Immunological effects of chimeric anti-GD3 monoclonal antibody KM871 in patients with metastatic melanoma

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We conducted an open label dose-escalation phase I trial of chimeric anti-GD3 mAb KM871 in patients with metastatic melanoma. Patients were entered into one of five dose levels (1, 5, 10, 20, and 40 mg/m²) and received three infusions of KM871 at 2-wk intervals. A metastatic melanoma site was biopsied at day 7-10. Pharmacokinetics, immune function, and mechanism of action of KM871 were analysed. A total of 17 patients were entered into the trial; 15 were evaluable. KM871 had a serum half-life (T½-beta) based on ELISA of 10.39 ± 1.12 d (mean ± SD). Trough levels >1.0 µg/mL KM871 at 2 wk postinfusion were seen with the 10 mg/m² and higher dose levels. There were no significant changes in white blood cell subsets or serum complement levels during KM871 treatment. KM871 was stable in vivo and maintained binding affinity and complement-dependent cytotoxicity (CDC) function up to 2 wk postinfusion. No significant trends in CDC or antibody-dependent cellular-cytotoxicity (ADCC) activity in patients were observed during treatment. Analysis of tumour biopsies demonstrated a significant increase in CD4+ T cell infiltrates compared to control patient tumours (P = 0.010), and in patients with either stable disease (2 patients) or a clinical partial response (1 patient) at restaging, a significant increase in CD3 and CD4 infiltrates in tumour over nonresponding patients was observed. The favourable immune properties of KM871, combined with this preliminary clinical data, indicate that KM871 has potential for the treatment of metastatic melanoma.

Introduction

The high expression of gangliosides on melanoma cells, and the responsiveness of metastatic melanoma to biologic therapies, has led researchers to select this antigen system for immunotherapeutic approaches in the treatment of metastatic melanoma (1, 2, 3, 4). For many years, interest has focused on developing antibody-based therapies against the ganglioside GD3 antigen expressed on metastatic melanoma. The most extensively studied anti-GD3 mAb studied has been the murine mAb R24 (4, 5, 6, 7). R24 has undergone numerous clinical trials in patients with metastatic melanoma, both as monotherapy and in combination with cytokines and chemotherapy agents (5, 6, 8, 9, 10, 11, 12, 13, 14). While some encouraging results and clinical responses were observed in these trials, the potential therapeutic value of R24 was limited due to the strong immunogenicity of R24 in humans (6, 12).

In order to target the GD3 antigen in metastatic melanoma, and to reduce potential immunogenicity in patients, a chimeric IgG1 anti-GD3 mAb KM871 has been developed (15). KM871 has potent immune effector functions, including CDC and ADCC, and has a minimal difference in affinity or effector function as compared to the parent murine IgG3 antibody KM641 (16, 17, 18). KM871 was also shown to have a substantial antitumour effect in animal models (15, 17). We have successfully labelled KM871 with 111In, with retention of binding affinity, and we have demonstrated successful targeting of GD3-expressing xenografts in a nude mouse model (19). In order to determine the targeting ability of KM871 to melanoma, to define its immunogenicity, and to evaluate the biodistribution of an anti-GD3 antibody in humans for the first time, we conducted a phase I trial of KM871 in patients with metastatic melanoma (20). We are expanding on that work now by reporting on the potent biologic properties of KM871 indicated by pharmacokinetic analyses, immunological assays, and tumour biopsy results from patients in this trial.

Results

Patients

Of the 17 patients entering the study, 2 (patients 12 and 16) were taken off the study due to symptoms of disease progression prior to the second infusion of KM871 and were not further evaluable. There were no grade 3 or 4 adverse events related to KM871 observed in any patient, and no maximum tolerated dose was defined (20).

Of the 15 evaluable patients, 1 had a clinical partial response (patient 4) and 2 had stable disease at restaging (patients 3 and 7). This response was maintained for 11 mo, and disease progression was observed only during cycle 6 of KM871 treatment. Three other patients all had measurable or evaluable disease removed at the time of biopsy and hence could not be evaluated for response. The remaining 9 patients all had progressive disease upon restaging.

Three patients had evidence of inflammation in tumour following infusion of KM871 (patient 2, 1 mg/m² dose level;
patient 5, 5 mg/m² dose level; and patient 15, 40 mg/m² dose level). Onset of erythema and pain in lymph nodes (patient 2) and soft tissue metastases (patient 15), and pain in a pelvic metastasis (patient 5) occurred 1-2 wk following the first infusion of KM871. Excellent targeting of these sites with ¹¹¹In-KM871 was observed using gamma camera imaging (20).

