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ASX Announcement

Compelling Preclinical Kidney Cancer Results for Zantrene

- **Zantrene found to kill a range of kidney cancer cells both on its own and in combination with existing cancer treatments**
- **When used in combination with Zantrene the kidney cancer drugs lenvatinib, cabozantinib and pazopanib showed greatly improved cell killing (synergy)**
- **These results support advancing Zantrene in human kidney cancer trials.**

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10 March 2022 – Race Oncology Limited ("Race") is pleased to share final results from the clear cell renal cell carcinoma (a dangerous form of kidney cancer) preclinical program led by eminent cancer researcher, Associate Professor Nikki Verrills of The University of Newcastle and Hunter Medical Research Institute (ASX announcement: 25 March 2021).

This research found that Zantrene on its own and in combination with known kidney cancer drugs can kill kidney cancer cells at clinically relevant concentrations. These results support advancing Zantrene into the clinic as a possible new treatment option for advanced kidney cancer patients.

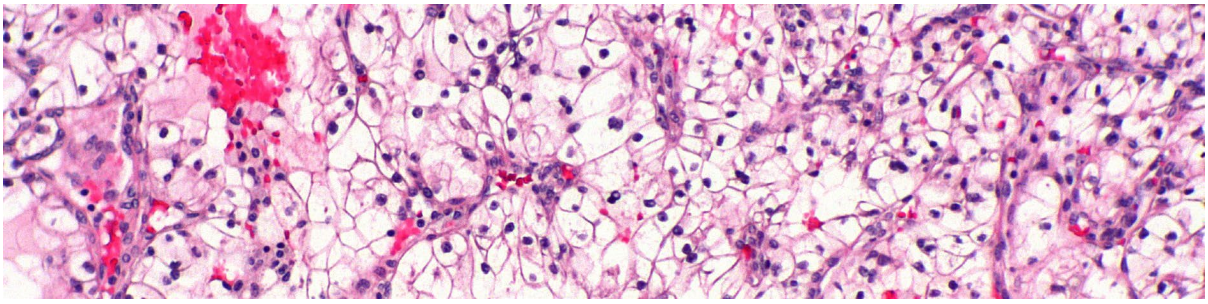

Figure 1. Human clear cell renal cell carcinoma (kidney cancer). *Image courtesy of Wikipedia.*

Chief Scientific Officer, Dr Daniel Tillett said: *"The results from Prof Verrills laboratory are highly encouraging and supportive of our clinical plans for Zantrene in kidney cancer. Advanced kidney cancer has a large unmet need for improved treatment options and Zantrene in combination with existing treatments may offer new hope for patients with this devastating disease."*

Chief Executive Officer, Mr Phillip Lynch said, *"We are again pleased to note Zantrene's effectiveness both in isolation and in combination with other known kidney cancer treatments. This result encourages clinical translation, and we look forward to determining an optimal approach for progressing clinical study."*

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Study Background

Clear Cell Renal Cell Carcinoma

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer, comprising over 70% of renal tumours (Figure 2). While a relatively rare cancer, accounting for approximately 2% of global cancer diagnoses and deaths, it has more than doubled in incidence over the past half-century, and today is the ninth most common cancer in the developed world[1].

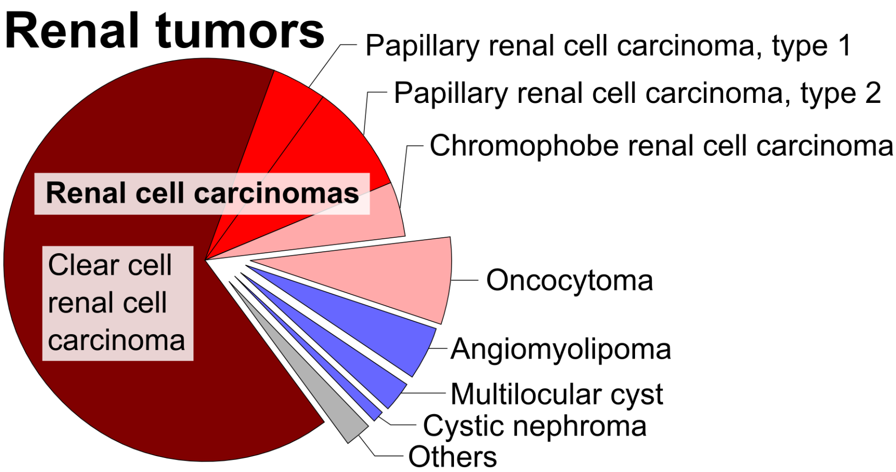

Figure 2. Kidney cancer types and relative incidence. *Image courtesy of Wikipedia.*

