitor sorafenib that demonstrated little efficacy with many off-target effects, highlighting the importance of selectivity when developing

BRAF inhibitors [19,20]. Recently, more potent and specific compounds have been established. Vemurafenib (PLX4032, Plexxicon) and dabrafenib (GSK2118436, GlaxoSmithKline) have both been extensively studied and were found to inhibit tumor growth in melanoma patients (**Table 1**). Although the initial responses were impressive, follow-up studies revealed that they were short-lived, with treated patients eventually acquiring resistance in every case [19,21,22].

Resistance to BRAF inhibition has been determined to occur as a result of reactivation of MAPK signaling [23]. In order to avert this onset of resistance, combination therapies targeting multiple pathways or multiple kinases in the same pathway are being investigated. For example, dual inhibition of BRAF and MEK has been found to increase apoptosis while preventing the acquisition of resistance in melanoma cells [22,23]. Preclinical studies performed on BRAF-resistant melanoma cell clones found that combination therapy using these two inhibitors suppressed MAPK signaling and reduced cellular proliferation, suggesting that the dual targeting could deter the outgrowth of resistant cells, as well as inhibit cell growth [24]. However, continued identification of additional inhibitors that could work synergistically with BRAF inhibitors to treat MM is urgently necessary and S100B has been determined to be a viable target. The process for S100B compound inhibitor discovery is illustrated in **Figure 1** and will be discussed in detail throughout this review.

The S100B calcium-binding protein

The S100 family of proteins is a unique class of **EF-hand** calcium-binding proteins composed of more than 20 members, making it the largest of the EF-hand super family. The first S100 protein was discovered in 1965 in a subcellular fraction from bovine brain tissue; however, it was later determined that this sample contained two similar, but distinct proteins that were designated S100 α and S100 β , and now referred to as S100A1 and S100B, respectively [25,26].

The S100 genes are only present in vertebrates and their coding sequences are approximately 50% homologous [27]. The highest sequence similarity is found in the Ca²⁺-binding domains, while the hinge region and the C-terminal loop share the least amount of sequence similarity, likely allowing for individual target specificity [27–29]. While S100 proteins have no inherent enzymatic activity, they are still important biological regulators through specific Ca²⁺-dependent protein–protein interactions [26,30]. S100 proteins have been found to have diverse functional roles and include involvement in calcium homeostasis, cell–cell communication, cell proliferation, differentiation, cytoskeletal dynamics and cell morphology [7,27,29,31].

One of the most extensively studied members of the S100 family is S100B, a 21.5-kDa symmetric homodimer that is found in melanocytes, glial cells, chondrocytes and adipocytes, exhibiting both intra- and extracellular functionality [7,29]. For example, S100B is present at high intracellular levels in glial cells and, when excreted, regulates neuronal cell activities. Low amounts of extracellular S100B promote growth and survival in nearby neuronal cells, while elevated amounts can lead to neuronal cell apoptosis [7,32]. Similarly, in melanocytes, low levels of S100B are sufficient for normal cellular function, but serious complications arise when the levels become elevated.

As with all S100 family members, each subunit of S100B contains four α helices (**Figure 2**). Helix 1, helix 2 and loop 1 comprise the first HLH motif of the ‘S100’ or ‘*pseudo*’ EF-hand (EF1), whereas helix 3, helix 4 and loop 2 make up the canonical EF-hand. These motifs are connected by the ‘hinge’ region, which consists of 10–12 residues and is crucial for target interactions. Upon binding Ca²⁺, S100B undergoes a significant conformational

