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Immunotoxins: A Promising Treatment Modality for Metastatic Melanoma?

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ABSTRACT

The incidence of melanoma is rising in the Western population, and melanoma is the most aggressive form of skin cancer with a very poor prognosis once it has progressed to metastatic stages. Patients with stage IV melanoma (metastases to distant lymph nodes and other areas of the body) are treated with the chemotherapeutic drug dacarbazine (DTIC). However, fewer than 5% of the patients treated with DTIC sustain long-term complete responses; hence, DTIC is administered with palliative purposes. New therapy is urgently needed. We are developing another therapeutic strategy, specifically targeting melanoma cells with the 9.2.27PE immunotoxin (IT). ITs bind to antigens overexpressed on cancer cells and are therefore tumor selective. This targeted approach may potentially cause fewer side effects in a clinical situation compared to conventional approaches like chemotherapy and radiotherapy.

INTRODUCTION

Melanoma arises from melanocytes, melanin-producing cells found primarily in the skin and eyes. The global incidence of melanoma continues to increase, and 132,000 new cases are diagnosed each year (World Health Organization; www.who.int; accessed May 21, 2010). The incidence rate of melanoma is highest in Australia/New Zealand, with a prevalence of 37.7 cases per 100,000 men and 29.4 cases per 100,000 women, as compared with 16.4 cases per 100,000 men and 11.7 cases per 100,000 women in North America (year 2002).[1] Norway is among the countries in Europe with the highest incidence rate of melanoma, with a prevalence of 17.6 cases per 100,000 men and 16.4 cases per 100,000 women in 2008 (www.kreftregisteret.no; accessed May 21, 2010). A major risk factor is exposure to ultraviolet radiation, especially in combination with fair skin types. Other risk factors are frequent sunburns, a history of previous melanoma, multiple benign and atypical moles, immunosuppression, and a family history of melanoma.[2–4] Metastatic melanomas are highly resistant to cytotoxic agents and radiotherapy, possibly because of high resistance to apoptosis (programmed cell death).[5] Dacarbazine (DTIC) is the approved chemotherapeutic drug for treatment of metastatic melanoma, but durable long-term responses are uncommon in these patients. Regimens combining several agents have been studied, but unfortunately polychemotherapy has failed to demonstrate a significant benefit in survival.[6] Interferon (IFN)-α-2b has been shown to significantly prolong disease-free survival and to improve overall survival,[7,8] and high-dose IFN-α-2b is approved by the US Food and Drug Administration (www.fda.gov; accessed May 21, 2010) for adjuvant treatment of high-risk melanoma (stage IIB, IIC, and III), which is associated with a 40% to 80% chance of relapse and death. However, data from pooled analyses from several trials administering high-dose IFN-α-2b to patients with high-risk resected melanoma showed no significant prolongation of disease-free survival or improved overall survival.[9]

Because of the dismal benefit of the approved therapeutic drugs for treatment of melanoma, new drugs with different working mechanisms are being developed. Of these are several protein kinase inhibitors, as some protein kinases show an increased activity leading to higher proliferation signals in cancer cells. Patients with melanoma have yet to benefit from protein kinase inhibitors, for example, sorafenib (originally synthesized to target B-RAF and C-RAF), imatinib mesylate (inhibitor of c-Kit, platelet-derived growth factor receptor, and BRC-ABL), and temsirolimus (inhibitor of mammalian target of rapamycin).[10,11] Addition of sorafenib to the chemotherapeutic drugs carboplatin and paclitaxel (CP) did not improve progression-free survival or overall survival when compared to the placebo and CP combination for patients with advanced melanoma.[12] New inhibitors, for example, B-RAF inhibitors[13] in combination with chemotherapy, immunotherapy, and vaccines

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are likely to be explored as treatment options for melanoma in the near future.

