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# Targeted Treatment of Prostate Cancer

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**Abstract** Over a half century ago, Charles Huggins demonstrated the response of prostate cancer to androgen deprivation therapy. Subsequently, many discoveries and evolving findings continued to support a research rationale focused on the androgen receptor (AR) as a key target for prostate cancer. More recently, preliminary trials have suggested that other targets could also be useful in the treatment of prostate cancer, and the proposed strategies for treatment have ranged from targeted toxins to immunotherapeutic agents. We provide an overview of some of these approaches, with an emphasis on those that employ prostate specific membrane antigen (PSMA) as a target. J. Cell. Biochem. 102: 571–579, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** prostate cancer; prostate specific membrane antigen (PSMA); prostate specific antigen (PSA)

Prostate cancer is highly treatable as long as it has not metastasized. Surgery and radiation therapy can achieve control of tumor localized to the prostate. Indeed the criticism is that we may be over-treating tumors that lack the potential to become a life threatening disease. Nevertheless, if we merely observe more aggressive forms of disease, there is a greater risk of metastatic spread, and the patient is more likely to die from the cancer [Scher et al., 2005]. The mainstay of treatment of advanced disease is androgen deprivation, with many of the tumors responding to a decrease in circulating androgens, anti-androgens or a combination of both. Androgen deprivation therapy is considered palliative. Even when the tumor is no longer responding to treatment, the tumor still expresses the androgen receptor (AR) which is driving expression of androgen responsive genes. In addition some receptors are mutated and are able to respond to other steroids or even anti-androgens [Culig, 2004; Bettoun et al., 2005; Scher et al., 2005; Dehm and Tindall, 2006; Wu et al., 2006]. Occasionally, AR may be increased in expression, making cells hypersensitive to low circulating amounts of androgens. Recent gene expression studies have found that the prostate cancer cells express increased levels of androgen metabolic enzymes enabling the production of androgenic steroids [Mostaghel et al., 2007]. Studies have shown that patterns of gene expression change dramatically in prostate cancer cells. Specifically, androgens initially stimulate genes that promote growth and differentiation, but later begin to downregulate differentiation and growth-suppression genes while promoting anti-apoptotic genes [Hendriksen et al., 2006]. Thus, developing new strategies for targeting androgen signaling to prevent activation has demonstrated resurgence. Groups are examining ways to block androgen activation by increasing the rate of AR degradation and these approaches were summarized following a recent symposium [Tindall et al., 2004].

Prostate cancer is similar to most solid tumors which exhibit a number of potential oncogenic changes that occur during its progression to cancer. The goal is to individualize tumor therapy, thus gene expression profiles of tumors are being characterized. However, genetic analysis in other solid tumors found an

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average of 90 mutations per patient sample; while it is believed that only a subset of these contribute to the oncogenic process. While this approach allows classification of individual tumors and potential individualized targeted therapy, it suggests that many different targets may need to be addressed [Sjoblom et al., 2006]. Recent findings of a fusion gene combining the androgen response element of the protease TMPRSS2 and the transcription factor ERG has further demonstrated the importance of androgen signaling in prostate cancer [Tomlins et al., 2005]. Furthermore, it has raised the possibility of targeting a specific oncogenic change, similar to those seen in hematological tumors [Tomlins et al., 2005].

Understanding of the genes expressed by the tumor cell population could provide potential targets for therapy. However, recent studies suggest that tumor cells have characteristics of stem cells and their differentiating daughter cells. If there is a tumor stem cell representing only a hundredth or a thousandth of the total cell population, experiments examining expressed genes and proteomic examinations may not provide information on the important stem cell population. A tumor stem cell theoretically is self-renewing, divides rarely, and generates differentiating cells that divide repeatedly but have a limited potential for continued proliferation. Because stem cell doubling time is less than the differentiating population, stem cells may be harder to eradicate since many chemotherapy agents are more active against cells in cycle. Stem cells also express membrane pumps that can serve to eliminate drugs, rendering the cells harder to eliminate. This is a critical consideration because eradicating the bulk of a tumor with a therapy could eliminate cells in the differentiating population without treating the stem cells that can re-populate the tumor at a later time. The potential presence of a stem cell-like population should be considered in investigations of prostate cancer targeting [Collins and Maitland, 2006; Wang et al., 2006; Nikitin et al., 2007].