Pharmacokinetics

The calculated clearance of KM871 measured by ELISA was found to be similar to ¹¹¹In-KM871, and could also be optimally fitted using a two compartment model. For the first infusion of KM871 there was no significant difference in T½-alpha or T½-beta between dose levels, with mean ± SD values of 0.58 ± 0.42 and 9.61 ± 4.16 d, respectively. In comparison to the ¹¹¹In-KM871 values, the ELISA measurements were slightly higher, but no significant differences were observed (P = 0.128). As measured by ELISA, no significant differences were observed for mean T½-alpha or T½-beta values for the second or third infusions in each cycle when compared to the first infusion (P > 0.45; Table 1).

The peak serum concentrations of KM871 after infusion were dose dependent, and the peak serum concentrations following each infusion of KM871 are shown in Table 2. Trough values were observed just prior to the subsequent KM871 infusion, and serum concentrations of >1 µg/mL at 1 wk postinfusion of KM871 were observed in all but one patient (patient 16) at dose levels of 5 mg/m² and higher. Trough levels increased with higher dose levels, although the 20 mg/m² dose level was not dissimilar to the 10 mg/m² dose level (Table 3). Similar values for peak and trough serum concentrations of KM871 were observed following infusions 2 and 3 in each cycle and dose level of KM871 (Tables 2 and 3).

Flow cytometry and serum complement

Blood from 16 patients (patient 16 was nonevaluable) receiving multiple doses of up to 40 mg/m² KM871 exhibited no significant changes in white blood cell subclasses (including neutrophils, macrophages, and T, NK and B cell populations) during the phase I study (Figure 1).

Table 1
Serum pharmacokinetics (ELISA) of KM871 for all dose levels

<table>
<thead>
<tr>
<th>KM871 Infusion Number</th>
<th>T₁/₂ α (days) Mean</th>
<th>T₁/₂ α (days) SD</th>
<th>T₁/₂ β (days) Mean</th>
<th>T₁/₂ β (days) SD</th>
<th>T₁/₂ α (hours) Mean</th>
<th>T₁/₂ α (hours) SD</th>
<th>T₁/₂ β (hours) Mean</th>
<th>T₁/₂ β (hours) SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion 1</td>
<td>0.58</td>
<td>0.42</td>
<td>9.61</td>
<td>4.16</td>
<td>13.96</td>
<td>10.11</td>
<td>230.72</td>
<td>99.87</td>
</tr>
<tr>
<td>Infusion 2</td>
<td>0.77</td>
<td>0.75</td>
<td>11.67</td>
<td>4.89</td>
<td>18.37</td>
<td>18.04</td>
<td>280.02</td>
<td>117.37</td>
</tr>
<tr>
<td>Infusion 3</td>
<td>0.85</td>
<td>0.92</td>
<td>9.90</td>
<td>4.59</td>
<td>20.49</td>
<td>22.17</td>
<td>237.58</td>
<td>110.06</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>0.73</td>
<td>0.14</td>
<td>10.39</td>
<td>1.12</td>
<td>17.61</td>
<td>3.33</td>
<td>249.44</td>
<td>26.70</td>
</tr>
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</table>

Table 2
KM871 peak serum levels determined by ELISA

<table>
<thead>
<tr>
<th>Dose Level (mg/m²)</th>
<th>Mean Peak Serum KM871 Concentration ± SD (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infusion 1</td>
</tr>
<tr>
<td>1</td>
<td>0.34 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>3.79 ± 1.76</td>
</tr>
<tr>
<td>10</td>
<td>6.95 ± 1.91</td>
</tr>
<tr>
<td>20</td>
<td>9.20 ± 2.92</td>
</tr>
<tr>
<td>40</td>
<td>25.83 ± 7.43</td>
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Table 3
KM871 trough serum levels determined by ELISA

<table>
<thead>
<tr>
<th>Dose Level (mg/m²)</th>
<th>Mean Trough Serum KM871 Concentration ± SD (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infusion 1</td>
</tr>
<tr>
<td>1</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.66 ± 0.35</td>
</tr>
<tr>
<td>10</td>
<td>1.68 ± 0.54</td>
</tr>
<tr>
<td>20</td>
<td>0.95 ± 0.24</td>
</tr>
<tr>
<td>40</td>
<td>4.25 ± 2.08</td>
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</table>
No significant changes were observed in patient complement components of C3, C4, or CH100 following treatment with KM871 (Table 4). In particular, no reduction in any complement parameter was observed in any patient who had observable tumor inflammation.

