Negative argininosuccinate synthetase expression in melanoma tumours may predict clinical benefit from arginine-depleting therapy with pegylated arginine deiminase

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BACKGROUND: Arginine-depleting therapy with pegylated arginine deiminase (ADI-PEG20) was reported to have activity in advanced melanoma in early phase I–II trial, and clinical trials are currently underway in other cancers. However, the optimal patient population who benefit from this treatment is unknown.

METHODS: Advanced melanoma patients with accessible tumours had biopsy performed before the start of treatment with ADI-PEG20 and at the time of progression or relapse when amenable to determine whether argininosuccinate synthetase (ASS) expression in tumour was predictive of response to ADI-PEG20.

RESULTS: Twenty-seven of thirty-eight patients treated had melanoma tumours assessable for ASS staining before treatment. Clinical benefit rate (CBR) and longer time to progression were associated with negative expression of tumour ASS. Only 1 of 10 patients with ASS-positive tumours (ASS+) had stable disease, whereas 4 of 17 (24%) had partial response and 5 had stable disease, when ASS expression was negative (ASS–); giving CBR rates of 52.9 vs 10%, \( P = 0.041 \). Two responding patients with negative ASS expression before therapy had biopsies after tumour progression and the ASS expression became positive. The survival of ASS– patients receiving at least four doses at 320 IU m\(^{-2}\) was significantly better than the ASS+ group at 26.5 vs 8.5 months, \( P = 0.024 \).

CONCLUSION: ADI-PEG20 is safe and the drug is only efficacious in melanoma patients whose tumour has negative ASS expression. Argininosuccinate synthetase tumour positivity is associated with drug resistance and tumour progression.

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Despite new agents, such as BRAF inhibitors and ipilimumab, the prognosis for most advanced melanoma patients remains poor and new therapies are still needed. Amino-acid depletion has been used to treat certain malignancies (Pinheiro and Boos, 2004). One such nonessential amino acid is arginine (Tapiero et al, 2002; Wheatley, 2004). Arginine is synthesised from citrulline via the enzyme argininosuccinate synthetase (ASS; Husson et al, 2003; Shen et al, 2003). Several tumours such as melanoma do not express ASS and are unable to synthesise arginine (Takaku et al, 1992; Ensor et al, 2002; Park et al, 2003; Dillon et al, 2004; Szlosarek et al, 2006; Kim et al, 2007). Pegylated arginine deiminase (ADI-PEG20), which degrades arginine to citrulline, has entered into clinical trials (Holtsberg et al, 2002; Curley et al, 2003; Izzo et al, 2003; Assiet et al, 2005; Feun et al, 2008a; Ott et al, 2009; Dinh et al, 2011). ADI-PEG20 has shown activity in melanoma in a phase I/II trial. The optimal biologic dose (OBD) sufficient to eliminate all detectable arginine from the circulation for at least 7 days was 160 IU m\(^{-2}\) IM given weekly. To investigate the role of ASS expression with tumour response, we conducted a clinical/pharmacodynamic study of ADI-PEG20.

