

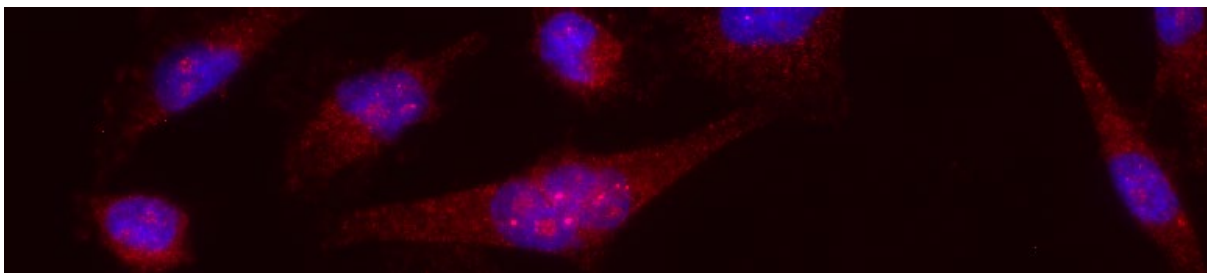
## ASX Announcement

### Zantrene Improves Cancer Immunotherapy in a Mouse Model of Treatment Resistant Melanoma

- Zantrene in combination with immunotherapy leads to shrinkage of mouse melanoma tumours that do not respond to immunotherapy alone
- Zantrene activates immune cells positively for better targeting of tumours
- Zantrene reduces resistance to immune therapy in human melanoma tumour cells
- Results are supportive of future clinical trials using Zantrene in combination with immune therapy treatments to potentially improve melanoma patient outcomes.

**22 June 2022** – Race Oncology Limited (“Race”) is pleased to share further interim results from its preclinical melanoma research program (ASX announcement: 19 March 2021). The program’s objective was to explore the use of Zantrene® (bisantrene dihydrochloride) in novel drug combinations for the treatment of drug and immunotherapy resistant melanomas using cell and animal models.

Used at low concentrations, Zantrene was found to enhance cancer immunotherapy in three distinct and complementary ways: (1) direct killing of melanoma cells; (2) activation of immune cells targeting the tumour, and (3) reducing the expression of immune evasion genes in the tumour.



**Figure 1. Human melanoma cells stained with BAK1 (red) and Dapi (blue).**

Race CSO Dr Daniel Tillett said: *“The results of this preclinical research demonstrate the potential of Zantrene to enhance immunotherapy outcomes in melanoma. While checkpoint inhibitors have revolutionised the treatment of advanced melanomas, far too many patients show no, or only a short-term response, to these treatments. Zantrene has shown its ability to work in three complementary ways to enhance the anti-melanoma immune response. I continue to be amazed by novel utility we’re seeing for Zantrene as a potential new cancer treatment.”*

Race CEO & MD Phillip Lynch said: *"Its pleasing to see continued reaffirmation of Zantrene's utility in suppressing and potentially improving cancer outcomes, in this case against Melanoma, which remains a challenging cancer. I look forward to us progressing clinical translation of this opportunity to assess improving patient outcomes."*

## Study Background

Checkpoint inhibitors immune therapies such as anti-PD-1 antibodies have emerged as a front-line treatment for many types of cancer, including melanoma. While these immune drugs have revolutionised the treatment of advanced melanoma, only a minority of patients show a long-term response to therapy and the five-year survival rate remains low due to treatment resistance.<sup>1</sup>

### ***Previous Human Trials of Zantrene in Melanoma Patients***

Zantrene showed significant historical *in vitro* activity against fresh human melanoma samples taken from patients in human tumor clonogenic assays.<sup>2,3</sup> In a subsequent historic Phase I trial of Zantrene administered weekly, one of four treated patients with metastatic melanoma achieved a complete response (complete shrinkage of tumour) which lasted 6 months.<sup>4</sup>

Despite these early successes, four subsequent historic Phase 2 studies of Zantrene in 100 melanoma patients using far longer dosing intervals and higher dosing levels (one maximally tolerated dose every three or four weeks) did not achieve any significant clinical responses.<sup>5-8</sup> Seventeen patients achieved disease stabilization, but no further complete responses were observed in any of these Phase 2 trials.