**CDC**

Due to the long circulating half-life of KM871, patients at dose levels greater than or equal to 5 mg/m$^2$ had not completely cleared all KM871 protein prior to the administration of subsequent infusions (Table 3). In the presence of approximately 1 µg/mL or more serum KM871, KM966 control or baseline cytolytic activity increased. This was evident in two patients receiving 5 mg/m$^2$ KM871 (patient 4, cycles 1, 3, and 5, and patient 6). At the 10, 20, and 40 mg/m$^2$ dose levels of KM871, increased baseline cytolysis was more evident with higher circulating trough levels of KM871 (Figure 2, panels B and C). Accordingly, the CDC data for each patient were corrected for the circulating KM871 in these samples as measured by KM871 ELISA (20) (Figure 2, panels D and E). The serum concentration of KM871 at 50% CDC activity (CH50), was determined for each infusion of each patient from the corrected data and is presented in Figure 3A. There was no significant difference in CH50 CDC results *in vivo* among dose levels of KM871. The CDC activity achieved by each patient’s serum in general did not markedly alter during the course of KM871 infusions. Maximal cytolytic activity was constant for all patients except patient 2 (1 mg/m$^2$ dose level), in whom a marked decrease was observed from samples taken prior to infusion 1 to samples taken prior to infusion 3.

Since active circulating KM871 interfered with the assay, CDC analyses in a non-GD3 system were performed to determine the specific CDC activity of preinfusion patient serum from the 20 and 40 mg/m$^2$ dose levels with anti-Lewis Y mAb hu3S193 on target MCF-7 cells. The high control or baseline cytolytic

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### Table 4

<table>
<thead>
<tr>
<th>Complement Factor</th>
<th>Pre-study Mean</th>
<th>Pre-study SD</th>
<th>Pre-final KM871 Mean</th>
<th>Pre-final KM871 SD</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 (g/L)</td>
<td>1.56</td>
<td>0.22</td>
<td>1.54</td>
<td>0.26</td>
<td>0.81 – 1.66</td>
</tr>
<tr>
<td>C4 (g/L)</td>
<td>0.31</td>
<td>0.10</td>
<td>0.39</td>
<td>0.37</td>
<td>0.12 – 0.42</td>
</tr>
<tr>
<td>CH100 (U/mL)</td>
<td>68.75</td>
<td>39.15</td>
<td>77.32</td>
<td>38.91</td>
<td>34 – 98</td>
</tr>
</tbody>
</table>

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**Figure 1**

A. FACS® analyses of the peripheral blood of patients after receiving KM871 exhibit similar profiles. (A) Proportion of neutrophils, macrophages, and lymphocytes expressed as the mean ± SD percentage of total white blood cells. Total lymphocytes were not determined in samples collected before and after 1 mg/m$^2$, nor before and after 5 mg/m$^2$ dose levels. (B) Proportion of lymphocyte subpopulations expressed as the mean ± SD percentage of total lymphocytes. Lightly dotted bars indicate a 1 mg/m$^2$ dose before the first infusion; heavily dotted bars indicate 5 mg/m$^2$ before the first infusion; cream bars indicate 10 mg/m$^2$ before the first infusion; black bars indicate 20 mg/m$^2$ before the first infusion; open bars indicate 1 mg/m$^2$ before the final infusion; (/\) indicates 5 mg/m$^2$ before the final infusion; (//) indicates 10 mg/m$^2$ before the final infusion; squares indicate 20 mg/m$^2$ before the final infusion; and hatchmarks indicate 40 mg/m$^2$ before the final infusion.

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activity observed in the GD3 CDC system was not present in the Lewis Y CDC system (Figure 2F). Typical increases in cytolysis were observed in the presence of increasing concentrations of hu3S193. The ability of patient serum to lyse MCF-7 breast carcinoma cell targets in the presence of exogenous hu3S193 did not alter over the period of KM871 infusions, and no significant differences in CH50 levels were observed in the Lewis Y CDC system among dose levels of KM871 (Figure 3B). Negligible CDC activity was observed for all six patients’ serum samples in the presence of control huA33 mAb in this system (data not shown).