change where helix 3 becomes perpendicular to helix 4, exposing a hydrophobic cleft where molecular targets can then bind [28]. Typically, the S100 EF-hand binds Ca^{2+} with a relatively low affinity ($K_D = 200\text{--}500\ \mu\text{M}$), while the true EF-hand binds Ca^{2+} with a much higher affinity ($K_D = 20\text{--}50\ \mu\text{M}$). Specifically, the affinity of a S100B monomer's true EF-hand for Ca^{2+} is fivefold tighter than that of its S100 EF-hand [33,34]. In addition to binding Ca^{2+} , S100B also binds Zn^{2+} at a distinct site; however, Zn^{2+} alone is not capable of inducing target binding, but functions in conjunction with Ca^{2+} by increasing S100B's affinity for its target proteins [35,36]. S100B binds Zn^{2+} with a relatively high affinity ($K_D = 94.2 \pm 16.7\ \text{nM}$), increasing S100B- Ca^{2+} binding by as much as tenfold [33,37]. In fact, S100B's affinity for intracellular Ca^{2+} may be too low to be activated without the addition of Zn^{2+} . This is unlikely to be an issue for extracellular functionality, since Ca^{2+} and Zn^{2+} are present at higher concentrations beyond the cell membrane [29]. In fact, as a result of this elevation in Ca^{2+} levels, extracellular S100B dimers can assemble themselves into larger complexes, creating tetramers, hexamers and even octomers. Additional molecules of Ca^{2+} bind to the protein interfaces of these structures to aid in stabilization, allowing them to perform cytokine-like activities, particularly in the brain [38].

Intracellular S100B has been discovered to interact with a variety of target proteins, and, thus, has been implicated in a wide range of cellular functions [39]. Several groups have previously shown that S100B directly interacts with the tumor suppressor p53, inhibiting tetramerization and PKC-dependent phosphorylation, consequently decreasing p53 DNA binding and transcriptional activity [33,40–42]. Hdm2, an important negative regulator of p53, has also been identified as an S100B target, suggesting the possibility that the two work in concert to regulate p53 function [43]. In addition to p53, S100B inhibits the phosphorylation of multiple other PKC targets, including MARCKS, τ -protein and caldesmon [44–46]. S100B's calcium-dependent interactions with τ -protein and caldesmon contribute to the modulation of cytoskeletal dynamics via microtubule assembly and/or disassembly, and regulation of actin and myosin, respectively [46,47].

There is also mounting evidence indicating that S100B acts to stimulate proliferation and migration, while suppressing differentiation and apoptosis [48–50]. Arcuri *et al.* showed that expression of S100B in neuronal cells results in increased proliferation and decreased responsiveness to the differentiation agent NGF, attributed to S100B-induced AKT activation [50]. Shortly thereafter Riuzzi and colleagues found that in myoblasts S100B stimulated ERK phosphorylation, promoting proliferation and, at low concentrations, also induced NF- κ B activity, resulting in protection from apoptosis. Concomitantly, but independently, S100B inhibited p38 phosphorylation and, as a result, differentiation [49]. It was also recently discovered that S100B prevents melanoma cell apoptosis as a result of its interaction with p53, underscoring the importance of elevated S100B and the detrimental downstream effects. By reducing S100B protein levels with siRNA constructs, it was possible to activate p53 and induce cell apoptosis via the extrinsic pathway [51]. With this knowledge, therapeutic strategies are underway to target Ca^{2+} -bound S100B with small-molecule inhibitors, blocking the Ca^{2+} -dependent S100B–target interactions with the hopes of inhibiting the progression of cancers, particularly MM.

Inhibition of S100B

In order to successfully identify compounds that will bind to S100B and inhibit target complex formation, a combination of several approaches have been applied (**Figure 1**). The use of computer modeling provided a way to virtually screen hundreds of thousands of compounds based on physical characteristics that complement a designated site on the target protein. In the case of S100B, inhibitors that were determined to bind to the hydrophobic cleft were considered hits. A fluorescence assay was also utilized through which a large

number of compounds were examined for their ability to compete a known target peptide off of calcium-loaded S100B. The structural interaction between S100B and a selected inhibitor was then validated using NMR and/or x-ray crystallography. Strategies are also described in the 'Future perspective' section where NMR can additionally be utilized to study the dynamic properties of S100B-target interactions and to provide a higher degree of specificity in identifying compounds capable of disrupting the p53–S100B association as another potential method of validation. Furthermore, biological assays were performed to demonstrate the capability of each compound to penetrate the cell membrane, inhibit target protein activity and decrease melanoma cell growth.