Advanced/metastatic clear cell renal cell carcinoma occurs in 25–30% of people before diagnosis. The clinical signs of ccRCC are often mild or non-existent until the disease has spread throughout the body (metastasis)[2]. The most common organs for ccRCC to metastasize to lymph nodes, lungs, bones, liver and brain[3]. Late diagnosis remains a major challenge in the effective treatment of ccRCC.

Treatment of Clear Cell Renal Cell Carcinoma

Advanced ccRCC has a poor prognosis compared to many other cancers. While there have been major improvements in kidney cancer treatment in recent years, including the recent approval of immune therapies, the five-year survival rate for advanced ccRCC is still as low as 12%[4]. New treatments and drug combinations remain urgently needed to address what is often a devastating disease.

Importance of FTO in Clear Cell Renal Carcinoma

A recent preclinical study identified a synthetically lethal interaction between the Von Hippel-Lindau (VHL) tumour suppressor protein and the m^6A RNA demethylase Fatso/Fat Mass and Obesity Protein (FTO), in ccRCC[5]. Synthetic lethality occurs when the loss of either one of a pair of genes or proteins has little or no effect on the survival of the cell, but the loss of both proteins (or their activity) at the same time is lethal.

VHL is inactivated in the majority of ccRCC (~90%)[6], suggesting that the loss of FTO activity could prove lethal to cells lacking a functional FTO protein. Xiao *et al* found that FTO expression is increased in VHL-deficient ccRCC tumours, and genetic inactivation of FTO reduced the growth and survival of VHL-deficient cells[5].

Zantrene has been recently identified as a potent inhibitor of FTO[7] so may prove efficacious in the treatment of ccRCC with inactive VHL genes. This hypothesis was tested using Zantrene on an isogenic VHL mutant and wildtype ccRCC cell line. The potential for synergies with Zantrene and existing kidney cancer treatments was also explored.

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Study Highlights

1. Zantrene kills clear cell renal cell carcinoma cells

The sensitivity of kidney cancer and normal kidney cell lines to Zantrene was tested as a single agent to determine the cytotoxicity IC_{50} (drug concentration required to kill 50% of cells). Zantrene cytotoxicity was measured using a resazurin assay combined with visual inspection of the cells at each dose level and the IC_{50} values calculated. Direct cytotoxicity IC_{50} values ranged from 242nM to 12,353nM in the kidney cancer cell lines tested (Table 1). With the exception of the ccRCC *A-704* cells, which were highly resistant, all other lines showed IC_{50} values below 1.4μM with 7 of the 12 lines displaying IC_{50} values below 1μM, a concentration achievable in patients based on prior human trials.

Table 1. Cytotoxic IC_{50} values of Zantrene in human renal cell lines.

Cell Line	Renal Cell Type	Zantrene IC_{50} (nM)
HK-2	Non-tumourigenic cortex/proximal tubule	1061
HEK293	Tumourigenic embryonic kidney	219
ACHN	Metastatic (pleural effusion)	242
Caki-1	Adenocarcinoma (metastatic)	659
Caki-2	Adenocarcinoma	331
769-P	ccRCC	1028
786-O	ccRCC	1309
A-704	ccRCC	12353
KMRC-1	ccRCC	506
A-498	ccRCC	325
RCC4 EV (VHL mutant)	ccRCC	907
RCC4 VHL (VHL widetype)	ccRCC	1170

Blue: non-cancer cell lines; Black: kidney cancer cell lines.

To determine if VHL status (i.e. wildtype or mutant/deleted) was associated with increased sensitivity to Zantrene, the isogenic cell lines *RCC4 EV*, which has a mutant VHL gene and the *RCC4 VHL* cell line which has been transduced with the wildtype VHL gene to rescue the VHL loss, showed that the VHL mutant cell line was more sensitive (1.3x) to Zantrene than the VHL rescue line (Figure 3).