Other targeted treatment options with novel mechanisms of action are ribosome-inactivating proteins and other toxins able to inhibit the protein synthesis and to subsequently cause cell death. The SLT-1A^{IYSNKLM} toxin variant showed antitumor effect in human melanoma cells in vitro and in vivo.[14] The toxin consists of the cytotoxic A subunit of the Shiga toxin harboring the peptide insertion IYSNKLM, which allows the toxin variant to selectively target and kill the melanoma cells; however, the targeted receptor is thus far unknown. Interestingly, SLT-1A^{IYSNKLM} in combination with DTIC resulted in tumor regression and increased survival in a mouse xenograft model in comparison to either SLT-1A^{IYSNKLM} or DTIC treatment alone. Toxin-containing molecules targeting melanoma cells should therefore be explored as a treatment option for melanoma. Immunotoxins (ITs) are agents designed to target and kill cancer cells and consist of a toxin linked to a monoclonal antibody.

IMMUNOTOXINS AND THEIR WORKING MECHANISMS

The dimeric ITs consist of toxins and monoclonal antibodies, either as unmodified or modified molecules, linked together by a peptide sequence or by a disulfide bond. The toxins used are primarily derived from plants and bacteria, and diphtheria toxin (DT) and Pseudomonas exotoxin A (PE) are 2 bacterial toxins commonly used in IT constructs. The DT and the PE are single-chain proteins with 3 functional domains: a cell-binding domain, a translocation domain, and a catalytic domain. The antibodies used in ITs target antigens overexpressed on cancer cells, enabling the ITs to bind specifically to these cells. PE-based ITs are internalized by endocytosis, and the IT is subsequently translocated to acidic compartments in the cell, where the catalytic domain of the toxin is cleaved off by the enzyme furin.[15] The catalytic domain is subsequently transported from the early endosomes via the late endosomes to the Golgi in Rab9-dependent manner, and from the Golgi to the endoplasmic reticulum under control of Src.[16–18] Cell death is subsequently caused by adenosine diphosphate (ADP)–ribosylation of elongation factor-2, resulting in inhibition of protein synthesis, and by induction of apoptosis (programmed cell death)[19–22] (Figure 1).

CHALLENGES LIMITING THE SUCCESS OF IMMUNOTOXIN

Immunogenicity, vascular leak syndrome (VLS), toxicity to nontumor tissue, and poor penetration into solid tumors are the major challenges of IT treatment.

Neutralizing antibodies will inhibit the IT and compromise its efficacy. Hematologic tumors are more ideal for treatment with ITs than solid tumors, since patients with these tumors often lack sufficient immunity to make antibodies against the IT.[23] Repeated doses can therefore be given to some patients, as the immunogenicity after a single cycle of IT ranges from 0% to 40% for patients with hematologic tumors, as compared to 50% to 100% for those with solid tumors.[23] PEGylation, a process in which polyethylene glycol is attached to another molecule to ''mask'' the molecule from the immune system, has been used to reduce the immunogenicity. Another advantage of PEGylation is the prolonged half-life of the molecule owing to reduced renal clearance. Despite the advantages, PEGylation can lead to decreased activity and increased size of ITs. Indeed, limited success with PEGylation of ITs has been achieved with the anti–Tac(Fv)-PE38 (LMB-2) IT and the mesothelin targeted IT SS1P.[24,25] A number of B- and T-cell epitopes have been identified on the Pseudomonas exotoxin A,[26,27] and removing the epitopes can result in less immunogenic PE-based ITs, as has been shown for HA22-8X (a BL-22 IT with B-cell epitopes removed, see Table).[28] Using immunosuppressive drugs to limit the production of the anti-IT antibodies is an option; in addition, the cytotoxic effect of cyclosporin A (CsA), in combination with different ITs, has been assessed both in vitro and in vivo, with promising results.[29]

VLS is caused by endothelial damage, characterized by increased vascular permeability, resulting in leaking of fluids and proteins from the circulation into the tissue.[30] VLS is a common side effect of ITs because of direct exposure of the endothelial cells to the intravenously administered drug, and VLS restricts the doses of IT administration. Specific residues in ricin (a plant toxin), PE, and interleukin-2 (IL-2) bind to and can damage endothelial cells, and VLS has been induced in rats administered PE-based ITs.[31–34] Baluna et al[34] have shown that these residues contain the conserved sequence motif xDy, where D is aspartic acid; x is leucine, isoleucine, glycine, or valine; and y is valine, leucine, or serine. More specifically, a tripeptide motif in ricin, (Leu-Asp-Val or LDV), and in IL-2, (Leu-Asp-Leu or LDL), causes VLS, as assessed by the in vivo model of skin transplants in mice with severe combined immunodeficiency, and it also mediates binding to HUVEC cells. Similar motifs in domain III of PE (Gly-Asp-Leu or GDL) and in DT (LDV) have been identified.[34] Results from a clinical trial with a PE-based IT containing an antibody targeting the Lewis^{Y} antigen showed that the antibody was responsible for the induced VLS.[35] Modifying the ITs to avoid the motifs