Computer-aided drug design

The discovery of the S100B–p53 interaction raised the possibility that compounds could be developed to inhibit S100B binding, thereby increasing p53 transcriptional activity and inducing cancer cell death (**Figure 2**). The hydrophobic binding pocket that is exposed upon the addition of calcium makes up part of the S100B–p53 binding interface and became the focus of the search for small-molecule S100B inhibitors [33]. Computer-aided drug design (CADD) was utilized in order to screen hundreds of thousands of known drugs and predicted compounds from multiple databases, taking into account the physical characteristics of the defined binding pocket in order to identify potential inhibitors. The most promising compounds identified in these assays were then further characterized structurally, as well as biologically [52,53]. This rational drug design program resulted in the identification of several putative S100B inhibitors, most notably **pentamidine isethionate**, which is also referred to as SB1[52]. This finding, once confirmed via biochemical and *in vivo* studies, coupled with the drug's prior FDA approval, facilitated a rapid transition to clinical trials at the University of Maryland Medical Center to determine its efficacy in patients [SAUSVILLE E, WEBER DJ, UNPUBLISHED DATA].

CADD can also be employed to optimize previously identified compounds in order to improve their binding affinity, specificity, absorption, distribution, and metabolism/excretion (ADME) properties. A new fragment-based drug optimization strategy has been developed, termed site identification by ligand competitive saturation (SILCS), which aids in identifying potential modifications that can improve binding affinity and specificity. SILCS is an *in silico* approach that computationally combines the target protein of interest with aliphatic and aromatic molecules in an aqueous solution to be used in molecular dynamics simulations. A 3D map of the protein surface is ultimately generated that depicts the probability of favorable protein interactions. As a result, small molecules, such as hydrophobic groups, aromatic groups, hydrogen bond donors and hydrogen bond acceptors, can be identified that have a strong affinity for a defined target protein region while incorporating atomic-level solvation, as well as protein flexibility [54–56]. This information can then be used to modify existing compounds with the addition of a strategically placed functional group or by linking two compounds together to span a greater area of the protein surface and increase binding affinity [54,55].

This methodology was particularly useful in the case of the protein S100B. There are three general sites that targets of S100B occupy, termed sites 1, 2 and 3 (**Figure 3**). For example, two known S100B targets, the C-terminal p53 peptide and CapZ peptide (TRTK-12), bind to Site 1 (**Figure 3A & 3B**), while pentamidine and other small molecules mainly bind to sites 2 and 3 (**Figure 3C**). It has been posited that small molecules engineered to span site 1 rather than 2 or 3 would directly compete with and inhibit p53, resulting in highly effective therapeutic drugs. Inhibitors that interact with multiple sites are also of interest as it is thought that they will exhibit synergistic binding and higher specificity in comparison to their single-site counterparts. The use of SILCS provided a computational method to direct

the chemical modifications of such small-molecule inhibitors to improve binding affinity and specificity. Investigations are continuously ongoing to identify compounds and consequently synthesize derivatives that act as S100B inhibitors (designated SBiX, where 'X' is the compound number) and, therefore, potential novel chemotherapy agents [57,58].

Biochemical high-throughput screening

Once promising candidate compounds have been identified, in-depth biochemical studies can be conducted to determine potency and define the parameters of use. Fluorescence polarization competition assays (FPCAs) and isothermal calorimetry can be performed to evaluate the binding affinity of each compound for S100B, while NMR and x-ray crystallography can provide detailed information on the structural interaction. The combination of such methods with CADD allows for binding validation and compound optimization with the goal of achieving drugs with low nanomolar binding affinities, adequate specificity, and the desired ADME properties. The top eight compounds generated through different combinations of these techniques are shown in **Figure 4**.

FPCA—The development of FPCAs and their implementation as a high-throughput screening system has been quite powerful. The binding affinity of predicted protein inhibitors can rapidly be measured with relative sensitivity and little time requirements and materials. The concept of FPCA makes use of a known interaction between two molecules with differing molecular weights, labeling the smaller molecule with a fluorophore. Upon exposure to polarized light, excitation can be measured both parallel and perpendicular to the source, and the degree of polarization can be determined. A fluorophore bound to a larger complex of molecules will tumble more slowly in solution, and, therefore, retain a higher degree of polarization, while smaller, faster molecules will exhibit lower polarization [59].