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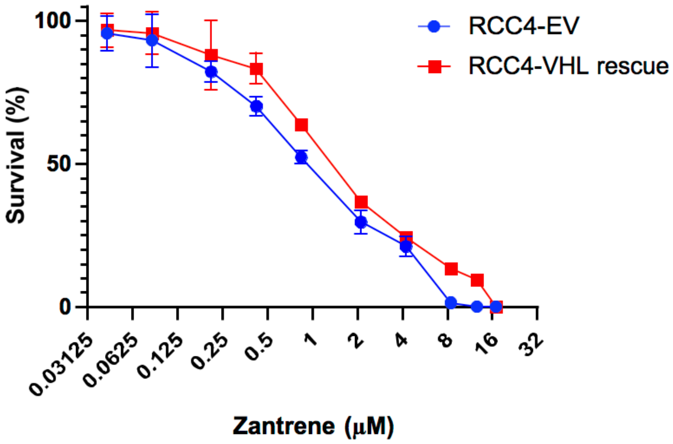

Figure 3. Association between Zantrene sensitivity and VHL status. Direct comparison of Zantrene sensitivity in the *RCC4* isogenic cell line pair. Cells were treated for 72h with indicated drug doses and cell viability determined. Mean +/- SEM, n=3.

2. Zantrene slows the growth of ccRCC cells

Greater lethality between VHL loss and Zantrene was observed using long term clonogenic cell growth assays (cell colony formation), which better measures a drug's effect on cancer cell growth rather than cell killing[5]. The clonogenic assays were performed on the same panel of renal cell lines. A representative example of the effect of Zantrene on ccRCC cell colony formation is shown in Figure 4.

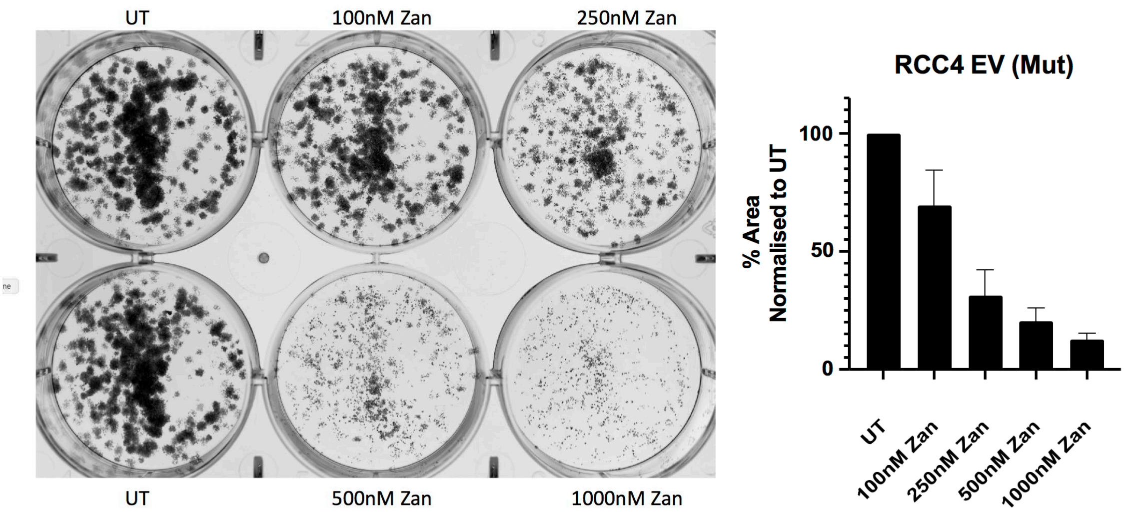

Figure 4. Clonogenic cell growth assay of Zantrene in *RCC4 EV* cells. *RCC4 EV* cells were seeded in 6-well plates at 1000 cells/well and left to adhere overnight before treatment with the indicated concentrations of Zantrene for 96h. Wells had fresh media added and were left for an additional 96h to assess drug recovery. Clonogenicity was assessed using crystal violet staining. Image of plate used for analysis (left). Colony area normalised to the untreated (UT) wells (right). Mean +/- SEM, n=3.