### ***Role of FTO in Melanoma***

Zantrene has been identified as a potent, targeted inhibitor of the Fat Mass and Obesity associated protein (FTO).<sup>9</sup> Previous studies have observed that FTO is over-produced in approximately 50% of metastatic melanomas<sup>10</sup> and that inhibition of FTO can overcome PD-1 immune checkpoint resistance in the B16-F10 (known to over-produce FTO) mouse melanoma model.<sup>10,11</sup>

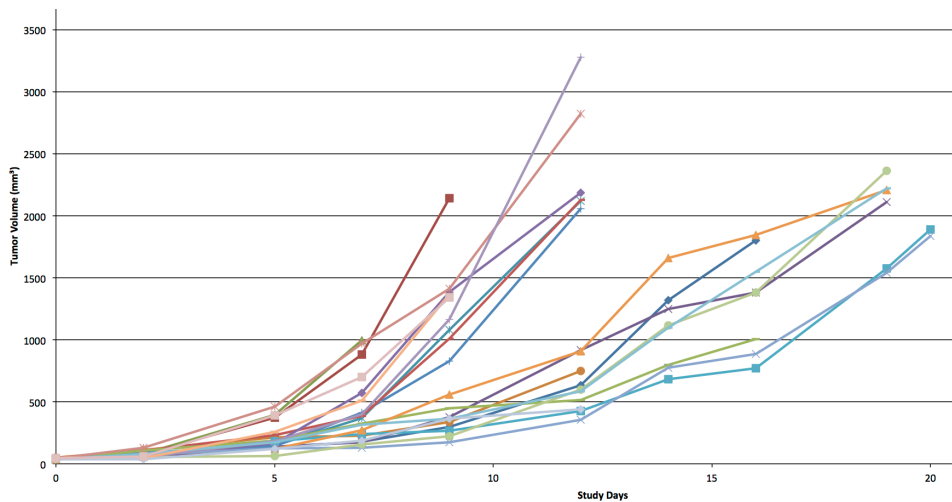
Further supporting these animal and cell studies, human genetic research has identified variations within the FTO gene that are associated with an increased risk of developing melanoma, with this increased risk being independent of body mass index.<sup>12,13</sup>

On the basis that Zantrene is a potent FTO inhibitor (IC<sub>50</sub> 142 nM)<sup>9</sup>, it was hypothesised that Zantrene and anti-PD-1 antibodies may synergise to overcome checkpoint inhibitor resistance in a treatment resistant mouse model of melanoma.

## Study Highlights

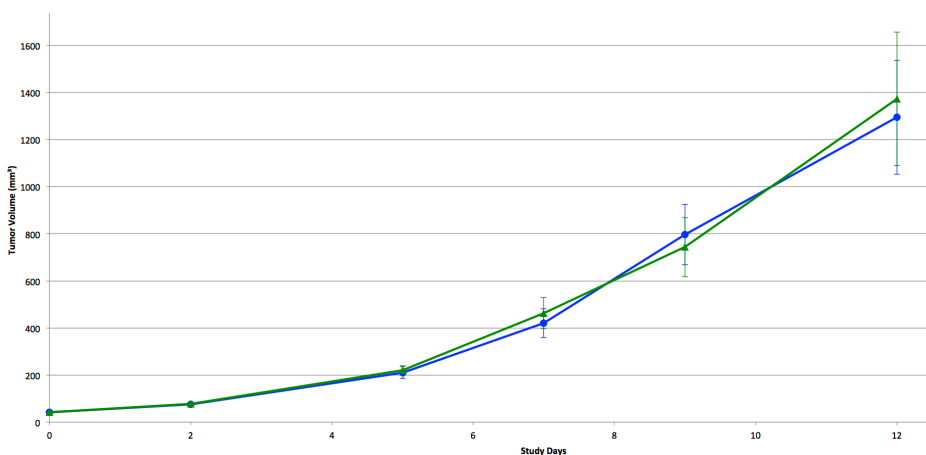
### 1. The B16-F10 mouse melanoma model is highly aggressive and unresponsive to current immunotherapy treatments

The murine B16 melanoma model is the most commonly used metastatic melanoma model for preclinical studies.<sup>14</sup> The B16-F10 cell line was generated as the 10<sup>th</sup> serial passage sub-clone of the B16 parent tumour line in C57BL/6 mice.<sup>15</sup> B16-F10 melanoma cells implanted subcutaneously into C57BL/6 mice result in highly aggressive (fast growing) tumours that double in size approximately every two days, although with significant variation between individual animals (Figure 2).