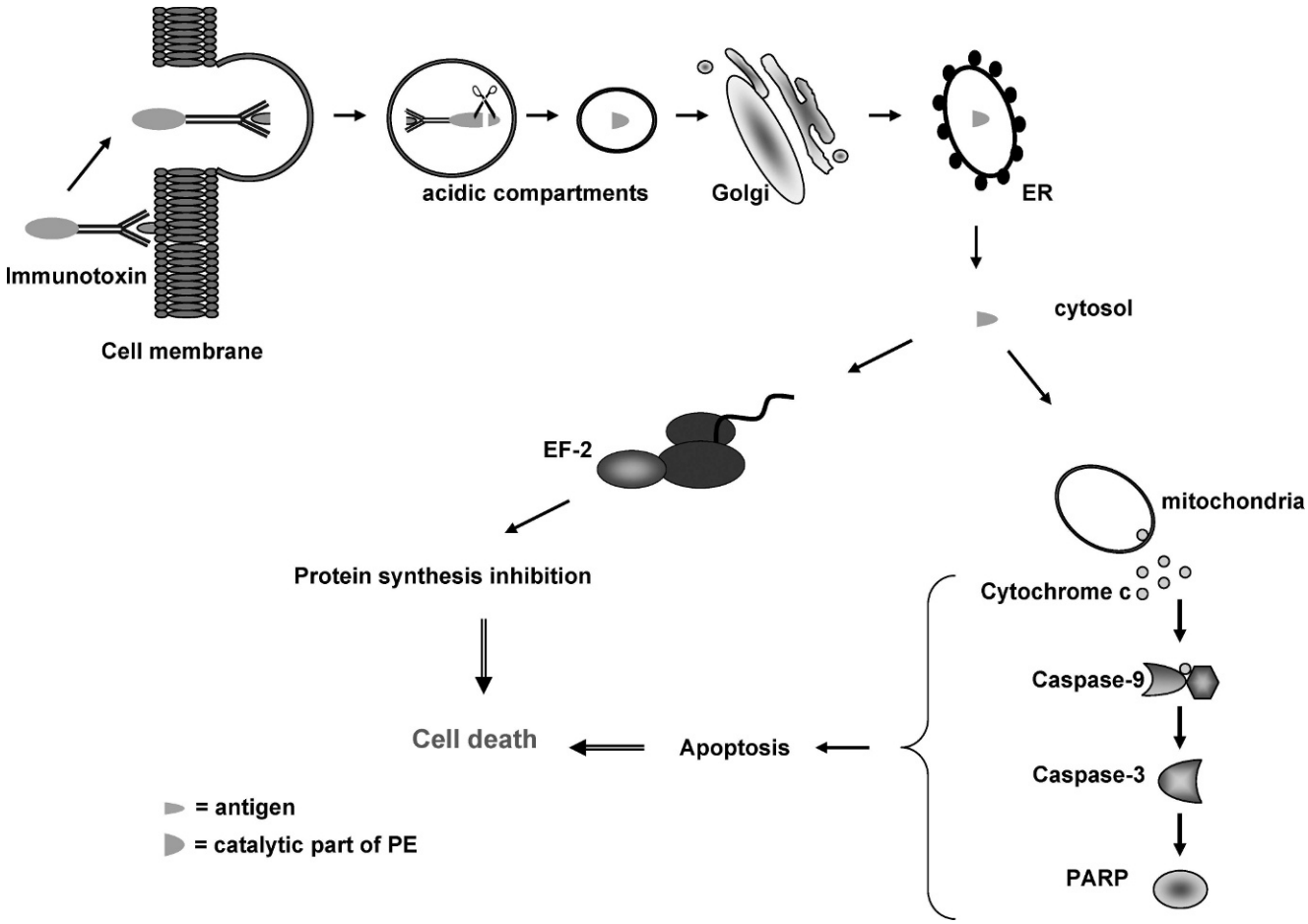

Figure 1. Schematic illustration of Pseudomonas exotoxin A (PE)–based immunotoxin (IT) targeting antigens overexpressed on cancer cells. The IT is subsequently taken up by endocytosis. The catalytic part of the toxin (PE) is cleaved off and transported from the acidic compartments via the Golgi and endoplasmic reticulum (ER) to the cytosol where it inhibits protein synthesis by adenosine diphosphate (ADP)–ribosylating elongation factor (EF)-2. The catalytic part has also been shown to induce apoptosis, involving depolarization of the mitochondrial membrane, resulting in release of cytochrome c, activation of caspase-9 and caspase-3, and inactivation of poly (ADP-ribose) polymerase (PARP).[56]

that cause binding to the endothelial cells might prevent VLS. Suppressing VLS with anti-inflammatory drugs, such as steroids or nonsteroidal anti-inflammatory drugs, which suppress cytokine actions, might also be an option to limit the problem.

Normal cells may, to some extent, express antigens used as targets for the ITs, and ITs may theoretically show toxicity to these cells. However, the same antigen might be distributed differently in normal cells and malignant cells, as is the case with the epithelial cell adhesion molecule (EpCAM) antigen. In normal colon gland epithelia, the EpCAM antigen is mainly distributed on the basolateral membrane and in the region between the basolateral membrane and the cytoplasmic part near the nucleus.[36] The expression pattern of colon malignancies is mainly on the whole surface of epithelia. The expression has been shown to be much higher in the malignant colon cells compared to normal colon tissue.[36] It is therefore important to choose antibodies that recognize antigens that are overexpressed or distributed differently on malignant compared to normal cells.