All ccRCC cell lines were more sensitive to Zantrene (i.e. lower IC_{50} values) in the clonogenic cell growth assay (Table 2 & Figure 4). Similar sensitivity trends were observed as those seen in the cytotoxicity assay (Table 1). The *A-704* cells remained the most resistant to Zantrene and the *HEK293, ACHN, KMRC-1* and *A-498* cell lines were the most sensitive. Interestingly, the *Caki-1* ccRCC cells showed more than 10 times greater sensitive to Zantrene in the clonogenic assay than in the cytotoxicity assay (60nM verses 659nM) suggesting Zantrene may be a potent inhibitor of ccRCC growth at concentrations below the cytotoxic level.

As seen in the cytotoxicity assays, the *RCC-4 EV* (VHL mutant) cells were significantly more sensitive (2.9x) to Zantrene than the wild-type *RCC-4 VHL* rescue cells (Table 2 & Figure 5).

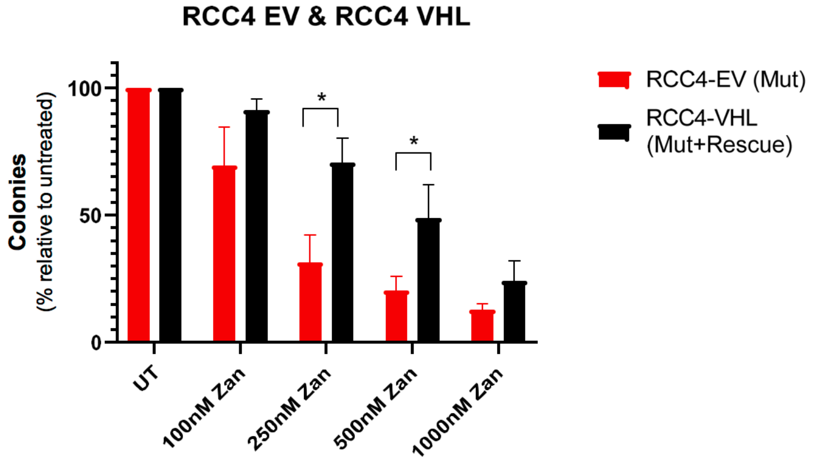

Figure 5. Association of Zantrene sensitivity and VHL status as assessed using clonogenicity assays. Colonies observed for the *RCC4 EV* and *RCC4 VHL* rescue cell lines. Cells were treated as described in Figure 4. NS, not significant, unpaired t-test. n=4, *p<0.05, paired t-test.

Table 2. Clonogenic IC_{50} of Zantrene in human renal cell lines.

Cell Line	Renal Cell Type	Zantrene IC_{50} (nM)
HK-2	Non-tumourigenic cortex/proximal tubule	217
HEK293	Tumourigenic embryonic kidney	46
ACHN	Metastatic (pleural effusion)	53
Caki-1	Adenocarcinoma (metastatic)	60
Caki-2	Adenocarcinoma	116
769-P	ccRCC	84
786-O	ccRCC	229
A-704	ccRCC	750
KMRC-1	ccRCC	60
A-498	ccRCC	46
RCC4 EV (VHL mutant)	ccRCC	167
RCC4 VHL (VHL widetype)	ccRCC	483

Blue: non-cancer cell lines; Black: kidney cancer cell lines.

3. The FTO inhibitor Dac51 is less effective at killing ccRCC cells than Zantrene

The cytotoxic sensitivity of five ccRCC cell lines to the structurally distinct FTO inhibitor, Dac51[9], was examined. A significantly higher concentration of Dac51 than Zantrene was required to kill all five ccRCC cell lines (Figure 6 & Table 3).