**Figure 2. Individual B16-F10 melanoma tumour growth rates in C57BL/6 mice inoculated with  $2 \times 10^5$  cells.** Time for the tumours to reach a size greater than  $2000 \text{ mm}^3$  ranged from 8 to 20 days.  $n=20$ .

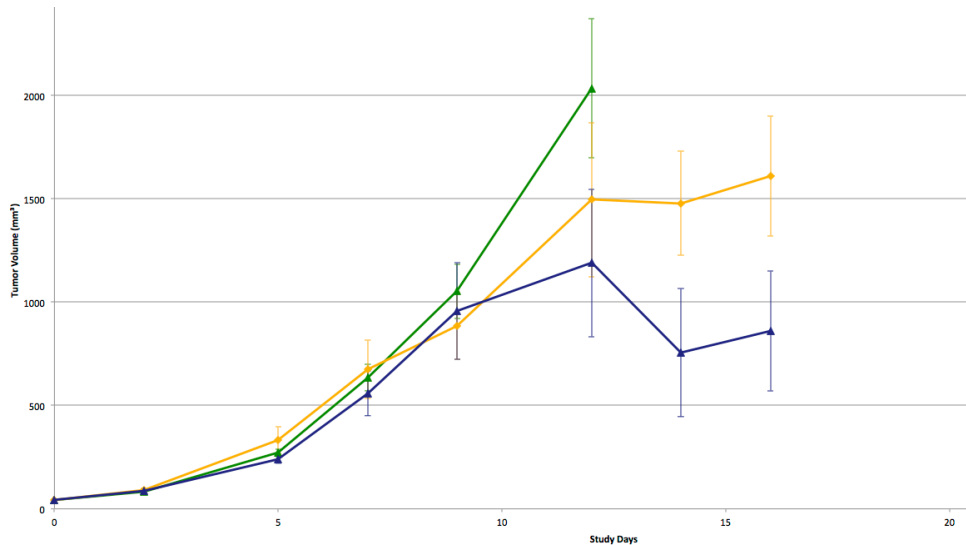
B16-F10 tumours are very resistant to immunotherapy treatments.<sup>14</sup> The checkpoint inhibitor anti-mPD-1 does not produce any response in subcutaneous B16-F10 tumours (Figure 3). Similarly, treatment with anti-mPD-L1, anti-mCTLA-4, anti-mLAG-3, or anti-TNF receptor family co-stimulatory receptor CD137 antibodies all fail to produce a response in this immunologically “cold” tumour model.<sup>14</sup>



**Figure 3. Treatment of B16-F10 tumours in C57BL/6 mice with either anti-mPD-1 antibody (green) or antibody control (blue).** Mice were dosed with 10 mg/kg of anti-mPD-1 antibody (RMP1-14) or control antibody (rat IgG2a) every 3 days starting on Study Day 0.  $n=20$ . Error bars = SEM.

## 2. Zantrene can overcome melanoma immune therapy resistance in mice.

To explore whether low dose Zantrene could enhance immunotherapy efficacy, groups of 20 mice were inoculated subcutaneously with  $2 \times 10^5$  B16-F10 cells and the tumour was allowed to grow for 6 days before initiation of treatment with either 10 mg/kg anti-mPD-1 every 3 days, 5 mg/kg Zantrene every 2 days, or a combination of 10 mg/kg anti-mPD-1 and 5 mg/kg Zantrene every 3 and 2 days, respectively. Mice with abnormally slow growing tumours (smaller than  $170 \text{mm}^2$  at Study Day 5) were excluded. The study ended when only 2 mice in each group were alive (Figure 4).