In vitro CDC activity of patient 13 serum. The serum was collected (A) before infusion 1, (B) before infusion 2, and (C) before infusion 3 of 20 mg/m² KM871 doses. CDC analyses were performed in the presence of 0.001–10 µg/mL exogenous KM871 (solid circles) and the isotype matched control antibody KM966 (gray squares) on target SK-MEL-28 cells. High background cytolytic activity was observed due to the presence of circulating KM871 in the patient sera. Based upon ELISA pharmacokinetic measurements, the data were corrected for KM871 concentration for (D) before infusion 2 and (E) before infusion 3 CDC analyses. (F) High background CDC activity of patient serum was not present in the control Lewis Y system in the presence of increasing hu3S193 (solid circles with lines) and isotype control (circles with white dot) antibodies.

Figure 2

In vitro CDC activity of patient 13 serum. The serum was collected (A) before infusion 1, (B) before infusion 2, and (C) before infusion 3 of 20 mg/m² KM871 doses. CDC analyses were performed in the presence of 0.001–10 µg/mL exogenous KM871 (solid circles) and the isotype matched control antibody KM966 (gray squares) on target SK-MEL-28 cells. High background cytolytic activity was observed due to the presence of circulating KM871 in the patient sera. Based upon ELISA pharmacokinetic measurements, the data were corrected for KM871 concentration for (D) before infusion 2 and (E) before infusion 3 CDC analyses. (F) High background CDC activity of patient serum was not present in the control Lewis Y system in the presence of increasing hu3S193 (solid circles with lines) and isotype control (circles with white dot) antibodies.

Figure 3

A. CH₅₀ KM871

B. CH₅₀ hu3S193

CDC analyses. Mean ± SD concentration of (A) corrected serum KM871 and (B) hu3S193 at 50% CDC activity (CH₅₀) at each dose level of KM871 before infusion 1 (squares), before infusion 2 (triangles), and before infusion 3 (inverted triangles).
Retention of KM871 immune effector function *in vivo*

The retention of KM871 immunoreactivity *in vivo* was further examined through additional CDC analyses. Results are presented in Figure 4 for patient 13 (20 mg/m² dose level) and indicate the retention of potent KM871 CDC activity *in vivo* 2 wk after infusion 2 when circulating levels were 0.93 µg/mL (20). Results from similar analyses with patients 10, 11, and 14-17 were in agreement with these observations (data not shown).

ADCC

ADCC analyses were performed on 17 patients receiving KM871 infusions (3 patients on the 1 mg/m², 5 mg/m², and 10 mg/m² dose levels; 4 each on the 20 mg/m² and 40 mg/m² dose levels). In contrast to the CDC measurements, no interference from circulating KM871 occurred during ADCC analyses due to the washing steps required to prepare the PBMC population. This lack of interference was demonstrated by observing an absence of cytotoxicity in the absence of KM871 (data not shown).

Examination of individual patient data indicated little change in the levels of ADCC (Figure 5), with the exception of patient 2 in whom a marked decline in ADCC occurred over the course of KM871 infusions. This patient experienced an inflammatory response at tumour sites following the second and third infusions of KM871 (20) and had a marked decline in CDC activity over the course of KM871 treatment. The decline in ADCC and CDC were not observed in patients 5 or 15, who also experienced inflammation at tumour sites.

Good correlation was shown between ADCC determinations from experiments with different effector-to-target (E:T) ratios with 1 µg/mL KM871 and results from measurements in which the E:T ratio was kept constant and the concentration of KM871 was titrated. Importantly, both sets of ADCC analyses indicated no alteration in KM871-mediated ADCC achieved with patient PBMCs over the course of KM871 infusions. In both sets of experiments, the mean ADCC tended to be higher in patients receiving doses of 10 mg/m² or more of KM871 (Figure 5). However, a great variation in effector NK cell numbers was observed among individual patients, rendering comparisons of ADCC between groups difficult. Furthermore, in agreement with ADCC measurements, no significant changes in mean NK cell levels were observed at any dose level between the initial and the final preinfusion samples (Student's *t*-test; *P* > 0.5).

Within each dose level, a trend for a reduction in ADCC achieved by patient effectors in the presence of KM871 was observed over the course of KM871 infusions (Figure 5). However, these changes were not statistically significant and did not correlate with changes in patient NK or cytotoxic T cell levels.

Repeat cycles of treatment with KM871 did not show any change in ADCC levels. No marked changes were observed in the ADCC mediated by the PBMCs of patient 4 over 6 cycles (11 mo) of KM871 infusion with 15-25% cytotoxicity (E:T ratio = 50:1; 1 µg/mL KM871) consistently measured during her disease stabilization. In addition, negligible differences were observed in the NK cell levels of patient 4 over the 6 cycles. Similarly, PBMCs isolated from patient 7 effected consistently high ADCC of target SK-MEL-28 cells over 2 cycles at the 10 mg/m² dose level, correlating with this patient's high NK cell levels as determined by FACS® (data not shown).