Treating solid tumors with IT remains a challenge, even though systemically administered ITs have shown some efficacy for patients with solid tumors.[37] The limitations include the large size of the ITs, high intratumoral pressure, and heterogenous blood supply within the tumors owing to abnormal or dysfunctional tumor vasculature.[38] Reduced size of ITs will potentially improve their ability to penetrate solid tumors.[39] Recombinant DNA technology and protein engineering enable the design of ITs that contain only the elements required to recognize and kill the tumor cells. However, small ITs are more rapidly cleared through renal elimination, which limits their cytotoxic effect. Local administration of ITs into solid tumors

Table. Studies With Immunotoxins as Listed in the ClinicalTrials.gov Web site (Recruiting Patients as of May 2010)[a]

Immunotoxin	Antigen	Basic Toxin	Disease	No. of Trials
LMB-2	CD25	PE	Leukemia Lymphoma	4
HA22	CD22	PE	Leukemia Lymphoma	5
SS1(dsFv)-PE-38	Mesothelin	PE	Malignant mesothelioma Lung cancer	3
Deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22	CD17 CD22	Ricin	Leukemia	1
DT2219ARL	CD19 CD22	DT	Lymphoma	1
BL22	CD22	PE	Leukemia	1
A-dmDT390-bisFv	CD3	DT	Leukemia Lymphoma	1
MR1-1	EGFRvIII	PE	Supratentorial malignant brain tumor	1
MOC31-PE	EGFR	PE	Carcinoma	1
LMB-2/HA22/BL22	CD25/CD22	PE	Leukemia Lymphoma	1

Abbreviations: DT, diphtheria; PE, Pseudomonas exotoxin A.

[a] Available at: www.clinicaltrials.gov.

might circumvent the above-mentioned challenges and has been used to treat patients with brain tumor, bladder cancer, head and neck cancer, and leptomeningeal neoplasia.[40–43] The incidence of immunogenicity was very low in these studies, and there were both partial and complete responders among the patients.

Treating solid tumors with IT is limited by the above-mentioned challenges, including poor penetration into solid tumors. Circulating metastatic cells disseminated from solid tumors are likely to be ''an easier target'' for IT treatment, as these cells are accessible in the blood or lymphatic system, much like hematologic tumors.