**Figure 4. Treatment of B16-F10 tumours in C57BL/6 mice with anti-mPD-1 antibody (green), low dose Zantrene (yellow), or anti-mPD-1 antibody plus low dose Zantrene (blue).** Mice were dosed with 10 mg/kg anti-PD-1 antibody (RMP1-14) every 3 days, or with 5 mg/kg of Zantrene every 2 days starting on Study Day 0. Green (n=13); Yellow (n=13); Blue (n=9). Error bars = SEM.

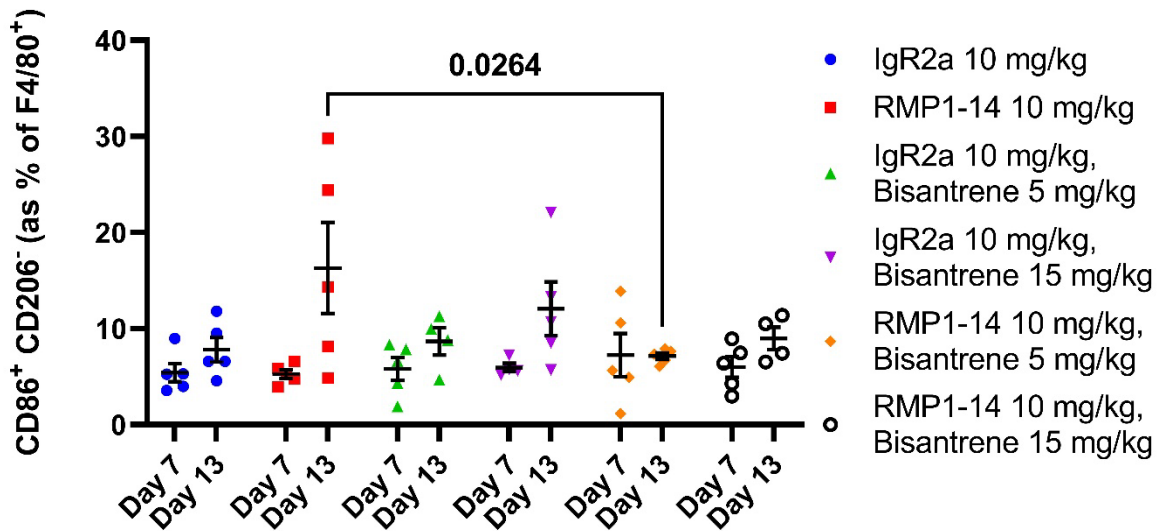
The anti-mPD-1-alone treated mice showed no inhibition or tumour shrinkage with the mice only surviving for 12 days after treatment initiation. The Zantrene-alone treated mice showed modest suppression of B16-F10 tumour growth and increased survival to Study Day 16. The combination of anti-mPD-1 and Zantrene resulted in additional tumour growth suppression after Study Day 12.

Of note, 4 of the 20 combination-treated mice showed sustained tumour regression (shrinkage) after Day 6 over more than four days. No sustained tumour regressions occurred in any of the 40 mice treated with anti-mPD-1-alone or Zantrene-alone.

## 3. Zantrene activates immune cells involved in melanoma tumour rejection

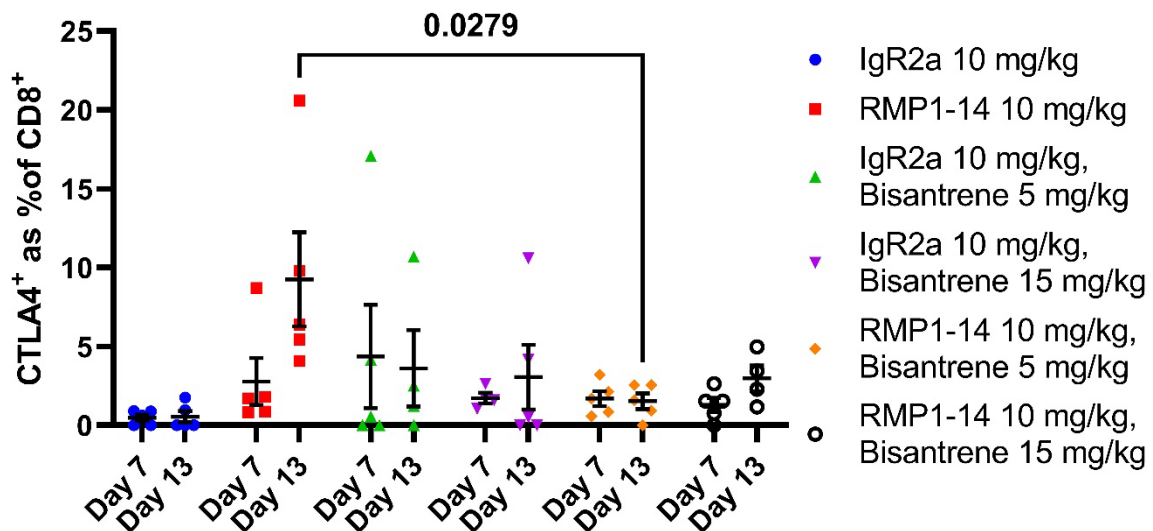
To explore the effect of the different treatments on immune activation, mice were euthanized on Day 7 or 13 (five mice per group at each time point) and the tumours and spleens excised for flow cytometric immune cell analysis.