Whether or not a protein found on glandular cells and considered a marker of differentiation can serve as a target for therapy depends on the characteristics of the potential target. Some markers associated with differentiation disappear in the tumor cell population. To be an ideal target for therapy, a marker must remain highly expressed in a majority of the tumor cells. For prostate cancer, one such potential target is prostate specific membrane antigen (PSMA). We will discuss its role as a target for prostate cancer and its possible function as a target for all solid tumors due to its expression in the neovasculature of all tumors. Recent reviews have thoroughly addressed vaccine strategies, so we will not address them in this review. Rather, we will focus on treating prostate tumors directly through targeting of PSMA.

## PROSTATE SPECIFIC MEMBRANE ANTIGEN

PSMA is a type two-membrane protein with a short intracellular amino terminal of 18 amino acids, a transmembrane region of 24 amino acids, and a glycosylated extracellular domain of 706 amino acids [Ghosh and Heston, 2004]. The extracellular domain has been crystallized and the three-dimensional structure of the protein determined [Davis et al., 2005; Mesters et al., 2006]. There is some sequence and structure homology with the transferrin and transferrin-like receptors, yet PSMA does not bind transferrin and it is not thought to play a role in iron metabolism. Like the transferrin receptor, the PSMA receptor does undergo endocytosis. However, unlike transferrin, which uses the YTRF internalization motif, PSMA has a unique motif for internalization, MXXXL [Liu et al., 1998; Rajasekaran et al., 2003a; Ghosh and Heston, 2004]. Despite the differences in motifs for internalization, the transferrin receptor and PSMA appear to undergo endocytosis and recycling in similar compartments. PSMA is also found in the lysosomes of prostate cancer cells, much like the lamp protein lysosomal marker. Because PSMA has a high rate of internalization, its behavior resembles cell-surface receptors. However, no ligand that impacts its internalization in the manner of a cell-surface receptor agonist has yet been identified.

## ENZYMATIC ACTIVITY

PSMA has been shown to have enzymatic activity as a carboxypeptidase. Small peptide substrates such as polygammaglutamated folates and N-acetylasparatylglutamate (NAAG) inhibitors have been identified, and their interaction site on the protein has been examined by X-ray crystallography [Ghosh and Heston, 2004; Davis et al., 2005]. The external domain of PSMA demonstrates that upon binding of low

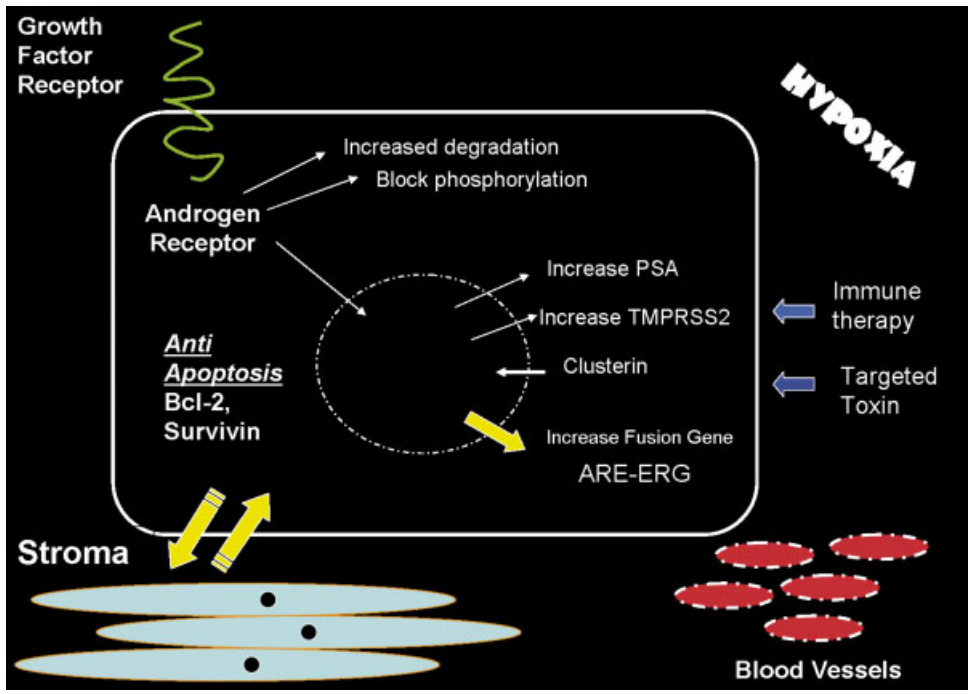


**Fig. 1.** There are a number of attack sites on tumor cells such as the androgen receptor, anti-apoptotic genes and the unique ERG fusion gene. Consideration for targeting should include the surrounding stroma and the recruited vasculature as well as direct targeting of proteins such as prostate specific membrane antigen (PSMA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

