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Clinical Experience of Treatment of Metastatic Melanoma and Solid Tumours Adopting a Derivative of Diphtheria Toxin: Cross-reacting Material 197

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Abstract. *Background: Diphtheria toxin (DT) has shown anticancer activity in both experimental models and humans but its adverse effects stopped further developments. Cross-reacting Material 197 (CRM197) is the product of a single missense mutation (Gly52 to Glu) within fragment A of DT. It has been shown to induce weak toxicity in some cell strains, but it shares immunological properties with native DT. CRM197 commonly acts as an immunological adjuvant, or as an inhibitor of heparin-binding epidermal growth factor. Recently, CRM197 was shown to have promising antitumor activity. To better-define this property, we planned a phase I-II study. Patients and Methods: Twenty-nine patients bearing advanced melanoma (18 cases), and other solid tumors (two ovarian cancer, two sarcoma, two gastrointestinal cancers, one urinary bladder carcinoma, one glioblastoma, one neuroblastoma, one ocular melanoma and one primitive neuroectodermal embriogenic tumor (PNET) were evaluated and 19 of them, sub-divided in cohorts, received the following levels of CRM197: Level 1, 0.3 mg; level 2, 1.0 mg; level 3, 2.5 mg; level 4, 3.5 mg; level 5, 5.0 mg; level 6, 7.5 mg. The drug was given once every two days for 4 times and then, after a 2-week rest period, once every 2 days for 4 times. CRM197 was administered subcutaneously in the abdominal wall. Results: grade 1-2 common toxicities included fever, chills, fatigue, dizziness, nausea, vomiting and headache, neutrophilia and skin painful reactions appeared regularly at levels 3 and 4 (2.5 mg and 3.5 mg). Vomiting and abdominal pain, skin reaction tachycardia and hypotension appeared in two patients at level 5. At 7.5 mg, we observed a severe grade 3 reaction with hypotension, dyspnea and grade 4 myalgia. This was considered the dose-limiting toxicity. Eleven patients (seven with melanoma and four with other tumors) were treated to evaluate anticancer effects at the maximum tolerated dose (5 mg). Only one patient reported a minor response, lasting eight weeks. Ten patients reported progressive disease. Conclusion: CRM197, injected subcutaneously at 5 mg, elicited a generic inflammatory response causing toxicity, and did not exert a significant degree of antitumor activity in patients with advanced melanoma and solid tumour.*

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Diphtheria toxin (DT) has long been known to provide some anticancer activity both *in vitro* and *in vivo* but its toxicity makes it non-suitable for clinical use (1-8).

A possible solution to avoid toxicity without compromising its efficacy could be to use variants of the DT, such as Cross-reacting Material 45 and Cross-reacting Material 197 (CRM197).

CRM197 is obtained through the substitution in position 52 of glutamic acid instead of glycine (9).

This substitution allows for the molecule to retain the same three-dimensional structure and cellular activity but strongly affects its toxicity. CRM197 has largely been used as adjuvant in different vaccines to improve immune stimulation (10).

It has also been shown to possess anticancer activity *in vitro*, similar to that reported for DT (6, 7). Two major mechanism of actions have been postulated to explain the anticancer activity of CRM197: non-specific stimulation of the immune system and the inhibition of heparing binding epidermal growth factor (HB-EGF). The first mechanism relates to the non specific stimulation of the immune system. Such stimulation can induce or reinforce an immune reaction against cancer. Cancer cells can be recognized and killed by

specific sub-populations of lymphocytes, such as cytotoxic T-lymphocytes (CTLs) and Natural-Killer cells (NK), through the previous identification of specific antigen present on the surface of neoplastic cells (11). These antigens can be presented as small peptides binded to major histocompatibility complex molecules (MHC) and are directly recognized by lymphocyte T-cell receptor (TCR) or present as intact molecules on the surface and are recognized by circulating antibodies. In the latter case, antibodies bind to the target molecule with their Fab fragment, and make a capping around the cell membrane. They are subsequently recognized by NK, neutrophils and macrophages, which possess receptors for the Fc fragment of the antibodies and which ultimately release perforin, granzyme and other toxic molecules killing the cancer cell. This process is called antibody-dependent cellular cytotoxicity (ADCC) and is under evaluation (12). However, immune reaction against cancer cells in patients is often weak and not efficient. It has been shown that DT and its variant CRM197, as well as other pathogens such as Bacillus-Calmette Guerin (BCG), can stimulate this process, with consequent activation of immune cells against specific neoplastic targets.