CLINICAL TRIALS WITH IMMUNOTOXINS

The number of ongoing clinical trials with IT, listed in the ClinicalTrials.gov Web site (www.clinicaltrials.gov; accessed May 21, 2010), are listed in the Table. As of May 2010, 14 of the 19 active recruiting trials are enrolling patients with leukemia or lymphoma, and most of these trials are conducted with PE-based IT. Hematologic malignancies are ideal for treatment with IT, as compared to solid tumors, as many patients with hematologic malignancies make limited neutralizing antibodies against the IT.[23] In addition, the hematologic cancer cells are readily accessible for the IT molecule, whereas the size of the IT (>200 kDa) and the high intratumoral pressure limit penetration of ITs into solid tumors.[38]

MELANOMA AND 9.2.27PE IMMUNOTOXIN

ITs developed to target melanoma cells demonstrate advantages for several reasons. ITs binding to antigens overexpressed on melanoma cells is a so-called targeted approach, theoretically leaving normal cells unaffected. Even though it has not been investigated, this mechanism of cell killing may be independent of mutations and alterations in melanoma cells, which drive the proliferation and cause resistance to therapy. The melanoma-targeting scFvMEL/rGel IT in combination with photochemical internalization showed promising cell-killing effect in vitro and in vivo,[44] and a recombinant IT consisting of a single-chain 9.2.27 antibody fragment targeting the melanoma-associated chondroitin sulfate proteoglycan/high molecular weight–melanoma associated antigen (MCSP/HMW-MAA), linked to a truncated variant of PE, has shown cell-killing effects in melanoma cells.[45] None of the above-mentioned ITs are currently in clinical trials, but their clinical potential warrants further evaluation. The effect of 2 ITs targeting $CD25^+$ cells is being tested in patients with melanoma (www.clinicaltrials.gov; accessed May 21, 2010). These trials are, however, designed to activate the immune system by targeting the regulatory T cells (T suppressor cells) expressing CD25, a subpopulation of T cells that act to suppress the activation of the immune system.

The 9.2.27 antibody targets HMW-MAA or MCSP, the human homologue of the proteoglycan NG2 found

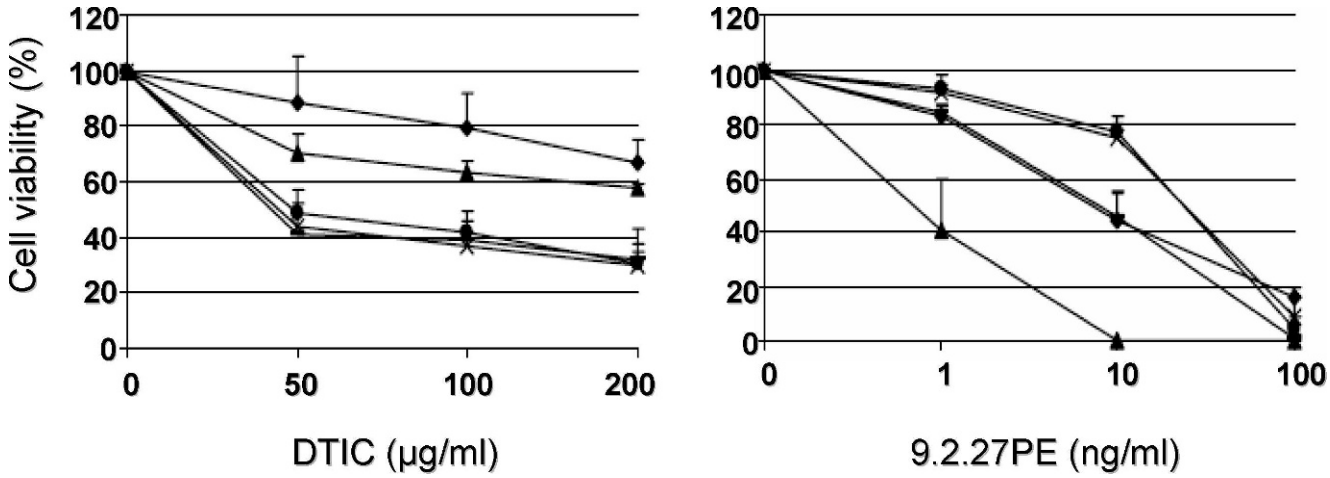

Figure 2. A panel of early passage melanoma cells was established from lymph node metastases. These Melmet cell lines were treated with increasing concentrations of dacarbazine (DTIC) and 9.2.27PE for 72 hours. The Melmet cell lines showed varied response to dacarbazine. These cell lines were also treated with increasing doses of 9.2.27PE, and the 9.2.27PE was effective in killing the Melmet cells, as cell viability was close to 0 for all cell lines. All data represent the mean ± SD of 3 independent experiments, each plated in triplicate. Melmet-1 (♦), Melmet-5 (●), Melmet-28 (x), Melmet-30 (▲), Melmet-44 (-). (The figure [slightly modified] was first published in Risberg K, et al. *J Immunother.* 2010;33(3):272–278.[53])