A wide range of anti-tumour immune cell changes were observed in animals treated with the Zantrene/anti-mPD-1 combination. In one example, the Zantrene/anti-mPD-1 combination produced a 56% increase in the number of anti-tumour M1-type macrophages, relative to mice treated with anti-mPD-1 antibody alone (Figure 5).



**Figure 5. Anti-tumour M1 macrophage cells.** Group 1. Rat IgG2a at 10mg/kg; Group 2. RMP1-14 at 10mg/kg; Group 3. Rat IgG2a at 10mg/kg plus Zantrene at 5mg/kg; Group 4. Rat IgG2a at 10mg/kg plus Zantrene at 15mg/kg; Group 5. RMP1-14 at 10mg/kg plus Zantrene at 5mg/kg; Group 6. RMP1-14 at 10mg/kg plus Zantrene at 15mg/kg. n=5 per group per day. *p* calculated using Šidák's multiple comparisons test.

In another example, the Zantrene/anti-mPD-1 combination produced an 83% decrease in the number of CD8+ T-cells co-expressing the immunosuppressive checkpoint protein CTLA4, relative to animals treated with anti-mPD-1 antibody alone (Figure 6). Both of these immune cell changes are associated with a better immune response against the tumours.

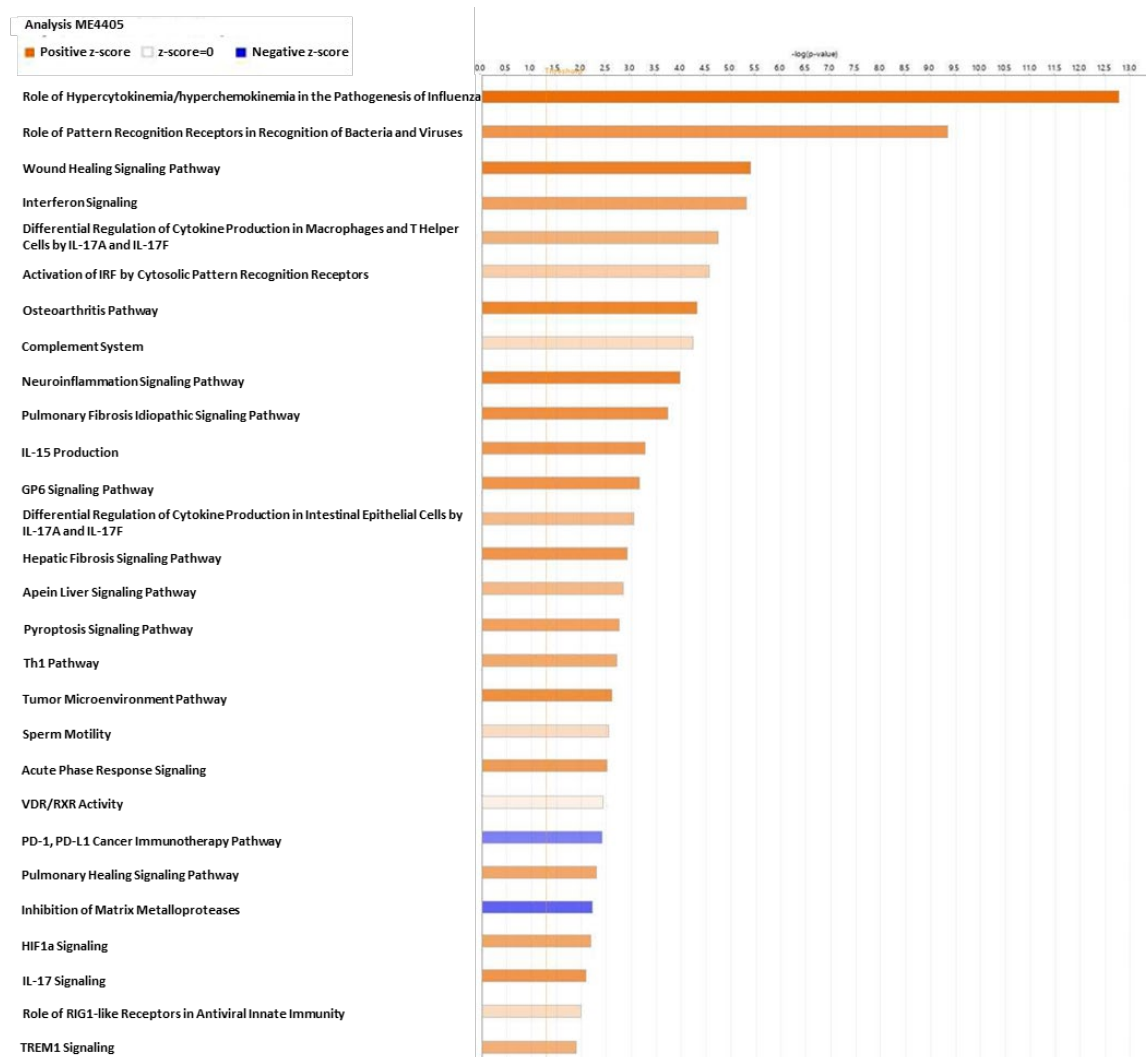


**Figure 6. Immunosuppressive CD8+ cells.** Group 1. Rat IgG2a at 10mg/kg; Group 2. RMP1-14 at 10mg/kg; Group 3. Rat IgG2a at 10mg/kg plus Zantrene at 5mg/kg; Group 4. Rat IgG2a at 10mg/kg plus Zantrene at 15mg/kg; Group 5. RMP1-14 at 10mg/kg plus Zantrene at 5mg/kg; Group 6. RMP1-14 at 10mg/kg plus Zantrene at 15mg/kg. n=5 per group per day. *p* calculated using Šidák's multiple comparisons test.

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#### 4. Zantrene reduces the expression of the immune evasion genes in melanoma

Previous studies identified that Zantrene is able to reduce the expression of immune evasion proteins such as PD-L1 and LILRB4 in Acute Myeloid Leukaemia (AML) cells via inhibition of FTO<sup>9</sup>. RNA sequencing analysis of the primary human melanoma cell line ME4405 exposed to Zantrene also showed down-regulation of the PD-1/PD-L1 pathway and up-regulation of a large number of pathways including those involved in immune activation (Figure 7).