Biopsy analysis

Tumour biopsy specimens were obtained from all 17 patients entered into the trial. Five specimens were from metastatic lymph nodes and twelve were from subcutaneous metastatic lesions. Quantitative uptake of ⁴⁸⁶In-KM871 in tumour biopsies was measured, allowing the KM871 antibody concentration in tumour to be calculated. KM871 antibody concentration in tumour increased with dose level (Table 5), with maximal uptake ranging from 0.11 µg/mL (patient 2, 1 mg/m² dose level) to 5.89 µg/mL (patient 17, 40 mg/m² dose level). The tumour-to-normal tissue ratios obtained from biopsy specimens ranged from 1:1 in sections showing substantial tumour necrosis to 15:1 in sections with predominantly viable tumour and uniform GD3 expression.

![Figure 4](https://example.com/figure4.png)

**Figure 4**

KM871 retains effector function *in vivo*. Patient 13 sera were collected before infusion 3 (20 mg/m² KM871 dose level), KM871 levels were determined, and sera were complement-inactivated by heat treatment. CDC analyses on target SK-MEL-28 cells were performed with heat-inactivated patient sera alone (triangles) or with healthy donor complement added (squares). Control healthy donor sera with equivalent levels of exogenous KM871 added are also shown (circles).

![Figure 5](https://example.com/figure5.png)

**Figure 5**

ADCC effected by PBMCs of patients receiving KM871. Patients received 1, 5, 10, 20, or 40 mg/m² KM871 biweekly infusions. Results (mean ± SD) from analyses in the presence of 1 µg/mL KM871 at an E:T ratio of 50:1 are shown.
The KM871-treated patients had a significantly higher number of tumour-infiltrating CD4+ lymphocytes than the control patients ($P = 0.010$) (Figure 6). There did not appear to be a strong correlation between the phenotype of the TILs and the treatment dose. Tumour-infiltrating CD4+ lymphocytes were observed in 94% of KM871-treated patients, whereas only 58% of control patient tumours showed CD4+ infiltrating lymphocytes (Figure 7). CD57+ infiltrating lymphocytes were observed in 71% of KM871-treated patient tumours compared with 53% of control patients; however, there was no significant difference in the numbers of these CD57+ TILs between the two groups (Figure 7). No statistically significant differences were observed for CD3+, CD8+, or CD20+ cells between KM871-treated and control patient tumour samples. There was no statistical difference in the number of CD4+ or CD8+ TILs between the KM871 treated patients with lymph node metastasis and the control group with lymph node metastasis, or the KM871-treated patients with subcutaneous/organ tumours and the control group with subcutaneous/organ tumours. However, patients 3 and 7 (partial clinical response of 11 mo), exhibited a statistically significant increase in CD3+ ($P = 0.016$) and CD4+ ($P = 0.015$) lymphocytic infiltrates compared with other evaluable nonresponding patients.

Three patients displayed clinical symptoms of inflammation in tumour sites following infusion of KM871 (20). Patient 2 (1 mg/m$^2$ dose level) following the second infusion, patient 5 (5 mg/m$^2$ dose level) 1 wk after the first infusion of KM871, and patient 15 (40 mg/m$^2$ dose level) beginning 1 wk after the first infusion of KM871. The biopsy samples were collected 7-10 d following the first infusion, and the samples from these 3 patients did not show any marked difference in TIL numbers or population compared with the other patients in the study.

The overexpression of the membrane-associated complement regulatory proteins CD55, CD46, and CD59 serve as an important mechanism of tumour self-protection by rendering cells insensitive to the action of complement. All biopsy samples from evaluable KM871-treated patients and control (non-KM871-treated) patients were observed to highly express these inhibitors of the complement system. There was no evidence of detectable complement complex deposition in biopsy samples from control and KM871-treated melanoma patients, as indicated by the lack of staining for ic3b and sc5b-9 (data not shown).