molecular weight agonists and antagonists of the enzyme, there is a structural change involving a region of the protein with a "glutamate sensing" site [Davis et al., 2005]. However, measurements of internalization of the full-length PSMA protein in intact cells demonstrate that these substrates or enzymatic inhibitors do not increase the rate of internalization (Heston, unpublished data). In contrast, certain antibodies bind to other areas of the protein and do increase the rate of internalization. This is true for the single chain antibody as well as the intact antibody, suggesting that it is not a phenomenon associated with antibody induced cross-linking [Liu et al., 1998]. Some aptamers that inhibit the enzymatic activity of PSMA have been found in the peri-nuclear lysosomal region of prostate cells, suggesting that they have also been internalized [Lupold et al., 2002]. Therefore, it appears to be recognized sequences apart from the small peptide ligands that will induce internalization. This is important for chemotherapeutic purposes since it is considered useful to deliver the therapeutic agent into the cell. Lupold and Rodriquez have also identified low molecular weight disulfide constrained peptides that bind to PSMA [Lupold and Rodriquez, 2004]. Thus, substantial arrays of potential targeting reagents that bind to PSMA have been identified.

## PSMA EXPRESSION IN PROSTATE CANCER

PSMA is expressed in normal prostate glandular cells on the acinar surface. PSMA has been described as the second most upregulated protein in prostate cancer when examined by gene array analysis and immunohistochemistry. Nearly all prostate tumors and prostate cancer cells express PSMA and are increased with aggressive tumors [Tasch et al., 2001; Ross et al., 2003; Perner et al., 2007]. Following prostatectomy, patients that have high expressing PSMA tumors tend to have higher rates of relapse, and shorter time to relapse.

The majority of PSMA is found outside of the cell and is thought to possibly play a role in cell adhesion. Research from Rajasekaran's group has demonstrated that PSMA expressing cells are resistant to proteolytic disassociation in the presence of bone marrow extract, whereas cells that do not express PSMA are readily disassociated [Barwe et al., 2007]. This is true only of bone marrow extracts as other types of matrix

did not show the same effect in their studies [Barwe et al., 2007]. This is potentially one reason why prostate cells are bone seeking, with bone being a preferential site for metastatic disease.

PSMA is also upregulated following androgen deprivation in vitro model systems [Israeli et al., 1994]. This would be a useful feature for a therapeutic target because many patients who will be treated have undergone androgen deprivation or will be taking agents to reduce androgen activity. In hormone treated patients, results of immunohistochemical evaluations have been mixed. Some reports indicate that PSMA is increased, while others indicate that the expression is unchanged following hormone deprivation therapy [Tasch et al., 2001]. Most importantly, PSMA expression is either increased or unchanged while other markers such as prostate specific antigen (PSA) are decreased following androgen withdrawal. Substantial experimental work with PSMA is derived from experimental pre-clinical models focused on LNCaP and its derivative cell lines. Other models may give different results. We also realize that tumor cells are heterogeneous and there will be variation of any one target, thus multiple tumor targets or excellent bystander activity of the therapeutic agent will be required. In preliminary experiments we have separated high or low expression of PSMA in LNCaP cells by flow cytometry. Despite a 100-fold difference in PSMA expression at the extremes, there was no difference in tumor clonogenecity in soft agar (Heston, unpublished data). Clonogenecity and formation of large colonies in soft agar is thought to reflect stem cell activity. If PSMA is a marker of differentiation, one would expect high expressing cells to have the least clonogenic potential, yet our group did not find this to be the case. In addition, we did not find cells that lacked expression of PSMA, which we would expect if stem cells were similar to basal cells, which do not express PSMA protein in immunohistochemical analysis. However as a target, PSMA has many desirable features that have been verified in patient samples.

## PSMA TARGETING

Antibodies have already been used in PSMA targeting and imaging. The first antibody against PSMA was a mouse monoclonal that received FDA approval for use as an imaging agent and is sold under the brand name of Prostascint©. This antibody only recognizes the intracellular epitope on PSMA, which are generally dead or necrotic cells, commonly found in lymph nodes. In bone marrow, where there is likely less cell death, Prostascint© is not useful for imaging. Because of this drawback, it is not useful to target living tumor cells but may be used to add bystander therapy to reach viable cells in proximity to dead and dying tumor cells, potentially imaging the region where therapy has caused elimination of prostate cancer cells [Meraney and Heston, 2007].