The other mechanism of action postulated to explain the anti-tumoral activity of CRM197 is the binding to HB-EGF, which represents the main target of DT. HB-EGF is expressed at different levels in a variety of different malignancies and recent evidence suggests that it plays a critical role in cancer progression and in acquired resistance to antineoplastic drugs; it has also been demonstrated that CRM197 is able to selectively bind and inhibit this target (13-15). Inhibition of HB-EGF seems to increase the activity of paclitaxel in xenografts of ovarian cancer and enhances activity of other anti-neoplastic drugs, as reported by other preclinical studies (16-19). Inhibition of HB-EGF could be in part responsible for the hypothesized anticancer activity of CRM197 (17, 18). Another possibility is that CRM197 binds to HB-EGF on the surface of cancer cells and enhances the immunization against neoplastic antigens (17, 19).

Metastatic melanoma is a very aggressive disease, with reported median survival time of less than one year. Among the treatments which have shown some efficacy, are immunotherapy with interferon-alpha (IFNα) or interleukin-2 (IL-2), chemotherapy with temozolomide, dacarbazine and other agents, or a combination of immunotherapy and chemotherapy. IL-2 and IFNα when used alone can induce responses in patients ranging from 10 to 40%. Interestingly, it has been reported that long-term survival of patients with metastatic melanoma can occur and relates strongly to the development of autoimmunity, arguing that stimulation of the immune system is linked to tumor eradication (21). Chemotherapy-alone can produce a similar proportion of responses but an impact on survival has not been demonstrated and long-term survivors are not reported. Finally, a combination of IFNα or IL-2 with chemotherapy has been shown to significantly increase the response rate with respect to chemotherapy or immunotherapy-alone but, again, no significant benefit on survival has been observed in clinical studies (21). Clinical activity of CRM197 has been reported by some treated patients with advanced cancer (22, 23) and others suggested interesting possibilities of cure for tumors (7, 13, 14, 17). On the basis of these results and on the immune-sensitive nature of melanoma, we started a phase I/II study with end-points of clarifying the maximal tolerated dose (MTD) and assessing the clinical activity of CRM197 in patients bearing mainly metastatic melanoma, as well as other solid tumors.

Patients and Methods

Phase I. The primary end-point of this part of the study was to determine the maximal tolerated dose (MTD) of CRM197. Previous clinical studies report that CRM197 could be administered to a dosage of up to 3.5 mg per subcutaneous injection with injections performed weekly but the side-effects have never been clearly reported. We planned six cohorts of three to five patients each: level 1, 0.3 mg; level 2, 1.0 mg; level 3, 2.5mg; level 4, 3.5 mg; level 5, 5 mg; level 6, 7.5 mg. The drug was given once every two days for 4 times and then, after a 2-week rest period, once every 2 days for 4 times. CRM197 was administered subcutaneously in the abdominal wall. Cycle administration and timing of the injections were identical across the different cohorts. The secondary endpoint of the phase I study were toxicities and clinical responses.