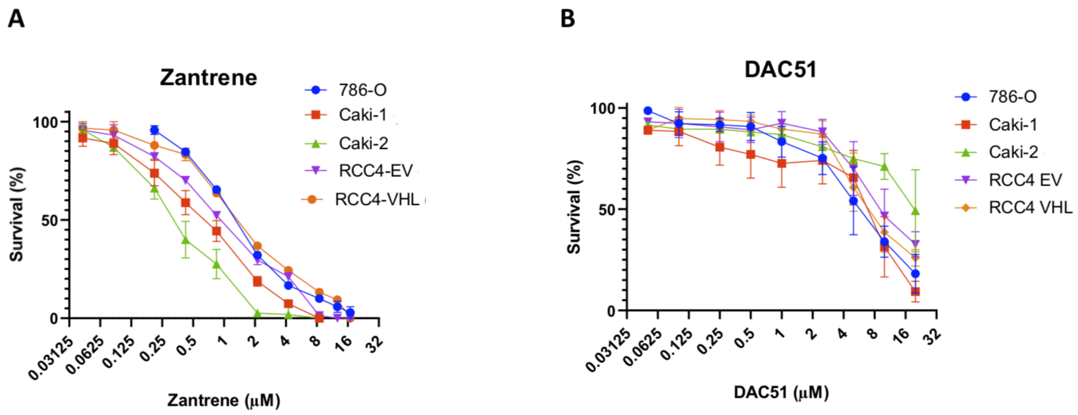

Figure 6. Cytotoxicity of single agent Zantrene and DAC51 in clear cell renal cell carcinoma cell lines. *786-O, Caki-1, Caki-2, RCC4 EV* and *RCC4 VHL* ccRCC cells were treated for 72h with the indicated drug concentrations of **(A)** Zantrene or **(B)** DAC51. Cell viability was determined using the resazurin metabolic assay and visual inspection. Cell viability is expressed as a percentage of untreated control cells. Mean +/- SEM, n=3.

A number of interesting differences were noted between the two FTO inhibitor agents. The *786-O* cell line was the most sensitive to Dac51, yet the least sensitive to Zantrene. In contrast, the *Caki-2* cells were the most sensitive to Zantrene, but the least sensitive to Dac51 (Table 3). Unlike Zantrene, Dac51 was more effective at killing the *RCC4 VHL* wildtype cell line than the corresponding mutant *RCC4 EV* cell line. In addition, the cytotoxic IC_{50} values for Dac51 ranged between 14x to 50x its reported IC_{50} values for FTO inhibition (0.4μM)[9], suggesting that the modest Dac51 cytotoxic activity may reflect an off-target effect.

Table 3. IC_{50} of DAC51 compared to Zantrene in RCC cell lines.

Cell Line	IC_{50} Dac51 (μM)	Rank sensitivity Dac51	IC_{50} Zantrene (μM)	Rank sensitivity Zantrene
786-O	5.53	1	1.31	5
Caki-1	7.12	3	0.66	2
Caki-2	19.73	5	0.33	1
RCC4 EV	9.00	4	0.91	3
RCC4 VHL	6.08	2	1.17	4

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4. Zantrene improves the killing of ccRCC cells when used in combination with other kidney cancer drugs

To enable synergy combination studies to be performed with Zantrene and a range of kidney cancer drugs, the single agent IC_{50} value for each drug was measured against five ccRCC cell lines; *786-O, Caki-1, Caki-2, RCC4 EV* and *RCC4 VHL* (Table 4).

Table 4. Single agent cytotoxic IC_{50} values of clinical kidney cancer drugs.

Cell Line	Ever	Suni	Soraf	Pazop	Len	Caboz
786-O	1.467	3.702	7.916	>10	>20	11.728
Caki-1	>20	>8	9.658	7.867	>20	10.442
Caki-2	>20	5.451	15.997	>10	>20	7.345
RCC4 EV	1.119	5.02	10.233	7.021	>20	6.586
RCC4 VHL	1.151	2.216	8.65	5.202	>20	7.326

Ever: everolimus; Suni: sunitinib; Soraf: sorafenib; Pazop: pazopanib; Len: lenvatinib; Caboz: cabozantinib. All values μM.

To determine the possible synergy effects of the Zantrene/drug combinations, two different synergy analysis methods were utilized, Webb[10] and Bliss[11].

Webb Analysis

Webb analysis revealed synergy across multiple drug doses for Zantrene and all drugs. An example of Webb analysis for the cell line *786-O* performed with Zantrene + everolimus, or Zantrene + sunitinib is shown in Figure 7.

The strongest Webb synergy was observed for Zantrene in combination with the VEGFR kinase inhibitors lenvatinib, cabozantinib and pazopanib. Synergy was also observed at some doses of sunitinib, sorafenib and everolimus, most often at lower drug doses. Similar results were observed across the other four ccRCC cell lines, with **lenvatinib, cabozantinib and pazopanib consistently displaying the greatest synergy with Zantrene** (data not shown).