in rats.[45–47] HMW-MAA is expressed at high density on melanoma cells and found in at least 80% of melanoma lesions with limited intralesional and interlesional heterogeneity, and it has a restricted distribution in normal tissues.[48–50] The 9.2.27 immunoglobulin (Ig) G antibody has previously been covalently linked to the toxins abrin and ricin; however, when conjugated to unmodified PE, higher cytotoxic effects were obtained in melanoma cells.[49,51] In a recent article by Risberg et al,[52] the effect of 9.2.27PE in HMW-MAA–positive melanoma cells was assessed. Cell death was primarily caused by inhibition of protein synthesis, followed by decreased cell viability and some apoptotic features, such as poly (ADP-ribose) polymerase inactivation and chromatin condensation. Caspase-3 activation and DNA fragmentation, typical apoptotic features, were not observed.[52] Melanoma cells are extremely resistant to apoptosis, which might explain why only few apoptotic features were observed in the melanoma cells. Importantly, 9.2.27PE caused decreased cell viability and cell death independently of the sensitivity level of DTIC in a panel of melanoma cell lines, including early passage melanoma cells (Figure 2).[53] The MOC31PE IT (targeting the EpCAM antigen)[29] consists of IgG antibodies and unmodified PE and did not cause VLS in any of the 36 patients enrolled in a phase I study (data not published); we speculate that the specific residues enabling PE to bind to the endothelial cells are not exposed owing to the folding of the protein. It is therefore likely that the 9.2.27PE IT mentioned above, which consists of 9.2.27 IgG antibody and unmodified PE, will not induce VLS, but this has yet to be investigated.

9.2.27PE is relevant in the development of novel therapeutic strategies for melanoma, as fewer side effects are likely to occur because of the tumor cell–specific approach. A possible option to treat melanoma is to target circulating melanoma cells, which have disseminated from the surgically removed primary tumor, as these cells are accessible in the blood or lymphatic system. Repeated administration of 9.2.27PE is likely to be needed to eradicate all tumor cells, which will generate neutralizing antibodies inhibiting the cytotoxic effect of 9.2.27PE. Combining 9.2.27PE with CsA is an option, not only because it allows repeated administration of 9.2.27PE but also because the combination of other ITs with CsA has shown synergistic cytotoxic effect in other cancer cell types.[29]

Melanoma cells show elevated protein levels of several of the antiapoptotic Bcl-2 family members when compared to melanocytes and normal cells. For example, the increase of metastatic potential and progression of melanoma is associated with decreased Bcl-2 expression,[54] but all stages of melanoma show significantly higher Bcl-2 protein levels than nevi and normal tissue samples.[55] Treating FEMX melanoma cells with the 9.2.27PE did not decrease the protein level of Bcl-2, despite inhibition of protein synthesis.[52] Decreased protein level of other Bcl-2 family members and inhibitors of apoptosis protein members were observed. Bcl-2 might therefore play an important role in the level of

apoptotic resistance seen in these cells, as the PE-based 9.2.27PE IT did not cause depolarization of the mitochondrial membrane, strong activation of caspase-3, or DNA fragmentation, features observed for other cancer types treated with PE-based ITs.[56] Combining inhibitors of Bcl-2 with the 9.2.27PE IT might therefore enhance the cell-killing effect of the IT and should therefore be investigated as a treatment option for melanoma cells. Combining 9.2.27PE with endolytic and lysosomic agents or with drugs targeting other signaling pathways in the cells might enhance the effect of the IT treatment. We are currently working on the above hypothesis to develop 9.2.27PE as an alternative treatment option for patients with melanoma.

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