MATERIALS AND METHODS

Inclusion criteria included: (1) histologically confirmed metastatic melanoma, (2) unresectable and measurable/evaluable disease, (3) progressive disease after chemotherapy, radiotherapy, surgery or immunotherapy, no longer responding to standard therapy or have refused standard therapy, (4) been off previous treatment for at least 4 weeks, (5) fully recovered from prior surgery, (6) age \( \geq 18 \) years old, (7) KPS \( \geq 70 \), (8) expected survival of \( \geq 12 \) weeks, (9) absolute neutrophil count \( > 1500 \) per \( \mu l \), platelets \( > 100 000 \) per \( \mu l \), serum bilirubin \( \leq 3 \) mg dl\(^{-1}\), albumin \( > 3 \) g dl\(^{-1}\), alkaline phosphatase \( < 1.5 \) ULN, glucose \( > 60 \) mg dl\(^{-1}\), amylase \( < 1.5 \) ULN, (10) signed an IRB approved informed consent, and (11) not enrolled in other IND studies. Patients with metastatic ocular melanoma were allowed but analysed separately. Exclusion criteria included: (1) significant cardiac disease (New York Heart Association Class 3 or 4), (2) serious infection requiring
antibiotics, (3) pregnancy or lactation, (4) clinical ascites or symptomatic pleural effusion, (5) expected non-compliance, and (6) known allergy to other *Escherichia coli* drug products (such as GM-CSF). Patients with stable brain metastases were not excluded. Patients continued to receive treatment until: (1) the patient developed progressive disease or (2) the patient had grade 3 or greater toxicity, except for local discomfort from injection or (3) the patient had grade 2 or greater allergic reaction or (4) the patient had a complete response. There was no dose de-escalation for toxicity. If a complete response was observed, the patient continued treatment for additional 4 weeks. If the patient had stable disease or less than a complete response, the patient was allowed to continue therapy as long as no major toxicity was observed. There was no maximum number of cycles required. Analysis of clinical outcomes was done by Sylvester Cancer Centre following closure of the study by the sponsor after 39 patients had been enrolled and treated. Clinical outcome data include overall response, disease progression and survival. ADI-PEG20 was administered at weekly intramuscular doses. The initial starting dose for the protocol was 160 IU m$^{-2}$. When it was noted that 160 IU m$^{-2}$ did not decrease peripheral blood arginine to nondetectable levels, the dose was increased to 320 IU m$^{-2}$. Twenty-one patients received a starting dose of 160 IU m$^{-2}$, based on the OBD. One cycle of therapy was considered 4 weekly doses.

Patients had radiologic evaluation, CT scan after 2 months and every 2 months while on study. If they tolerated the drug well (without grade 3 or greater toxicity or grade 2 allergic reaction) and clinical progression after 2 cycles of therapy were treated as censored observations using the date of last disease assessment. Overall survival was defined as the elapsed time from treatment start to earliest documented evidence of disease progression or death from any cause. Patients who remained alive without progression were treated as censored observations using the date of last disease assessment. Overall survival was defined as the elapsed time from treatment start to death from any cause with follow-up for surviving patients censored at the date of last contact. In the subset of patients for whom baseline ASS was measured, Fisher’s exact test was used to compare response rate in ASS-positive (ASS $+$) vs ASS-negative (ASS $-$) patients, and the log-rank test was used to compare PFS and OS.

ASS expression was performed by immunohistochemical staining (Dillon *et al*, 2004). The criteria for determining ASS positivity and negativity was as follows: normal endothelial cells in the section were used as positive control, any positive staining regardless of the intensity of staining was considered as positive. The reason is that our *in-vitro* data strongly suggest that for primary culture or cell lines with positive staining regardless of the intensity, ASS can be induced rather rapidly (Savaraj *et al*, 2007). Examples of ASS-positive melanoma and ASS-negative melanoma are shown in Supplementary Figures 1S and 2S. When feasible, RT–PCR or qRT–PCR was performed in the primary culture (see Supplementary Method). At the beginning of the study, RT–PCR was performed (Savaraj *et al*, 2007) while we developed the assay for qRT–PCR (see Supplementary Method). Normal human fibroblasts (BJ-1) which express ASS and are resistant to ADI-PEG20 were used as a positive control and the value was set at one. The arbitrary value of $<0.01$ was considered as negative (Dinh *et al*, 2011).

### RESULTS

#### Patient characteristics

A total of 39 patients were enrolled and treated, 37 at the SCCC and 2 at the VA Medical Center. The primary analysis set consisted of 38 patients, excluding 1 patient deemed ineligible who received study treatment <4 weeks before the completion of radiation therapy.

The characteristics of the 38 patients who constitute the primary analysis set are given in Table 1. In all, 2 patients had ocular and 36 had cutaneous melanoma. Of the 36 cutaneous melanoma patients, 2 failed to complete one cycle (4 weeks) of therapy and return for follow-up. One experienced trauma from a fall unrelated to the study drug and could not return weekly and was taken off study. One patient with malignant pleural effusion required a chest tube...