Phase II. The primary objective of this part of the study was the response rate defined as the percentage of PR plus CR, calculated on an intention-to-treat basis. Secondary end-points were progression-free survival, overall survival and safety. The best overall response from each patient was reported. All results were reviewed by an independent radiologist. Progression-free survival (PFS) was calculated from the first day of treatment to first evidence of progression or death. Overall survival (OS) was calculated from the first day of treatment until death from any cause. Both OS and PFS were estimated using the Kaplan Meier method. The minimax two-stage sequential design, described by Simon (24) was used to determine the number of patients to be included. We assumed a 15% chance of response, obtained by the most active treatments evaluated in advanced melanoma (22) and pre-treated solid tumors. According to this, a response rate of 20% or more would be considered promising. The design parameters p0 (response rate in null hypothesis) and p1 (response rate in alternative hypothesis) selected were 0.10 and 0.20. Considering an alpha error of 0.05 and a beta error of 0.20 respectively, the first stage of the phase II study thus required 11 patients, and if at least two responses were observed, an additional 16 patients had to be enrolled in the second step of the phase II study. The treatment was considered interesting for further investigation if more than six responses were observed. IBM SPPS Statistics (International Business Machines Corporation, New York, NY, USA) was used for all calculations and plots.

Patient population. Patients aged 18 years or older, with tissue diagnosis of refractory malignant melanoma or other solid cancer,

Table I. *Patients' characteristics, response, survival and toxicity – Phase I.*

Tumor type	Measurable	Prior therapy	Ab reactivity to DT	CRM 197 (mg)	Response	Survival (mo)	TOX (sites)	TOX (grade)	T >38˚
Melanoma	Lymphnode, liver	S, CHT, I	Ab –	0.3	PD	6	null	0	no
Melanoma	Lung, liver	S, CHT, I	Ab –	0.3	PD	7	null	0	no
Melanoma	Lung, bone	S, CHT, I	Ab –	0.3	PD	4	null	0	no
Sarcoma	Lung, abdomen	S, CHT	Ab –	1.0	PD	5	Skin	2	no
Melanoma	Lung, lymphnode	S, CHT, I	NE	1.0	PD	12	Skin	2	yes
Melanoma	Lymphnode, liver	S, CHT, I	Ab –	1.0	PD	6	Skin	2	no
Urinary ca.	Lymphnode, lung	S, CHT	Ab +	1.0	PD	13	Skin	2	no
Sarcoma	Lung, abdomen	S, CHT	NE	1.0	PD	5	Skin	2	no
Neuroblastoma	Bone, lung	S, CHT	Ab –	2.5	PD	6	Skin	2	yes
Gastric ca.	Lymphnode, liver	S, CHT	Ab –	2.5	PD	5	Skin	2	yes
Melanoma	Lymphnode, skin	S, CHT, I	NE	2.5	PD	14	Skin	3	yes
Melanoma	Lymphnode, lung	S, CHT, I	NE	2.5	PD	13	Skin	3	yes
Melanoma	Lymphnode, lung	S, CHT, I	Ab –	3.5	PD	13	Skin	3	yes
Glioblastoma	Relapse	S, RT,CHT	Ab +	3.5	PD	9	Skin	3	yes
Melanoma	Lung, brain	S, CHT, I, RT	Ab –	3.5	PD	5	Skin	3	yes
Melanoma	Lymphnode, skin	S, CHT, I	NE	5.0	PD	14	Skin, cardiac	3	yes
Melanoma	Lung,brain	S, CHT, I, RT	Ab –	5.0	PD	5	Skin, myalgia	3	yes
Neuroblastoma	Bone, lung	S, CHT	Ab –	5.0	PD	6	Skin, vomiting, pain	2	yes
Melanoma	Lymphnode, lung	S, CHT, I	NE	7.5	PD	13	Cardiac, myalgia	3, 4	yes

S=Surgery, CHT=chemotherapy, I=immunotherapy, Ab Reactivity to DT=titer of antibodies to diphteria toxin, TOX=toxicity observed, T=fever.