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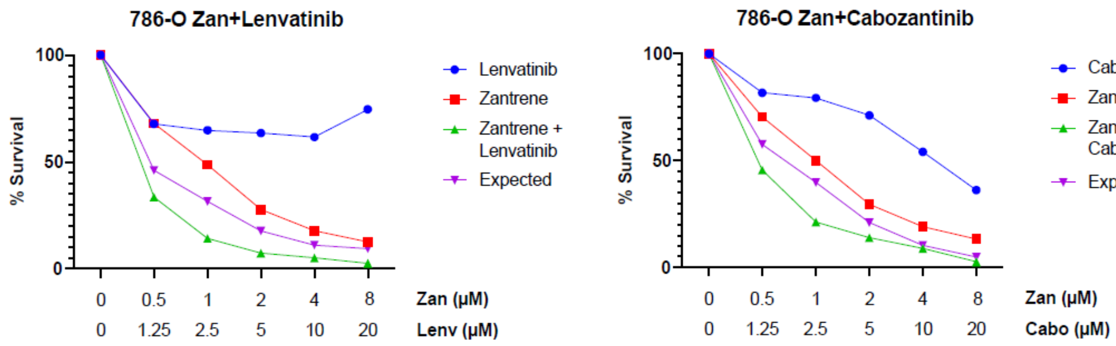

B

786-O					
Zan (µM)	Lenv (µM)	Webb Result	Zan (µM)	Cabo (µM)	Webb Result
0.5	1.25	-0.125	0.5	1.25	-0.120
0.5	2.5	-0.274	0.5	2.5	-0.254
0.5	5	-0.333	0.5	5	-0.314
0.5	10	-0.348	0.5	10	-0.237
0.5	20	-0.470	0.5	20	-0.156
1	1.25	-0.067	1	1.25	-0.078
1	2.5	-0.173	1	2.5	-0.185
1	5	-0.223	1	5	-0.202
1	10	-0.235	1	10	-0.142
1	20	-0.330	1	20	-0.093
2	1.25	0.030	2	1.25	-0.042
2	2.5	-0.048	2	2.5	-0.081
2	5	-0.103	2	5	-0.072
2	10	-0.111	2	10	-0.065
2	20	-0.173	2	20	-0.046
4	1.25	0.064	4	1.25	-0.007
4	2.5	0.007	4	2.5	0.001
4	5	-0.044	4	5	-0.015
4	10	-0.059	4	10	-0.015
4	20	-0.102	4	20	-0.024
8	1.25	0.140	8	1.25	0.028
8	2.5	0.071	8	2.5	0.041
8	5	0.006	8	5	-0.020
8	10	-0.030	8	10	-0.012
8	20	-0.068	8	20	-0.021

Figure 7. Webb analysis of 786-O ccRCC cells. (A) Cell viability in response to different dose ranges of lenvatinib and cabozantinib in combination with Zantrene, as indicated. Experimental data is shown for each drug alone and the combinations. The 'Expected value' is calculated using the method of Webb and shows the expected value if the drug combination was additive. Experimental observed values below this line show synergy, at or near the line is additive, and above the line is antagonistic. **(B)** Webb analysis for all drug combination doses tested. A result of <-0.1 indicates drug synergy (red), between -0.1 to 0.1 is additive (green), and >0.1 is antagonistic (yellow).

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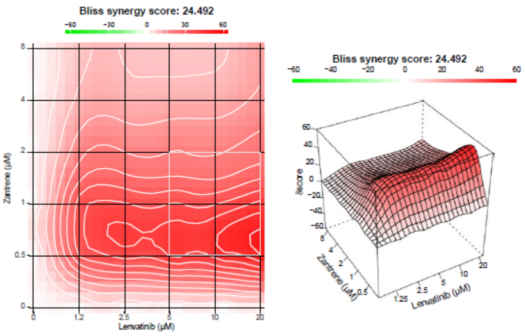
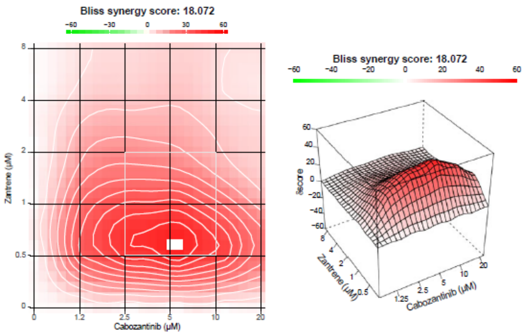
Bliss Analysis

Analysis using the Bliss method similarly revealed overall **synergistic effects of Zantrene with lenvatinib, everolimus and pazopanib in all five ccRCC cell lines** (Table 5). An example of Bliss analysis on an individual cell line and the drug combinations is shown in Figure 8.