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**Table 1** Characteristics of the 38 patients

| Characteristic                        | No. of patients (%) |
|--------------------------------------|---------------------|
| **Sex**                              |                     |
| Male                                 | 25 (66)             |
| Female                               | 13 (34)             |
| **Age, years median (range)**         |                     |
| 69 (29–85)                           |                     |
| **AJCC stage at entry**               |                     |
| Stage III                            | 2 (5)               |
| Stage IV M1a                         | 5 (13)              |
| Stage IV M1b                         | 8 (21)              |
| Stage IV M1c                         | 23 (61)             |
| **Baseline KPS**                     |                     |
| 100                                  | 24 (63)             |
| 90                                   | 9 (24)              |
| 80                                   | 4 (11)              |
| 70                                   | 1 (3)               |
| **Baseline LDH**                     |                     |
| > ULN                                | 12 (32)             |
| Normal                               | 26 (68)             |
| **Primary site**                     |                     |
| Cutaneous                            | 36 (95)             |
| Ocular                               | 2 (5)               |
| **ASS staining**                     |                     |
| Negative                             | 17 (45)             |
| Positive                             | 10 (26)             |
| Not assessed                         | 11 (29)             |
| **Sites of disease (some patients had multiple sites)** | |
| Visceral                             | 7 (18)              |
| Liver                                | 10 (26)             |
| Lung                                 | 22 (58)             |
| Nonvisceral                          | 29 (76)             |

**Prior therapy (patients may have had more than one type of prior therapy)**

| Treatment                           | No. of patients (%) |
|-------------------------------------|---------------------|
| Radiation therapy                   | 19 (50)             |
| Chemotherapy                        | 14 (37)             |
| Systemic vaccine or biologic therapy| 15 (39)             |
| Cytokine therapy                    | 4 (11)              |

**Abbreviations:** AJCC = American Joint Committee on Cancer; ASS = argininosuccinate synthetase; KPS = Karnofsky performance status; LDH = lactate dehydrogenase; ULN = upper limit of normal.
and declined further therapy after the first two weekly doses. Both patients were included in the response analysis. Toxicity data were collected on all patients treated. In terms of dosing for ADI-PEG20 received, the median was 8 doses (two cycles) and the range was 2–71 doses.

ASS expression

There was an association between ASS expression of the melanoma tissue obtained before treatment and the clinical benefit from ADI-PEG20 therapy. Pretreatment tumour samples were available in 27 cutaneous melanoma patients. The remaining patients either declined to have pretreatment biopsies performed or these were not available.

Although heterogeneity does occur, however, once staining is positive in certain cells, these cells will not respond to arginine deprivation and will continue to proliferate. Subset analysis of 27 patients with known baseline ASS status is presented in Table 2.

All of the responders in this study were ASS negative. In all, 4 of 17 ASS− patients achieved PR and 5 had SD, giving a clinical benefit rate (CBR) rate of 52.9%, compared with no PRs and one SD among 10 ASS+ patients. Although the difference in PR rate did not attain statistical significance, 23.5% vs 0%, P = 0.264, the difference in CBR did, 52.9 vs 10.0%, P = 0.042. The duration of PR was 6, 6, 12 and 16 months. One patient had PR of a cutaneous lesion and after 6 months had surgery to remove the lesion. Pathology showed no viable tumour, only melanosis. Sites of disease included soft tissue/nodal (three patients) and lung (one patient). Interestingly, two patients who were ASS negative by immunohistochemistry and RT–PCR at the start of therapy had a response to ADI-PEG20. When these two patients developed progression of disease, repeat biopsy of the melanoma showed ASS positive. The first patient had lung metastases and had a PR to ADI-PEG20. At the time of progression, a lung resection showed ASS-positive tumour. The second patient had multiple subcutaneous masses and had a PR. Sixteen months later at the time of tumour progression, a repeat biopsy showed ASS positive (see Supplementary Data). One patient was ASS negative by immunohistochemical staining but qPCR value of 0.09 for ASS and failed to respond. Repeat biopsy was ASS positive by immunohistochemical staining at the time of failure. Two patients with ocular melanoma were treated. One patient was ASS positive and did not respond. The other patient did not have ASS expression tested and had mixed response. Overall for the entire population treated, 4 out of 36 (11%) patients with cutaneous melanoma showed response, and the disease control rate including responding and stable patients (clinical benefit) was 10 out of 36 (28%).