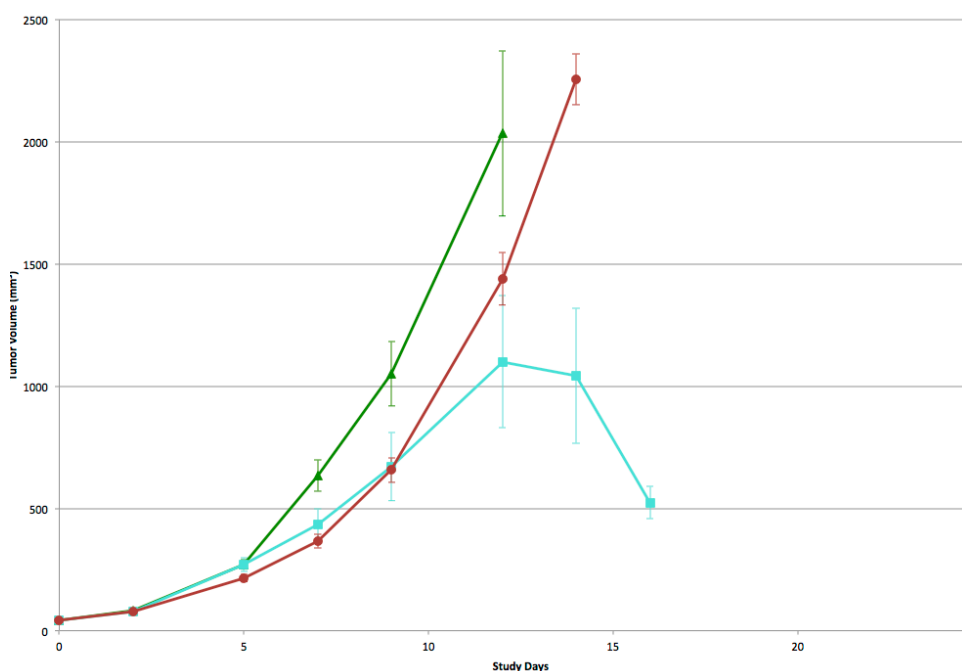


**Figure 7. RNA-Seq Analysis of ME4405 Melanoma cells exposed to Zantrene for 48h.** Pathways that are up regulated are shown in orange and those down regulated are shown in blue. Dark colour = greater up- or down- regulation. Longer bars = greater significance. Pathways with non-significant expression changes have been excluded.

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### 5. Zantrene dosage level is important for immune therapy response

To investigate the effect of higher doses of Zantrene on immunotherapy, groups of 20 mice were inoculated subcutaneously with  $2 \times 10^5$  B16-F10 cells and the tumour allowed to grow for 6 days before initiation of treatment with either 10 mg/kg of anti-mPD-1 every 3 days, 15 mg/kg Zantrene every 2 days, or a combination of 10 mg/kg anti-mPD-1 and 15 mg/kg Zantrene every 3 and 2 days, respectively. Mice with abnormally slow growing tumours (smaller than  $170\text{mm}^2$  at Day 5) were excluded from the study analysis. The study ended when only 2 mice in each group were alive (Figure 8).



**Figure 8. Treatment of B16-F10 tumours in C57BL/6 mice with anti-mPD-1 antibody (green), high dose Zantrene (teal), or anti-mPD-1 antibody plus high dose Zantrene (red).** Mice were dosed (IP) with 10 mg/kg of anti-PD-1 antibody (RMP1-14) every 3 days, or with 15 mg/kg of Zantrene every 2 days (IV) starting from day 0. Green (n=13); Teal (n=12); Red (n=11). Error bars = SEM.

The mice treated with high dose Zantrene (15 mg/kg) showed tumour shrinkage from Study Day 6 reflecting the known direct cytotoxic (cell killing) efficacy of Zantrene on B16 melanoma tumours.<sup>2</sup>

Interestingly, the inclusion of an anti-mPD-1 antibody substantially abolished this anti-tumour effect of Zantrene with the tumour displaying little growth slowing compared to the no Zantrene control, or when low dose Zantrene (5 mg/kg) was used in combination with an anti-mPD-1 antibody (Figures 4 & 8). Similar effects of chemotherapy (i.e. high doses of Zantrene) on the efficacy of anti-PD-1 immunotherapy treatments have been observed previously and are thought to be due to chemotherapeutic induced up regulation of COX-2 creating an pro-tumour inflammatory environment that limits the efficacy of chemo-immunotherapy combinations.<sup>16</sup>

This “dose” effect may explain why Zantrene showed efficacy in melanoma during the Phase 1 trial where frequent dosing was used, but failed to show any significant efficacy when used at a high and infrequent (chemotherapeutic) doses in the Phase 2 trials.<sup>5-8</sup>

## Conclusions

- Zantrene used in combination with an anti-PD-1 antibody can induce an effective immunotherapy response in the highly aggressive B16-F10 mouse model of immunotherapy treatment resistant (“cold”) melanoma.
- Zantrene when used at low, non-chemotherapeutic doses can activate immune cells to aid in the creation of an anti-tumour immune response.
- Zantrene reduces the expression of genes involved in immune evasion by human melanoma cells.
- Zantrene displays a dose effect on anti-PD-1 immunotherapy where low doses of Zantrene are more efficacious than high chemotherapeutic-level doses.
- A provisional patent has been lodged protecting this discovery.