Drug1	Drug2	Conc1	Conc2	Relative inhibition	Synergy	concUnit
Zantrene	Lenvatinib	0	0	0	0	µM
Zantrene	Lenvatinib	0.5	0	31.9804	0	µM
Zantrene	Lenvatinib	1	0	51.2914	0	µM
Zantrene	Lenvatinib	2	0	72.3114	0	µM
Zantrene	Lenvatinib	4	0	82.1208	0	µM
Zantrene	Lenvatinib	8	0	87.4741	0	µM
Zantrene	Lenvatinib	0	1.25	32.2331	0	µM
Zantrene	Lenvatinib	0.5	1.25	66.4324	33.097	µM
Zantrene	Lenvatinib	1	1.25	83.2899	33.127	µM
Zantrene	Lenvatinib	2	1.25	90.0954	18.309	µM
Zantrene	Lenvatinib	4	1.25	92.9001	10.917	µM
Zantrene	Lenvatinib	8	1.25	96.2079	9.1686	µM
Zantrene	Lenvatinib	0	2.5	35.1739	0	µM
Zantrene	Lenvatinib	0.5	2.5	73.6445	39.065	µM
Zantrene	Lenvatinib	1	2.5	85.7621	33.937	µM
Zantrene	Lenvatinib	2	2.5	91.3989	18.639	µM
Zantrene	Lenvatinib	4	2.5	93.5102	10.831	µM
Zantrene	Lenvatinib	8	2.5	96.6441	9.1202	µM
Zantrene	Lenvatinib	0	5	36.4675	0	µM
Zantrene	Lenvatinib	0.5	5	78.2846	43.578	µM
Zantrene	Lenvatinib	1	5	86.8523	34.296	µM
Zantrene	Lenvatinib	2	5	92.7333	19.762	µM
Zantrene	Lenvatinib	4	5	94.0079	11.089	µM
Zantrene	Lenvatinib	8	5	96.6739	8.8904	µM
Zantrene	Lenvatinib	0	10	38.4061	0	µM
Zantrene	Lenvatinib	0.5	10	81.4912	45.52	µM
Zantrene	Lenvatinib	1	10	87.7538	33.893	µM
Zantrene	Lenvatinib	2	10	92.992	19.207	µM
Zantrene	Lenvatinib	4	10	94.9264	11.697	µM
Zantrene	Lenvatinib	8	10	96.8904	8.767	µM
Zantrene	Lenvatinib	0	20	25.3596	0	µM
Zantrene	Lenvatinib	0.5	20	77.5542	55.141	µM
Zantrene	Lenvatinib	1	20	84.7335	40.524	µM
Zantrene	Lenvatinib	2	20	91.4757	23.234	µM
Zantrene	Lenvatinib	4	20	95.2612	15.988	µM
Zantrene	Lenvatinib	8	20	97.473	12.221	µM