Second generation antibodies are currently in pre-clinical and clinical trials. A mouse monoclonal antibody was generated by Dr. Neal Bander and is being developed by the BZL Company. This antibody has been genetically modified to replace certain regions of the mouse protein with human sequences that are more likely to reduce the possibility of a human anti-mouse antibody response, or HAMA reaction. This antibody has been given to patients; with few side effects and no HAMA responses have occurred. In addition the antibody has been modified with a metal chelating agent to allow delivery of therapeutic radionuclides, including Lutecium-177 ($^{177}$Lu) and Yittrium-90 ($^{90}$Y) [Milowsky et al., 2004; Bander et al., 2005; Vallabhajosula et al., 2005a,b] $^{90}$Y is a pure beta emitter while $^{177}$Lu emits both beta and gamma rays. An advantage of $^{177}$Lu is that the gamma ray emission can be imaged. In clinical trials of $^{177}$Lu-J591 antibodies versus $^{90}$Y-J591 antibodies, $^{177}$Lu-J591 had better and more predictable responses, even though $^{90}$Y has the shorter half-life (2.7 days vs. 6.7 days) and greater range of energy disposition 28–42 mm versus 1.2–3 mm.

In clinical studies of $^{177}$Lu-J591, 30 patients with metastatic disease were examined. Of the population, 21 had bone only disease, 6 had soft tissue only disease, and 3 had both bone and soft tissue metastatic disease [Bander et al., 2005]. All sites of metastatic disease were successfully imaged with $^{177}$Lu-J591. Additionally, more bone sites were identified with the radio-labeled antibody when compared with a bone scan. Twenty-one patients had demonstrable anti-tumor activity and four patients exhibited greater than 50% decrease in PSA values. Dose-limiting toxicity was myelotoxicity and the treatments did not reach the dose limiting

toxicities for other tissues such as liver. In the trial using $^{90}$Y-J591 (29 patients), more toxicity was seen and the dose causing myelotoxicity was not as predictable. However, six patients experienced stabilization and two had measurable tumor responses as well as declines of 85% and 70% in their PSA values [Milowsky et al., 2004].

Despite the delivery of relatively low doses of radiation, radio-immunotherapeutic targeting (RIT) can cause prolonged total body exposure to radiation due to long-term circulation of plasma levels of antibodies. Therefore, how can the dose be increased without increasing the toxicity to the marrow? One possibility derives from the findings of Gudkov and coworkers that specific p53 inhibitors can prevent bone marrow toxicity caused by single doses of otherwise lethal radiation [Burdelya et al., 2006]. Specifically, this group attempted to block p53 because it plays a more active role in normal tissues than tumors in which p53 is inactivated. Whether that approach would work in the case of RIT, remains to be seen.

Another approach would be to treat patients with a drug that has synergistic activity with radiation, such as a taxane [Milas et al., 1999]. Further considerations should include the use of agents that act at osteoblastic sites of tumor deposition; an example is 153-Samarium lexidronam [Sartor et al., 2007]. It can target tumor with antibody-linked agent and the associated stromal site with an osteoblastic seeking agent such as 153-Samarium lexidronam. 153-Samarium lexidronam rapidly clears and thus would not have the prolonged total body exposure that is associated with antibodies. Stromal targets might also be defined for soft tissue metastasis when specific proteins that are activated at those sites are identified. Neil Bander's team has also been exploring antibody directed toxins by linking an antibody with the drug maytansinoid-1 using a readily hydrolyzed linker. There has been evidence of anti-tumor activity, but they have also found a substantial release of drug in the serum, causing a high level of neurotoxicity, which is known to be associated with the free drug.

Unlike Bander's antibody, which is a mouse monoclonal antibody genetically engineered to eliminate the more antigenic regions; other antibodies have been generated in genetically engineered mice that have had mouse antibody genes removed and human antibody producing genes inserted. Using these mice, others have generated fully human anti-PSMA antibodies that are at different stages of development for clinical use [Ma et al., 2006]. One of the antibodies has been linked to a toxin derived from the potent anti-microtubule agent dolostatin [Ma et al., 2006]. Interestingly, this linkage strategy requires that the antibody be endocytosed into the tumor where it can be acted upon by the lysosomal protease cathepsin B. This is in contrast to those that have acid labile linkers that theoretically will not be hydrolyzed until exposed to the acid compartment of the endsome/lysosomes but also have a rate of hydrolysis at neutral pH. The enzymatic cleavage site of the PSMA-linked dolostatin toxin releases a pro-form of the drug that undergoes spontaneous rearrangement to release active dolostatin. This then diffuses into the cell and causes cell death. The toxin linkage is very stable in serum, reducing the likelihood of free drug and resultant untargeted toxicity for normal tissues [Ma et al., 2006]. The pre-clinical activity of the drug was dose dependent, with higher doses providing long lasting control of tumor growth [Ma et al., 2006]. It is anticipated that by 2008, this anti-PSMA human monoclonal antibody toxin conjugate will be in clinical trials.