Negative baseline ASS was also associated with longer PFS, P = 0.025 (log-rank test); the median time to progression for ASS− patients was 3.6 months (95% CI: 1.6–12.7) compared with 1.8 months (95% CI: 0.7–3.4) for ASS+ patients. Median OS was also longer for ASS− patients, 14.6 months (95% CI: 4.4 to < not attained >) as compared with 9.3 months (95% CI: 2.5 to < not attained >) for ASS+ patients; however, the difference was not statistically significant, P = 0.374 (log-rank test).

Table 2  Best overall response of cutaneous melanoma patients by baseline ASS status

| Best overall response | ASS− | ASS+ | P  
|---|---|---|---
| Partial response | 4 | 23.5 | 0 | 0 | 0.264 |
| Stable, including 4 pts with minor response | 5 | 29.4 | 1 | 100 | 0.1 |
| Clinical benefit | 9 | 52.9 | 1 | 100 | 0.041 |
| Progression, including 2 pts with mixed response | 8 | 47.1 | 9 | 90.0 | 0.003 |

Abbreviation: ASS = argininosuccinate synthetase.

ADI-PEG20 dosing

Fifteen patients had an initial starting dose of 320 IU m⁻² as did the two ocular melanoma patients. Twenty-one patients with cutaneous melanoma received a starting dose of 160 IU m⁻² (four of whom had dose escalation up to 320 IU m⁻²). Of the 19 cutaneous melanoma patients treated with 320 IU m⁻² (either as starting dose or as escalated dose at any time), 4 had response, whereas none of the 17 patients had response when the dosage was always <320 IU m⁻², P = 0.65 (Fisher’s exact test). The incidence of ASS expression was similar in both groups (data not shown). However, the survival of ASS− patients receiving at least four doses at 320 IU m⁻² was significantly better than the ASS+ group at 26.5 vs 8.5 months, P = 0.024. Thus, the dosage appears to be important, independent of ASS expression. Of the 10 cutaneous melanoma patients with ASS− tumours who received 320 IU m⁻², 4 (40%) had PR and 3 had SD with the CBR of 70%. In contrast, of seven patients who were ASS negative and had doses <320 IU m⁻², no partial responses were observed and only two had stable disease/minor response, P = 0.08 (Fisher’s exact test). Taken altogether, the data suggest that a higher dose of ADI-PEG20 may be needed to keep arginine levels down in the long term.

Toxicity

Serious side effects from ADI-PEG20 were uncommon. The most common side effect thought to be drug related was mild/moderate discomfort at the intramuscular injection site, which occurred in virtually all the patients but did not require discontinuation of therapy. Other side effects included grade 3 allergic reaction, occurring during the fourth cycle (one patient), grade 4 neutropenia and thrombocytopenia (one patient), grade 3 neutropenia (three patients), grade 3 anaemia (one patient) and grade 3 fatigue (two patients). Four of the five patients who had myelosuppression had prior radiation therapy or radiation therapy with chemotherapy. The patient who had grade 4 neutropenia and thrombocytopenia had recent prior cranial-spi nal radiation therapy.