were eligible for the study. Other eligibility criteria included documentation of evaluable tumor, Eastern Cooperative Oncology Group performance status ≤2, life expectancy of 12 weeks or longer, and adequate bone marrow, hepatic, and renal function. Prior chemotherapy, immunotherapy or radiation therapy was acceptable if they were completed ≥4 weeks prior to entry, with recovery from any toxicities (to grade 1 or grade 0). Toxicity was classified and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (25). Standard World Health Organization (WHO) criteria were used to define response and progression (26). Patients were considered evaluable for toxicity once therapy was started. The treatment responses for therapy were evaluated on every cycle. Patients with progressive disease (PD) were removed from the study. Patients with complete (CR) or partial responses (PR) or stable disease (SD) continued treatment until evidence of progression, unacceptable toxicity, or desire to withdraw from the study. The study was approved by the Institutional Review Board and by Regional Ethical Board of Tuscany, Italy. The study was carried out between June 2007 and June 2009 and was conducted in compliance with the protocol and the principles laid down in the Declaration of Helsinki, in accordance with the ICH Harmonized Tripartite Guideline for Good Clinical Practice (GCP). Written informed consent was obtained from each participant prior to evaluation, screening and treatment. The study was initiated applying the criteria for appraisal of the quality of a study. We present a well-reported patient population, with high quality data and quality control, with good clinically significant follow-up, without loss of patients.

Treatment. CRM197 was a gift of Novartis Vaccines, Siena, Italy. The preparation was diluted to a concentration of 432 μg/ml in a sterilized phosphate buffer (10-mM sodium phospate buffer, pH 7.2) containing 10% sucrose as stabilizer. The final product, tested for sterility and general safety on the skin of New Zealand rabbits, was aliquoted in pyrogen-free vials and stored at –20˚C. For preparation of the product the Authors followed detailed indications reported by Buzzi *et al*. (22). Treatment schedule and dose levels were defined on the basis of previously published trials (22, 23). The patients received CRM197 subcutaneously in the abdominal wall at the dose for the level they were assigned.

In 13 out of 19 patients in the phase I study, the titer of diphtheria antitoxin that resulted unprotective (<0.01 IU/ml) was assessed using an ELISA method (Genzyme Virotech, Russelsheim, Germany). These patients with no or low antibody titer were checked again for this parameter two months later after the first administration of CRM197.

In four patients status of HB-EGF and EGFR expression was obtained by immunochemistry. Paraffin sections were analyzed immunohistochemically for HB-EGFR expression using a standard avidin-biotin technique. Out of them one sarcoma and two melanoma showed high positivity.

Results

Phase I. We enrolled 19 patients. The cohorts were treated with CRM197 at 6 dose levels (Table I). The common toxicities (grade 2) included painful skin reactions, fever, chills, fatigue, dizziness, nausea, headache and neutrophilia. More intense side-effects appeared regularly at level 3 and 4. Grade 2 vomiting and abdominal pain, tachycardia and hypotension appeared in a patient (32 years old) one hour after administration of 5 mg CRM197. Twelve hours later,

Table II. *Patients' characteristics, response, survival and toxicity – Phase II.*

Tumor type	Measurable	Prior therapy	Ab reactivity to DT	CRM 197 (mg)	Response	Survival (mo)	TOX (sites)	TOX (grade)	T >38˚
Ovarian cancer	Lymphnode, peritoneum	S, CHT	Ab –	5.0	PD	4	Skin	2	yes
Sarcoma HBEGFR+	Lung, bone	S, CHT	Ab –	5.0	PD	5	Skin, Cardiac	2	yes
Ocular Melanoma	Liver, peritoneum	S, CHT, I	Ab –	5.0	RM	8	Skin	2	yes
Melanoma HBEGFR+	Liver, lung, bone	S, CHT, I	Ab +	5.0	PD	5	Skin	2	yes
Melanoma HBEGFR+	Liver, lung	S, CHT, I	Ab +	5.0	PD	4	Skin, Cardiac, Myalgia	2	yes
PNET	Brain	S, CHT	Ab –	5.0	PD	3	Skin, Myalgia	2	no
Melanoma	Lymphnode, lung	S, CHT, I	Ab –	5.0	PD	13	Skin	3	yes
Glioblastoma	Relapse	S, RT,CHT	Ab +	5.0	PD	9	Skin	3	yes
Melanoma	Lymphnode, skin	S, CHT, I	Ab –	5.0	PD	14	Skin, Cardiac	3	yes
Melanoma	Lymphnode, lung	S, CHT, I	Ab –	5.0	PD	13	Skin, Myalgia	3	yes
Melanoma	Lung, brain	S, CHT, I, RT	Ab –	5.0	PD	5	Skin, Myalgia	3	yes