In 2010, Wilder *et al.* designed a high-throughput FPCA to examine the interactions between S100B and several different inhibitors. In this study, a peptide derived from the CapZ protein, termed TRTK-12, was chosen to act as the probe, labeled with the TAMRA fluorophore, and bound to calcium-loaded S100B. TRTK-12 was previously determined to bind S100B at Site 1 (**Figure 3b**), competing with the p53 peptide, and TAMRA-TRTK was found to bind S100B with an ideal affinity for this large-scale screen ($K_D = 1.19 \pm 0.65 \mu\text{M}$). Upon the addition of successful small-molecule inhibitors, the TAMRA-TRTK probe will be displaced, or competed off, from the S100B protein decreasing the measurable fluorescence polarization (**Figure 5**). Initial screens are performed with four drug concentrations, followed by a more robust titration for the resultant lead compounds, ultimately producing an accurate binding affinity estimate for each. Three excellent candidates were identified in this way (SC0067, SC0332, SC0844) and advanced to additional validation steps [59].

NMR—Confirmation of the S100B-compound interactions is achieved through the use of NMR spectroscopy, providing important residue-specific information and eliminating any false-positives. Lead compounds are titrated in excess into ^{15}N -labeled S100B and monitored by NMR spectroscopy using the HSQC experiment, which allows for visualization of the backbone amide peaks for each residue in the protein. Chemical shift perturbations resulting from compound addition can be observed upon comparison to calcium-loaded S100B alone, indicating residues directly involved in the compound interaction, and from which a rough map of the binding site can be created [59].

Due to the high degree of similarity between S100B and S100A1, compound specificity can easily be evaluated by HSQC titrations of the compounds into ^{15}N -labeled S100A1 and

monitoring perturbations, as was performed in the case of SBI132, SBI279 and SBI523. If no perturbations are observed with S100A1, then the compounds are considered to be highly specific for S100B. Likewise, the calcium dependency of the interactions can be determined by adding compounds to ^{15}N -labeled apo-S100B in the presence of a calcium chelator such as EGTA; a lack of perturbations then indicates the necessity of calcium [60].

A second approach known as saturation transfer difference NMR often compliments HSQC data and provides information on the protons that are located at the protein–compound interface. In this way, group epitope mapping can be achieved, qualitatively representing the relative proximity of the protons associated with the inhibitor to those on the S100B protein [60]. It is also possible to quickly differentiate compounds based on their binding strengths and infer structure/function relationships [57]. Saturation transfer difference NMR data sets were obtained for the majority of the S100B–compound interactions (SC0067, SC0332, SC844, SBI132, SBI279, SBI523 and SBI1) (**Figure 6; Table 2**), and the proton assignments were confirmed using 2D TOCSY NMR experiments [60]. In order to determine the precise conformation and orientation of each inhibitor, however, the structure of the S100B–compound complex must also be solved, and for this x-ray crystallography is often employed.

x-ray crystallography—Crystallization of S100B in complex with candidate compounds allows for detailed determination of the exact position of potential inhibitors at atomic resolution. This not only provides another level of validation for the interactions, but also the opportunity to identify potential means of modifying the compounds to improve binding and/or specificity.

The x-ray structure of pentamidine bound to calcium-loaded S100B was solved at 2.15-Å resolution and revealed that two molecules bind to each S100B monomer, one each in sites 2 and 3 (**Figure 3C**) [61]. Further modeling and analysis indicated that increasing the linker length between the amidine moieties of pentamidine could create derivatives with increased affinity for S100B. Subsequently, SBI4211, also termed heptamidine, was synthesized with seven methylene units as opposed to the five units of pentamidine (**Figure 4**), and the S100B-SBI4211 crystal structure was determined at 1.65 Å. As a result, it was discovered that despite the structural similarity, only one molecule of SBI4211 binds to each monomer of S100B (**Figure 6; Table 2**), and this single SBI4211 molecule is able to span both sites 2 and 3 of S100B, signifying that additional modifications may enhance binding even further [McKnight LE, Prabhu Raman E, Bezawada P *et al*, Unpublished Data].