### Table 5

<table>
<thead>
<tr>
<th>Dose Level (mg/m$^2$)</th>
<th>Mean KM871 Concentration ± SD (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.82 ± 0.16</td>
</tr>
<tr>
<td>10</td>
<td>1.86 ± 0.75</td>
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<tr>
<td>20</td>
<td>2.38 ± 0.74</td>
</tr>
<tr>
<td>40</td>
<td>3.09 ± 1.30</td>
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### Discussion

This study has clearly demonstrated the stability in vivo of KM871 in patients with metastatic melanoma, with retention of affinity for the GD3 antigen up to 2 wk postinfusion. The terminal half-life of KM871, based on ELISA, was more than 10 d, consistent with other studies of humanised antibodies (21, 22, 23). Trough levels of KM871 were maintained at more than 1 µg/mL at dose levels of 10 mg/m$^2$ and higher, and were consistent with multiple infusions. The lack of immunogenicity of KM871 (20), combined with the retention of target antigen affinity in vivo, provides clear evidence of the physicochemical stability of KM871 in patients. Our results clearly demonstrate the retention of potent immune effector function (CDC) activity of KM871 in melanoma patients up to 2 wk postinfusion. This was confirmed by detailed analysis of the effects of patient native complement and with appropriate controls. This observation of prolonged Fc function in vivo is novel for antibodies to metastatic melanoma and provides further evidence of the functional activity of
Quantitation of tumour-infiltrating lymphocytes. (A) CD3, (B) CD4, (C) CD8, and (D) CD57-positive TILs observed in KM871-treated patient biopsy samples (P1-P17) and age-matched, sex-matched non-KM871-treated melanoma patients (C1-16). Mean (± SD) cell numbers were calculated from observations of 5-10 high-power field views of each biopsy sample.
KM871 in this patient population. Direct measurement of CDC and ADCC activity of patients entered into the trial was also performed, and no trends of definite increase or reduction in immune function following repeat infusions of KM871 were observed. A significant difference was observed in the KM871 CH50 over the course of the 40 mg/m² infusions, but this result was attributed to interference from circulating KM871, as the specific CDC activity was unaltered in a non-GD3 antigen system using preinfusion patient serum from 6 patients at the 20 and 40 mg/m² dose levels. The exception to the lack of change in CDC or ADCC during treatment was patient 2, in whom a reduction in CDC activity and ADCC activity with repeat infusions was observed, and who was one of three patients in whom an inflammatory response at tumour sites was seen (20). The cause of this effect on immune function is not clear, as it was not associated with any reduction in white cell subsets or complement serum components, although some functional inhibition of innate immune response must have occurred.

Detailed analysis of white cell subsets by flow cytometry did not reveal any changes following repeat infusions of KM871. Previous studies had shown a reduction in NK cell numbers in patients treated with R24 who subsequently demonstrated clinical responses, and NK cell numbers have also been shown to be prognostic in patients with high-risk melanoma (6, 24). In our study, there were no significant changes in NK cell numbers in any patient following KM871 treatment, including one patient who had a partial clinical response (20). GD3 has been shown to be expressed in a subset of T cells, and induction of T cell proliferation by R24 has also been reported (25, 26). We found no effect of KM871 on T cell numbers or subsets following repeat infusions.

Analysis of complement factors in tumour biopsies revealed no clear evidence of detectable complement complex deposition (iC3b and sc5b-9). The timing of biopsies may have had an impact in view of the clear retention of CDC activity (iC3b and sc5b-9). The timing of biopsies may have had an impact in view of the recent impressive results with protein-adenovirus vectors. In this study, KM871 has demonstrated to have potent immune effector functions, including CDC that is retained in vivo and inducing T cell infiltrates in metastatic melanoma. While enhancement of the antitumour efficacy of KM871 by cytokines (for example, IL-2) and chemotherapy has been demonstrated in tumour models (18, 31), optimising the efficacy of KM871 in patients with metastatic melanoma remains to be achieved in clinical trials. The treatment of patients with recently resected disease in an adjuvant setting would also be an attractive therapeutic option for future trials, in view of the in vivo properties of KM871. The immunological results of this study also raise the possibility that KM871 may have a role in enhancing the efficacy of vaccines through T cell activation and infiltration of tumours, which is timely in view of the recent impressive results with protein-based vaccines (for example, NY-ESO-1 ISCOM®) in this setting (32, 33, 34).