S=Surgery, CHT=chemotherapy, I=immunotherapy, Ab Reactivity to DT=titer of antibodies to Diphteria toxin, TOX=toxicity observed, T=fever.

the patient returned to the Emergency Room for diffuse skin rash, itch, grade 2 vomiting and myalgia. At the dose of 7.5 mg, we recorded a grade 3 toxicity with hypotension, dispnea, vomiting, myalgia, itch and fever >39˚C. This level was considered the DLT. The MTD was defined at 5 mg, and for greater safety of patients we decided to adopt pre-medication with levocetirizine at 5 mg given orally, 1 hour before CRM197 injection. We also observed changes in laboratory assays: neutrophilia was common, with an increase from 50 to 280%. Neutrophilia recovered within several days (18-51) after treatment.

Phase II. We enrolled 11 patients: six with melanoma, one with ocular melanoma and four with other tumor types: sarcoma, ovarian cancer, primitive neuroectodermal embriogenic tumor (PNET), glioblastoma (Table II). We observed one minor response and ten cases of PD. The patient with minor response had liver and peritoneal metastases from ocular melanoma. After therapy, an independent radiologist reported at MR scan a reduction of –20% in the diameters of the peritoneal nodules and appearances of necrosis in the liver metastases. After eight weeks, we documented evidence of PD; the patient died 8 months after starting CRM197 therapy. The median survival was 4 months (Figure 1).

Due to poor clinical activity and for ethical reasons (lack of effective therapy, presence of side-effects) the phase II study was closed in June 2009.

Discussion

Treatment with CRM197 in patients with advanced melanoma and cancer induces a general inflammatory reaction, with a significant increase in the number of circulating neutrophils. It is known that an increase of white blood cells is a usual feature of diphtheria (27) and correlates with the clinical outcome. CRM197 behaves as a powerful inflammatory-immunogenic agent. Moreover, its binding to HB-EGF results in inhibition of the strong mitogenic activity of this growth factor (13-15). The vast majority of our patients had zero levels of antibodies against DT but after two months from the first injection, we recorded very high titers of protective antibodies. The substantial modest results that we obtained are probably due to the excessive number of circulating antibodies that might prevent contact between CRM197 and the HB-EGF receptor of cancer cells. This factor probably limits the clinical results and excludes CRM197 from the possibility of being a real tool against cancer cells.

Buzzi *et al*. recognized that for patients with humoral immunity to DT, the subcutaneous route allowed only a partial absorption of CRM197 into the bloodstream, the molecule largely being neutralized by specific antibodies at the injection sites. To prevent this waste of product, they started a trial based on a 10-min intravenous infusion of CRM197. They noted fever from 37.5˚ to 40˚C. This side-effect started with a shorter time lag than previously observed for patients treated by the subcutaneous route (23). Due to these side-effects, no other Authors have, to our knowledge, adopted the intravenous route of administration. Another reason for poor results is that our patients presented with advanced and pre-treated cancer and as a result their immune system was probably less responsive. We observed only one minor response short lasting (eight weeks) in a patient with ocular melanoma. Buzzi *et al*. reported, in the most favorable study carried out so far, three responses out of 25 patients, with an overall response rate of 12%. One patient with neuroblastoma had CR of a small para-vertebral nodular relapse lasting more than 45 months, one patient with brain metastases from breast cancer had CR lasting four