A compilation of several S100B–compound surface structures solved via NMR spectroscopy or x-ray crystallography are shown in **Figure 6**. This collection highlights the differences and similarities between the inhibitor binding sites and that of the p53 peptide. The structure/function relationship of each interaction provides invaluable insight and represents the future of S100B inhibitors. All of these structures will be useful in performing further CADD simulations for the continuous creation of novel inhibitors, building on the structural information as it becomes available.

Cellular high-throughput screening

The compounds confirmed as S100B inhibitors *in vitro* must also be examined in cellular assays to determine toxicity, membrane penetration, and elucidate the mechanism of action. Previously, such compounds have been evaluated for their ability to inhibit primary MM cell growth, as well as the extent of the effects in normal melanocytes [52]. Ideally, compounds would selectively inhibit melanoma cell growth, while having little effect on the melanocytes, demonstrating that the inhibition is not simply due to extreme toxicity. Pentamidine, for instance, was found to accomplish just that, significantly impeding

C8146A melanoma cell growth while exhibiting lesser effects on melanocytes [52]. Recently, a more elegant approach to this experiment has been developed that employs siRNA directed against S100B to create matched cell lines. MALME-3M cells that are p53 wild-type and express elevated levels of S100B were transfected with vector containing scrambled siRNA to act as the control, continually expressing high S100B, or vector containing anti-S100B siRNA to act as normal melanocytes with low S100B levels [McKnight LE, Prabhu Raman E, Bezawada P *et al*, Unpublished Data].

Typically, each compound is tested in both the high S100B line and the low S100B line at four concentrations to establish an LD₅₀. Compounds are designated as 'S100B specific' when a larger effect on cell growth is observed on the high S100B cell line than for the low S100B cell line. This assay minimizes variability by comparing effects on cell lines that have the same genetic background, attributing differences specifically to the inhibitors rather than unidentified mutations.

Future perspective

As another means to help validate and discover novel MM therapeutics, **relaxation dispersion** and other NMR dynamic experiments have been recently used to determine the site-specific dynamic effects of S100B molecular targets. As previously shown, calcium-bound S100B is dynamic at multiple time scales (nanoseconds–milliseconds) [62–64] and upon binding a molecular target, S100B becomes less dynamic and is able to achieve a more stable conformation (i.e., extension of helix 4 and 4') [62]. In general, calcium-loaded S100B has fast and slow time scale motions in back bone amides found in the hinge region, helix 3 and helix 4 (i.e., hydro phobic pocket), which are essential for S100–target binding. Once bound to a target, the majority of these backbone dynamics are quenched in the microsecond–millisecond time scale, where S100B can now bind calcium with a much higher affinity and is able to attain a less disordered state [62]. In order for any S100B inhibitors to achieve a therapeutic level of specificity, they must also mimic the dynamic-stabilizing effect that molecular targets have on S100B, which includes this increase in calcium affinity effect. Work is underway to complement 3D-structures of S100B–target complexes with site-specific dynamic information to help accomplish this goal.

Although there is still much work to be completed in advancing melanoma therapies, the immediate next steps are clear and will produce definitive results in the near future. The eight compounds presented here, as well as additional S100B inhibitors that are continuously being developed and characterized, will undergo stringent studies in mice to identify those that safely reduce tumor progression and would be best suited for human clinical trials. Each compound will be assessed on the extent of suppression achieved in both primary and secondary tumor sites. The information gathered through this work will aid in focusing the design and synthesis on the most efficacious compound classes. Moreover, strict attention will be paid to how tolerant the mice are of each compound, and modifications will be made as necessary to compounds that are not well tolerated. This iterative approach allows for the creation of derivatives, each superior to the former. It is expected that successful S100B inhibitors will stimulate tumors to exhibit decreased proliferation and increased apoptosis as a result of reactivation of the p53 pathway.