Abbreviations
CH50, concentration of antibody effecting 50% cytotoxicity

References
Materials and methods

Trial design

The trial was an open label dose-escalation phase I study, as previously described (20). Briefly, three patients with advanced metastatic melanoma who had not undergone any therapy for 4 wk prior to the trial were enrolled at each dose level. Five dose levels of KM871 were studied (1, 5, 10, 20, and 40 mg/m²). Each patient received three infusions of KM871 administered at 2-wk intervals, with the first infusion of KM871 trace-radiolabelled with 111In to allow the determination of biodistribution and pharmacokinetics. The primary objectives were to establish the safety of repeated doses of intravenously administered KM871 in patients with metastatic melanoma and to determine the biodistribution, pharmacokinetics, and immunogenicity of KM871 in these patients. The clinical protocol and informed consent forms were approved by the Austin Hospital Human Research Ethics Committee, Victoria, Australia. Informed consent was obtained from each subject.

mAbs

The chimeric anti-GD3 mAb KM871 (IgG1) and control isotype matched chimeric anti-GM2 mAb KM966 were prepared by the Division of Immunology, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Patients received three infusions of KM871 at 2-wk intervals, as previously described (20). Immune effector function analyses with the Lewis Y antigen system utilised the humanised anti-Lewis Y mAb hu3S193 (35) and IgG1 class-matched control huA33 (36) antibodies produced by the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne, Australia). Anti-CD3-FITC/CD4-RPE, anti-CD3-FITC/CD8-RPE, anti-CD19-FITC, anti-CD20-RPE, anti-CD14-RPE, anti-CD15-FITC, mouse IgM-FITC negative control, mouse IgG1-FITC/IgG1-RPE negative control, mouse IgM-FITC negative control, and mouse IgG2a-RPE negative control were purchased from DAKO (Glostrup, Denmark). Anti-CD3-FITC/CD16+56-PE was purchased from Immunotech (ME, USA).

For immunohistologic studies, the following antibodies were used: anti-GD3 KM641 (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), PS1 (anti-CD3) and IF6 (anti-CD4) were obtained from Novocastra Laboratories (Newcastle, NSW, Australia); C8/144B (anti-CD8) and L26 (anti-CD20) were purchased from DAKO (Glostrup, Denmark); NK-1 (anti-CD57) was purchased from Zymed Laboratories (San Francisco, CA, USA), and for complement deposition analyses, iC3b and SC5b-9 antibodies were purchased from Quidel (San Diego, CA, USA).

Cell lines

The GD3-positive SK-MEL-28 melanoma cell line (ATCC HTB-72) and the GD3-negative MCF-7 breast carcinoma cell line (ATCC HTB-22) were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI+1640 medium supplemented with 5% heat-inactivated bovine serum (TRACE Biosciences Pty Ltd., Australia), 100 units/mL penicillin, and 100 μg/mL streptomycin.

Pharmacokinetics

Pharmacokinetic parameters for unlabelled KM871 and 111In-KM871 were estimated from data obtained from patients’ sera collected prior to, and following, each infusion of KM871 (20).
Flow cytometry

Patient PBMCs were analysed by direct immunofluorescence for reactivity with a series of mAbs using standard techniques on a Coulter Epics Elite ESP flow cytometer (Coulter Corporation, Miami, Florida, USA). Briefly, 3 × 10⁶ PBMCs were incubated for 30 min at 4°C with mAbs conjugated to FITC or PE, washed three times, and resuspended in fixative solution for analysis. PBMC phenotypes were classified as helper T cell (CD3+/CD4+), cytotoxic T cell (CD3+/CD8+), macrophage (CD14+), NK cells (CD3-/CD16+CD56+), and CD19+ or CD20+ B cells. The percentage of macrophages, neutrophils, and lymphocytes were determined as a percentage of total PBMCs analysed. CD3+/CD4+ T cells, CD3+/CD8+ T cells, and CD3-/CD16+CD56+ NK cells were determined as a percentage of total monocytes observed by FACS® analysis of forward and side scatter.

Serum complement measurements

Patient blood was collected prior to each KM871 infusion for biochemical screening, complete blood counts, and serum complement level determination (Austin Hospital, departments of biochemistry and immunology, respectively).

CDC

Preinfusion serum samples were collected from 17 patients receiving KM871 infusions (3 patients on the 1 mg/m², 5 mg/m², and 10 mg/m² dose levels; 4 patients each on the 20 mg/m² and 40 mg/m² dose levels). The complement (serum) was prepared on the day of collection and dispensed into 1.0 mL aliquots for storage at -70°C. Except for patients on the first dose level (patients 1, 2, and 3), sera from each cycle were analysed concurrently. The cytolyis of target GD3-positive SK-MEL-28 melanoma cells by patient serum was monitored with a 4-h ⁵¹Cr release assay based upon the previously published method (37). Briefly, antibody (0.00315-10 µg/mL) was added in triplicate to 1 × 10⁷ ⁵¹Cr-labelled target SK-MEL-28 cells and incubated on ice for 5 min. Fifty microliters of complement (serum) were added to yield a 1:3 final dilution of the serum. The microtitre plates were incubated for 4 h at 37°C. Following centrifugation, the ⁵¹Cr released in the supernatant was counted (Cobra II automated Gamma Counter, Canberra Packard, Melbourne, Australia). Percentage-specific lysis was calculated from the experimental, the total (50 µL target cells + 100 µL 10% Tween 20) and the spontaneous (50 µL target cells + 100 µL medium) ⁵¹Cr release. The anti-GD3 mAb KM871 was the test antibody, with KM966 included as an IgG1 class-matched control. The percentage cytotoxicity was plotted versus concentration of antibody (µg/mL), and the concentration of antibody at 50% cytotoxicity (CH50) was determined.