## PROSTATE SMART BOMBS

The enzymatic activity of PSMA has been the basis for the development of "pro-drug" strategies which require the enzymatic activity of prostate specific protein to activate a non-toxic pro-drug to a fully active toxin. Active toxins would act at the site of tumor, sparing normal tissue. We investigated this concept in demonstrating that PSMA can serve to activate non-toxic polygammaglutamated anti-folates. Specifically, we showed that PSMA could remove the gamma-linked glutamates from polygammaglutamated methotrexate to release the cytotoxic anti-metabolite methotrexate [Heston et al., 1997]. This approach has been pursued further by investigators such as Isaacs and Denmeade's group, who have been developing "smart bombs." Smart bombs rely on the enzymatic activity of targeted designer drugs. The toxic agent thapsigarin inhibits the endoplasmic reticulum calcium ATPase pump, inducing cell death, even in non-proliferating cancer cells.

A derivative of thapsigarin has been identified as a substrate for a designer "targeted bomb" that can be activated by PSMA [Mhaka et al., 2006]. This was achieved by identifying dipeptide and pentatpeptide using aspartate and glutamate derivatives of 8-0-(12-aminododecanoyl)8-0-debutanoylthapsigarin (12ADT). An analogue was found that had anti-tumor activity when activated by PSMA, releasing an active form of 12ADT that potently inhibited the $Ca^{++}$ pump and resulted in cell death in vitro and tumor regression in vivo. The additional benefit of a PSMA based "smart bomb" is that it will also target tumor neovasculature, of other solid tumors, all of which highly express PSMA.

Isaacs and Denmeade's group is also identifying pro-drug substrates of PSA that similarly produce tumor killing toxins such as the channel pore forming proaerolysin [Williams et al., 2007]. Serum PSA is enzymatically inactive since it is bound to serum protease inhibitors, so it does not release the active toxin. However, PSA is active as it is being released from the cell, where the toxin is desired for treatment. Pre-clinical testing has been very impressive in the ability to eliminate PSA expressing tumors. A concern would be the heterogeneity of PSA and its relatively decreased expression following androgen ablation and with increasing tumor grade. Nevertheless, many androgen resistant tumors still express PSA as its measurement is used to help monitor disease progression.

While the primary focus has been on antibodies, RNA aptamers targeting PSMA have also been developed by Lupold et al. [2002]. Aptamers are considered to have advantages over antibodies, including greater stability, ease of synthesis, and lower production costs. One group has taken the toxin gelonin and linked it to an aptamer for PSMA using the linker SPDP (Pierce Biotechnology, Rockford, IL) and found that it had excellent properties for killing PSMA expressing cells with a 600-fold to 10,000-fold difference depending on how one calculated the maximal kill dose [Chu et al., 2006]. Like the second generation antibodies being developed, these aptamers were found to be internalized and highly toxic. It was not clear to what extent these conjugates would remain intact in serum. Studies of xenograft tumors treated with the aptamer-gelonin toxin were not performed.

Bigger is not better when attempting to target tumors with the highest affinity. Small molecules will have better diffusion and achieve equilibrium and optimal binding at a faster rate. We thought it might be possible to generate a low molecular weight toxin by linking a glutamate thiourea derivative with doxorubicin as a potential targeted toxin. We were successful in creating the glutamate-linked toxin and it had excellent binding properties to PSMA, however it lacked adequate toxicity [Jayprakash et al., 2006]. It is not clear whether this was due to the linkage eliminating doxorubicin's toxicity, or whether it was due to the fact that binding did not induce endocytosis. We have found that the rate of endocytosis is not increased when these low molecular weight ligands bind to PSMA (Heston, unpublished data). Also, Rajasekaran et al. [2003b] reported that the internalized PSMA moiety lacked enzymatic activity. We continue to investigate the toxic potential of other low molecular weight linked toxins.