The assemblage of the *in vivo* mouse data with the previous biochemical and cellular results, along with the predicted ADME profile culminate to decide the future of each compound. The compounds with the highest potency and tolerability will be subjected to advanced *in vitro* testing, while others will undergo additional rounds of medicinal chemistry optimization or will be excluded altogether. Performance of *in vitro* ADME/toxicology/safety testing will provide information about potential human toxicity/metabolism/

permeability that cannot be obtained through animal studies, since none of the currently available models recapitulates the genetics and biology of human melanoma. Once past these milestones, the small-molecule inhibitors of S100B will progress to human clinical trials.

The drugs discovered as a result of these studies are likely to also be beneficial to patients suffering with cancers other than melanoma, as elevated S100B is also observed in thyroid carcinoma and renal cell carcinoma [65–67]. In addition, overexpression of S100B has long been known to be detrimental in brain tissue and has been linked to several neurological disorders [68]. The S100B inhibitors could potentially be repurposed for the treatment of Alzheimer's disease, Down's syndrome and schizophrenia [68–70]. Due to the assortment of multimeric forms of S100B that could be present depending on the proteins' subcellular location, particularly in the brain [38], studies must first be conducted to examine how the use of inhibitors designed to target the homodimer form effects the activities of the larger S100B complexes. Differences in how the inhibitors physically associate with the S100B forms, as well as the stoichiometry, could impact the efficacy of the compounds and/or cause adverse patient responses. As previously stated, S100B is normally expressed in certain cell types at relatively low levels and is sufficient to produce trophic effects [39]. The use of overly potent inhibitors to treat MM could interfere with the essential functions of S100B, perhaps resulting in reduced neuronal cell growth and survival, possibly resulting in more detrimental health issues for the patient. Many factors need to be taken into consideration when evaluating S100B inhibitors, however, thorough research and testing will yield viable treatment options for individuals suffering from MM. Undoubtedly, the development of novel inhibitors that are capable of targeting proteins outside of the MAPK pathway to combat disease will be invaluable to the medical community.

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Key Term

EF-hand motif: Domain comprised of a helix-loop-helix bound to calcium; using parvalbumin as an example, the E and F helices resemble the thumb and forefinger pair of the hand.

Key Term

Pentamidine isethionate:

US FDA-approved antimicrobial agent for the treatment of *Pneumocystis carinii* pneumonia

Key Term

Relaxation dispersion:

NMR experiment that measures site-specific dynamic information in the millisecond – microsecond time scale in isotopically enriched proteins (i.e., ^{15}N or ^{13}C).

Executive summary

Targeting human malignant melanoma

- While current therapies for the treatment of malignant melanoma, the deadliest of skin cancers, are lacking, the evolution of high-throughput screening to identify protein inhibitors makes for a promising future.

S100B calcium-binding protein

- The calcium-binding protein S100B is not only a diagnostic biomarker for malignant melanoma, but has also been determined to contribute to cancer progression by binding and inhibiting the tumor suppressor p53.

Inhibition of S100B

- Computer-aided drug design provides a rational and rapid method of screening millions of virtual compounds based on physical characteristics to identify potential S100B protein inhibitors that bind at any of three defined sites.
- The combination of several biochemical experiments, including fluorescence polarization competition assays, NMR dynamics and x-ray crystallography result in an accurate, detailed representation of the structure and orientation of the S100B–compound complexes, possibly identifying strengthening modifications that can be made to the compounds.
- Cellular high-throughput assays, though still in the early stages of development, readily distinguish true melanoma cell inhibitors from those that are unable to transverse the cell membrane or bind its target, as well as determine toxicity and specificity to cells expressing low levels of S100B.

Future perspective

- The top eight compounds presented will continue to undergo rigorous experimentation, including *in vivo* mouse studies, with the goal of ultimately producing novel therapeutic drugs to enter into human clinical trials.

Figure 1. S100B inhibitor design and validation

Strategies and results to be discussed in this paper are shown in green, while topics of future research aims are represented in orange. CADD: Computer-aided drug design; HTS: High-throughput screening.