The cytolyis of target Lewis Y-positive MCF-7 human breast carcinoma cells by the sera of 6 patients (2 at 20 mg/m² and 4 at 40 mg/m² dose level) was also monitored with a 4-h ⁵¹Cr release assay as described above. The anti-Lewis Y mAb hu3S193 was the test antibody, with huA33 included as an IgG1 class-matched control and CD3-/CD16+CD56+ NK cells over a range of E:T cell ratios was also determined where the ADCC effected by 1 µg/mL KM871 on target SK-MEL-28 cells with healthy donor effector cells. Accordingly, the ADCC effected by 1 µg/mL KM871 on target SK-MEL-28 cells over a range of E:T cell ratios was also determined where sufficient patient blood was available for PBMC isolation. Controls included in the assay corrected for spontaneous release (medium alone) and for total release (10% Tween20/PBS). The anti-GM2 mAb KM966 was included as an IgG1 class-matched control. The percentage cell lysis (cytotoxicity) was calculated according to the formula:

% Cytotoxicity = ([Sample Counts - Spontaneous Release]/(Total Release - Spontaneous Release)) x 100

Fluorescent-activated cell sorting of patient lymphocytes was used to determined the ratio of CD3-/CD16+CD56+ NK cells in the population, enabling a correlation with measured ADCC measurements to be determined.

Biopsy analysis

Biopsy specimens from patients were obtained 7-10 d after the first infusion of KM871. An anatomical pathologist examined all specimens. Following fixation and paraffin embedding, sections of tumour were taken for standard histological examination (hematoxylin and eosin staining) and for frozen sections. The histologic appearance of the tumour specimens, including the presence of cellular infiltrates, was determined. Sections were evaluated for GD3 antigen expression on tumour cells as previously described (20), in addition to cluster of differentiation markers, complement regulatory proteins, localization of ¹¹¹In-KM871 to tumour, and standard histological examination (hematoxylin and eosin staining). Since no pretreatment biopsy specimens were obtained from patients entered into the trial, tumour biopsies from age-matched and sex-matched melanoma patients not infused with KM871 were obtained from an archive tissue bank and studied for comparative purposes.

Biopsy specimens were either frozen in isopentane cooled in liquid nitrogen or fixed in 10% neutral buffered formalin and embedded in paraffin. GD3 and the membrane-associated complement regulatory proteins CD46 (MCF, membrane cofactor protein), CD55 (DAF, decay accelerating factor), and CD59 (protectin) expression were evaluated in frozen sections from tumour biopsy specimens that were fixed in acetone prior to immunohistochemical staining. The cluster differentiation markers, CD3 (T cells), CD4 (T-helper cells), CD8 (cytotoxic T cells), CD20 (B cells), and CD57 (NK cells) were evaluated in formalin-fixed, paraffin-embedded tumour biopsy tissue. Endogenous peroxidase was quenched prior to incubation with primary antibodies. Antibody staining was detected using a secondary biotinylated antibody (LSAB Kit; Dako, Glostrup, Denmark or Silenus, Melbourne, Australia) followed by incubation with horse radish peroxidase conjugated alpha-biotin.
(LSAB Kit; Dako, Glostrup, Denmark or Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) and visualized by immersing in chromogen substrate AEC (Sigma-Aldrich, St. Louis, MO, USA). GD3 antigen expression was described in terms of percentage of positively stained melanoma cells. TILs in at least 5, but up to 10, high-power fields were counted and an average number of lymphocytes was calculated.

**Statistical analyses**
Statistical analysis of results was performed using Sigmastat for Windows (SPSS Inc., Chicago, IL, USA). The differences in lymphocyte numbers between the patient group for lymph node and subcutaneous biopsy site and the corresponding control group were analysed using a Student's t-test. Analyses of variance (ANOVA) were used to assess any differences observed in pharmacokinetic parameters. \( P < 0.05 \) was considered statistically significant.

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