## Next Steps

- Further explore the dose effect of Zantrene on anti-PD-1 immunotherapy in new preclinical models of melanoma to determine the optimal dosage for use in human melanoma clinical trials.
- New preclinical studies in diverse model systems to understand the importance of FTO over-production in anti-PD-1 immunotherapy resistance.
- Additional preclinical studies of Zantrene on human immune cells to better understand the effects of Zantrene and dose levels with the aim of achieving optimal immune cell anti-tumour activation.
- Publication of results in a high quality, peer reviewed journal.
- Further consultation with key opinion leaders to explore initiation of a clinical study utilising the optimal dosage of Zantrene to enhance the immunotherapy response in treatment resistant melanoma patients.



## Q&A

### *Is this a good result?*

**Yes.** The FTO over-producing B16-F10 mouse melanoma is a very difficult and aggressive cancer model in which most clinically effective immunotherapy treatments fail<sup>14</sup>. To see any tumour directed immune response in this model is outstanding. Interestingly, the combination of Zantrene and anti-mPD-1 was able to shrink B16-F10 tumours, whereas previous studies using genetic knockdowns of FTO in combination with anti-mPD-1 only slowed the growth of the B16-F10 tumours.<sup>10</sup> It is possible the combined effects of Zantrene are responsible for this difference.

### *Why was such a difficult melanoma model used in this study?*

**Better to fail in the lab than fail in the clinic.** While a range of mouse models of melanoma exist, many have little clinical relevance. It is all too easy in preclinical studies to choose a mouse cancer model that responds well to almost any experimental treatment, but which fails to work in the clinic.

Melanoma is a highly competitive clinical space with more than 600 clinical trials recruiting around the world.<sup>17</sup> For a new treatment to have any chance of recruiting patients into a clinical trial, it needs to show outstanding preclinical results to gain the interest and support of key opinion leaders and clinicians.

### *Why do you need to do additional preclinical work?*

**To maximise the chance of clinical success.** This study has shown that the effect of Zantrene on immunotherapy depends on the dose used. It is appropriate to understand the optimal Zantrene dosing before moving on to treating patients. Rushing into the clinic with a new immunotherapy without a robust preclinical data package can prove problematic.

### *How soon could you start a human trial of Zantrene in melanoma patients?*

**2023**, however, the answer to this question depends on the additional preclinical work that is underway to optimise the dose levels of the combination of Zantrene and anti-PD-1.

### *Is ccRCC a better choice for a human Zantrene trial in solid tumours?*

**Possibly.** Race currently has the resources to run one solid tumour Phase 1/2 clinical trial. At this stage the choice is between melanoma and renal cancer. The initial renal cancer preclinical study showed impressive cell-based synergy of Zantrene with a range of kinase inhibitors (ASX announcement: 10 March 2022), but additional renal cancer studies (currently underway) are needed to know if these combinations will work in animals.

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**About Race Oncology (ASX: RAC)**

Race Oncology is an ASX listed precision oncology company with a Phase 2/3 cancer drug called Zantrene®.

Zantrene is a potent inhibitor of the Fatso/Fat mass and obesity associated (FTO) protein. Overexpression of FTO has been shown to be the genetic driver of a diverse range of cancers. Race is exploring the use of Zantrene as a new therapy for melanoma and clear cell renal cell carcinoma, which are both frequent FTO over-expressing cancers.

In breakthrough preclinical research, Race has also discovered that Zantrene protects from anthracycline-induced heart damage, while in tandem acting with anthracyclines and proteasome inhibitors to improve their ability to target breast cancer. Race is evaluating this discovery.

The Company also has compelling clinical data for Zantrene as a chemotherapeutic agent and is in clinical trial in Acute Myeloid Leukaemia (AML).

Race is pursuing outsized commercial returns for shareholders via its 'Three Pillar' strategy for the clinical development of Zantrene.

Learn more at [www.raceoncology.com](http://www.raceoncology.com)

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