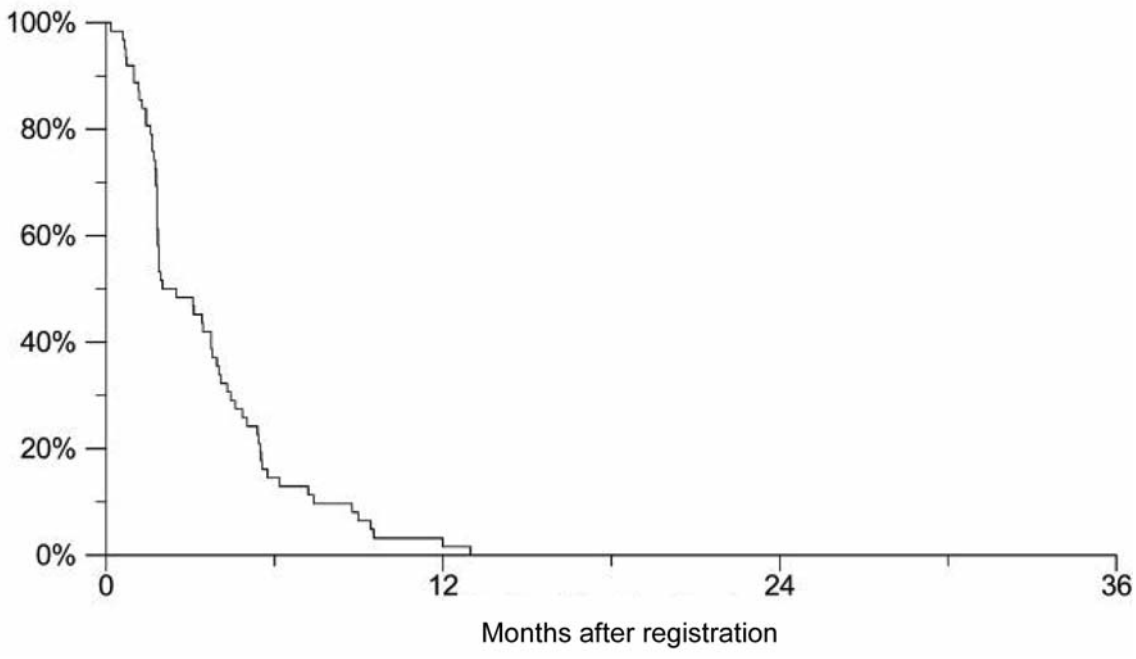

Figure 1. *Overall survival (Kaplan-Meier).*

months, and one patient with spine bone metastases from breast cancer had bone re-calcification lasting 15 months (22). In other studies, a role of chaperone for CRM197 in increasing the efficacy of chemotherapy has been suggested (16-18). From these data it is not clear, up to now, if any anticancer activity for CRM197 as a single-agent exists, as suggested (22, 23). We treated one patient with advanced sarcoma and two with melanoma expressing high positivity to HB-EGFR. They did not respond to therapy neither had any symptomatic benefit. We believe that the hypothesis of anticancer activity in tumors with positivity for HB-EGFR is uncertain and still doubtful.

In our experience CRM197 was administered at a high dose, from 1 to 5 mg for each injection. At this dose, it presents side-effects, even grade 2 and fever >39˚C, related to the dose administered and to the host immunological response. Of note, in vaccines there is a minimal amount (from 8 to 15 ng) of CRM197 when it is used as a carrier protein in conjugated vaccines to induce immunogenicity to some saccharide haptens. For this reason only minimal skin side-effects have been observed frequently.

Myiamoto *et al*. studied intraperitoneal administraton, directly into the ascitic fluid, reporting low toxicity (13). Buzzi *et al*. used intravenous infusion but then abandoned it due to rapid onset of high fever and then adopted subcutaneous injection (23). We selected subcutaneous injection and reported local and systemic side-effects even of grade 2 and 3.

In conclusion, the actual role of CRM197 appears to be a non-specific immunotherapy similar to the one which BCG can induce. The best route of administration of CRM197 seems to be subcutaneous.

In our study CRM197 did not produce clinical utility in melanoma and advanced tumors, further studies are warranted to better-define the type of activity of CRM197 and its mode of action.

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