Figure 2. Inhibition of the S100B–p53 Interaction

Ribbon diagrams of Ca^{2+} -bound S100B (x-ray, PDB: 1MHO) and p53³⁶⁷⁻³⁸⁸-bound Ca^{2+} -S100B (NMR, PDB: 1DT7). The helices in each S100B are colored blue, while the p53³⁶⁷⁻³⁸⁸ peptide is colored red. The two calcium ions per subunit are represented as green spheres. Small-molecule inhibitors are being developed that bind to the p53 binding site and inhibit formation of the S100B–p53 complex.

Figure 3. Depiction of the three binding sites on S100B

Structures of Ca²⁺-S100B bound to (A) the C-terminal negative regulatory domain of p53 (PDB: 1DT7) [71], (B) TRTK-12 (PDB: 1MWN) [72], and (C) pentamidine, also referred to SBi1 (PDB: 3CR4) [61]. The protein is depicted as a blue surface, and regions within 3 Å of the peptide or small molecule bound are colored yellow. TRTK-12, p53 peptide and pentamidine are shown in red.

Figure 4. S100B inhibitors

Top eight compounds confirmed to bind S100B.

Figure 5. Ribbon diagrams illustrating the fluorescence polarization competition assays One TAMRA–TRTK peptide (red) binds to each monomer of S100B–Ca²⁺ (blue) in the same region as p53 (site 1). Bound TAMRA–TRTK exhibits high polarization values; however, once displaced by a compound, the polarization values decrease measurably. Reproduced with permission from [59] © 2010 Dove Medical Press Ltd.

Figure 6. x-ray and NMR structures of peptides and small molecules bound to Ca^{2+} -S100B (A) A surface/ribbon diagram of Ca^{2+} -S100B (x-ray, PDB: 1MHO) is illustrated with Ca^{2+} ions depicted as green spheres. In panels b-k, the surface diagrams of the various compound and peptide complexes of Ca^{2+} -S100B are illustrated with peptides and compounds colored in red; (B) p53³⁶⁷⁻³⁸⁸ peptide (NMR, PDB: 1DT7), (C) TRTK-12 peptide (NMR, PDB: 1MWN), (D) SC0067/chlorpromazine (x-ray, PDB: 3LK0), (E) SC0332/thimerosal (x-ray, PDB: 3LK1), (F) SC0844/sanguinarine (x-ray, PDB: 3LLE), (G) SBI132 (x-ray, PDB: 3GK1), (H) SBI279 (x-ray, PDB: 3GK2), (I) SBI523 (x-ray, PDB: 3GK4), (J) SBI1/pentamidine (x-ray, PDB: 3CR4), (K) SBI4211/heptamidine (x-ray, PDB: 4FQO).

Table 1

Frequent mutations in melanoma and current therapies.

Gene [†]	Alteration	Frequency [‡] (%)	Drug [§]
<i>NRAS</i>	Mutation	15–30	—
<i>BRAF</i>	Mutation <i>V600E</i>	50–70 >90	PLX4032, GSK2118438
<i>MITF</i>	Amplification	10–20	—
<i>CDKN2A</i>	Mutation	<5	—
<i>PI3K</i>	Mutation	3	GDC0941, XL147
<i>PTEN</i>	Deletion, mutation	40	—
<i>p53</i>	Deletion, mutation	10–20	—

[†] Several gene mutations that frequently arise in melanoma are presented, although this is far from an exhaustive list.

[‡] Percentages are estimates based on previously reported observations.

[§] Drugs are in various stages of evaluation [15 – 18].

Table 2

Summary of biochemical studies on S100B inhibitors.

Compound	NMR [†]	Crystal structure (Å)	Ref.
SC0067 [‡]	Yes	2.04	[59]
SC0332 [‡]	Yes	1.79	[59]
SC0844 [‡]	Yes	1.85	[59]
SBi132 [‡]	Yes	2.1	[60]
SBi279 [‡]	Yes	1.98	[60]
SBi523 [‡]	Yes	1.9	[60]
SBi1 [‡]	Yes	2.15	[61]
SBi4211	Yes	1.65	[MCKNIGHT LE, PRABHLJ RAMAN E, BEZAWADA P ET AL., UNPUBLISHED DATA]

[†]Compounds confirmed to bind S100B via NMR HSQC experiments.[‡]Compounds verified using saturation transfer difference NMR.