## RE-TARGETING IMMUNE CELLS

Another cell therapy involves cytotoxic cells such as T-lymphocytes. With regard to PSMA this has been investigated by Sadelain's team [Gade et al., 2005]. They found that they could eliminate tumor cells expressing PSMA in vitro by exposing them to peripheral blood lymphocytes that they infected with a retrovirus expressing a chimeric antigen receptor (CAR). The CAR they used was the T-cell receptor in which the extracellular domain expressed an anti-PSMA single chain antibody fused with the internal activating CD3 zeta chain domain. This allowed these T-cells to recognize their antigen in the absence of human leukocyte antigen expression, designated $Pz1^{+}$ cells. Expansion of the $Pz1^{+}$ cells in vitro, in the presence of LNCaP cells engineered to express B7, enabled them to maintain their anti-tumor activity when given in vivo without additional stimulation. In vivo, PSMA expressing tumor cells were eliminated when implanted either orthotopically or subcutaneously and 50% of the animals were cured. In a lung metastasis model in which the tumor cells expressed PSMA, similar cures rates were seen. It took at least a week for the administered $Pz1^{+}$ T-cells to eliminate the implanted tumors; however the T-cells remained for that period of time. Injection

of excess T-cell controls did not interfere with the tumor elimination process, suggesting that such modification in tumor expansion and redirection should be readily achievable. Infusion of IL-2 or dendritic cells or other methods to maintain the in vitro expanded genetically retargeted T-cells was not required [Gade et al., 2005].

## IMAGING

One aspect of treatment relates to how one can visualize tumor, size, location, and response to treatment. PSMA is an appropriate target for imaging modalities. The second generation antibodies that recognize the extracellular domain of viable tumor cells have greater sensitivity in imaging, especially for bone metastases [Meraney and Heston, 2007]. These antibodies have also proven useful for imaging other solid tumors which do not express PSMA, while their neovasculature does express PSMA [Milowsky et al., 2007]. In general, radiolabeled antibodies have problems as imaging agents because of the extended time that it takes to diffuse from the vasculature into sites of tumors (days). Moreover, the long half-lives in circulation lead to prolonged total body exposure to radiation and increased non-specific binding to Fc receptors in the liver. These problems are not associated with low molecular weight ligands.

Pomper and co-workers have examined the role of radionuclide modification of low molecular weight ligands for PSMA and have found that they have excellent activity in terms of signal to background for imaging using positron emission scanning, PET, in pre-clinical models [Foss et al., 2005]. We have identified additional derivatives of low molecular weight ligands that can also be used in imaging studies. Hopefully, low molecular weight ligands will become available for imaging to aid in cancer staging and monitoring of tumor response to therapy.

## SUMMARY

Is PSMA a perfect target for therapy? It does have many desirable features such as strong expression in prostate cancer, upregulation in aggressive forms of the disease and expression in metastatic sites. PSMA is also found to be expressed in the neovasculature of all non-prostate solid tumors making it an ideal target for all solid tumors. However non-tumor targeting concerns exist. There has been expression of PSMA detected in non-malignant tissues such as brain, kidney, small intestine, and liver. In the brain, immunohistochemical reactivity has been found to be associated with a minority subset of non-neuronal glial cells [Sacha et al., 2007]. In other areas, PSMA expression is low, compared with prostate cancer and the results of targeting studies suggest that other sites are not likely to have significant uptake. In the liver, some immunohistochemical studies have found no expression and while others have suggested some expression may be present. Other groups have shown staining to be of a cytoplasmic nature, indicating that the PSMA would not likely be a target. Unfortunately, most of these studies are done postmortem, often following trauma, and it is not clear whether some of these findings may be occurring as a result of the trauma. Imaging with antibodies targeted to the internal domain and second generation antibodies targeted to the external domain show similar uptake patterns in the liver, consistent with this signal being due to non-specific antibody binding to Fc receptors. Additionally, sites on normal tissues are often not accessible due to the lack of leaky vasculature and the integrity of cell–cell adhesions. Some of these specificity issues will be resolved with small molecule imaging. Studies to date did not show PSMA imaging of the brain or neural tissue in mice [Foss et al., 2005]. Also mice in which PSMA was knocked out had normal birth numbers and were normal by almost all measures, suggesting that PSMA can be eliminated without toxicity [Bacich et al., 2005]. However, it does appear that adult knockout mice have difficulty regarding the recruitment of new blood vessels [Conway et al., 2006]. We believe that PSMA is not flawless perfection, but it remains an excellent target for treatment and imaging of prostate cancer.

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