DISCUSSION

This is the first clinical study to demonstrate that response to arginine-depleting therapy did correlate with lack of ASS expression, with the CBR being statistically significant between ASS-negative and ASS-positive patients. All the responders and five of six patients with stable disease occurred in the ASS-negative patients. Only one patient had stable disease when ASS was positive in the melanoma tumour. One limitation of this study is the small number of patients who had ASS staining performed. However, this is the first and only study to our knowledge that has investigated and correlated ASS staining with tumour response and CBR. As the CBR was statistically significant, we believe that future clinical trials with ADI-PEG20 should consider targeting only melanoma patients with negative ASS expression and so a future trial in ASS-negative melanoma patients is planned. In this regard, we plan a follow-up study in which only advanced melanoma patients whose ASS is negative will be treated with ADI-PEG20 at 320 IU m⁻². This will help to give more definitive results on the CBR in ASS-negative patients, while our preliminary study looked at both ASS-negative and ASS-positive patients. Currently, there are clinical trials in progress with ADI-PEG20 in hepatocellular carcinoma, small-cell lung carcinoma and mesothelioma. No clinical correlation has been performed yet in these tumours between clinical response and ASS expression. However, given our experience in melanoma patients with ADI-PEG20 and the scientific rationale for depleting arginine with this agent, it seems logical to target only patients with ASS-negative deficiency.
Another important observation from our trial is that the dose of ADI-PEG20 used to deplete arginine may be important for response. In a previous phase I/II trial, the dose of 160 IU m\(^2\) per week was considered to be the OBD, defined as the dose that lowered the blood arginine level to nondetectable levels (Ascierto et al, 2005). In that trial, all of the responders and those with stable disease were treated at a dose of 160 IU m\(^2\) or higher. The response rate was 25% in 24 patients. Patients were entered into one of six cohorts (doses ranged from 40 to 640 IU m\(^2\) per week). That trial did not state which cohort had most of the responses, which may partially explain the discrepancy with our results. Although 160 IU m\(^2\) per week was considered the OBD, blood arginine levels did not necessarily reflect tissue arginine levels. In our phase II trial, all of the responding patients had received ADI-PEG20 at 320 IU m\(^2\) per week and no patient responded when the dose was less. The incidence of ASS expression was similar at both doses of ADI-PEG20. Interestingly, of the 10 patients who received a dose of 320 IU m\(^2\) and were ASS negative, 4 (40%) had a partial response and 3 had stable disease for a CBR of 70%, as well as prolonged survival compared with ASS+ patients. Thus, the dose of ADI-PEG20 may also be important to obtaining response. This may explain the different results in another phase I/II trial in melanoma in which the maximum dose was limited to 160 IU m\(^2\) per week (Ott et al, 2009). No responses were observed but stable disease and time to tumour progression appeared to correlate with ASS expression. Taken together, the data suggest that higher doses of ADI-PEG20 than the OBD may be needed in certain ASS-negative patients for response and patients who benefit have tumours which are negative for ASS expression. Importantly, serious toxicity from ADI-PEG20 was uncommon. ADI-PEG20 has been well tolerated in clinical trials with other cancer types (Izzo et al, 2004; Ascierto et al, 2005; Feun et al, 2008a; Ott et al, 2009).

We have found re-expression of ASS occurs at the time of relapse in two patients, which could explain the cause of treatment failure. This finding also was observed in our laboratory in which ASS expression in certain melanoma cell lines can be induced on arginine deprivation (Savaraj et al, 2007; Dinh et al, 2011) and is one of the mechanisms of ADI-PEG20 resistance. To circumvent this form of resistance, it is important to understand how ASS is regulated. In this respect, we have found that c-Myc and HIF-1\(\alpha\) can positively and negatively regulate ASS, respectively (Tsai et al, 2009). Melanoma cells are sensitive to arginine deprivation by arginine deiminase. Although ASS-negative patients for response and patients who benefit have tumours which are negative for ASS expression. Importantly, serious toxicity from ADI-PEG20 was uncommon. ADI-PEG20 has been well tolerated in clinical trials with other cancer types (Izzo et al, 2004; Ascierto et al, 2005; Feun et al, 2008a; Ott et al, 2009).

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Conflict of interest

Wes Sisson and John Bomalaski are employees of Polaris. The remaining authors declare no conflict of interest.

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