Drug1	Drug2	Conc1	Conc2	Relative inhibition	Synergy	concUnit
Zantrene	Cabozantinib	0	0	0	0	µM
Zantrene	Cabozantinib	0.5	0	29.519	0	µM
Zantrene	Cabozantinib	1	0	49.8545	0	µM
Zantrene	Cabozantinib	2	0	70.454	0	µM
Zantrene	Cabozantinib	4	0	80.8559	0	µM
Zantrene	Cabozantinib	8	0	86.6419	0	µM
Zantrene	Cabozantinib	0	1.25	18.211	0	µM
Zantrene	Cabozantinib	0.5	1.25	54.4014	31.918	µM
Zantrene	Cabozantinib	1	1.25	69.5239	25.143	µM
Zantrene	Cabozantinib	2	1.25	81.23	13.807	µM
Zantrene	Cabozantinib	4	1.25	85.5257	6.0833	µM
Zantrene	Cabozantinib	8	1.25	90.0966	4.4877	µM
Zantrene	Cabozantinib	0	2.5	20.662	0	µM
Zantrene	Cabozantinib	0.5	2.5	66.7645	44.577	µM
Zantrene	Cabozantinib	1	2.5	78.7486	34.681	µM
Zantrene	Cabozantinib	2	2.5	84.5321	16.786	µM
Zantrene	Cabozantinib	4	2.5	87.0696	7.2756	µM
Zantrene	Cabozantinib	8	2.5	91.1938	5.3442	µM
Zantrene	Cabozantinib	0	5	28.8306	0	µM
Zantrene	Cabozantinib	0.5	5	80.0231	52.167	µM
Zantrene	Cabozantinib	1	5	84.6197	35.671	µM
Zantrene	Cabozantinib	2	5	86.1357	15.071	µM
Zantrene	Cabozantinib	4	5	90.4566	9.0718	µM
Zantrene	Cabozantinib	8	5	93.9393	7.0708	µM
Zantrene	Cabozantinib	0	10	45.8689	0	µM
Zantrene	Cabozantinib	0.5	10	85.0215	39.945	µM
Zantrene	Cabozantinib	1	10	84.7456	22.727	µM
Zantrene	Cabozantinib	2	10	87.8785	9.5112	µM
Zantrene	Cabozantinib	4	10	91.0943	4.8606	µM
Zantrene	Cabozantinib	8	10	95.489	5.5003	µM
Zantrene	Cabozantinib	0	20	63.7993	0	µM
Zantrene	Cabozantinib	0.5	20	86.2899	22.141	µM
Zantrene	Cabozantinib	1	20	85.2823	9.6074	µM
Zantrene	Cabozantinib	2	20	92.5319	7.1511	µM
Zantrene	Cabozantinib	4	20	93.9506	3.1347	µM
Zantrene	Cabozantinib	8	20	97.2834	4.0501	µM

Figure 8. Bliss Synergy Analysis in 786-O cells with Zantrene + lenvatinib or cabozantinib. 2D and 3D visualisation of predicted Bliss scores at each dose point, with red to green scale indicating areas of synergy to antagonism, and the average synergy score. A table of Bliss scores for each individual dose combination is shown. Values >10 are considered synergistic (red); values below -10 are considered antagonistic. Values between -10 to 10 are additive.

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Table 5. Synergy scores as determined by Bliss analysis[1]

Drug Combination	786-O	Caki-1	Caki-2	RCC4 EV	RCC4 VHL
everolimus + Zantrene	18.15	19.88	15.45	17.58	17.74
sunitinib + Zantrene	12.26	6.42	5.26	8.01	5.21
sorafenib + Zantrene	10.40	7.86	5.07	5.09	4.41
pazopanib + Zantrene	21.86	16.24	13.95	22.34	17.84
lenvatinib + Zantrene	24.49	20.68	17.93	36.42	29.32
cabozantinib + Zantrene	18.07	15.08	8.64	19.15	15.21

[1]Values >10 are considered synergistic (red); values between -10 to 10 are additive (green); values below -10 are considered antagonistic.

Bliss analysis further revealed Zantrene to be synergistic with cabozantinib in all cell lines except for *Caki-2*. Limited synergy was observed for Zantrene and sunitinib or sorafenib in *786-O* cells (Table 5).

Conclusions

- Zantrene can kill kidney cancer cells at clinically relevant concentrations as a single agent.
- Zantrene can slow the growth of kidney cancer cells at sub-cytotoxic drug concentrations.
- Zantrene shows strong and robust synergy when used with a number of existing kidney cancer drugs, especially those that inhibit VEGFR or the mTOR pathways.
- The synergistic combinations have high clinical relevance and potential for rapid translation into the clinic.
- A new patent protecting these findings has been submitted. If granted, the patent would be valid until 2042.

Next Steps

- ccRCC studies testing the best Zantrene drug combinations in relevant animal models.
- Further preclinical studies to explore the mechanism of action of the Zantrene synergies discovered.
- Discussions with key opinion leaders in renal cancer to explore advancing Zantrene for use in ccRCC patients in a Phase 1/2 treatment combination human clinical trial. If positive, such a trial could begin as early as end calendar 2022.

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Q&A

What do these kidney cancer results mean for Race?

We have identified a number of strong and robust drug combinations for Zantrene that can be rapidly translated into the clinic with potential for treating advanced kidney cancer. In addition, Zantrene proved to be effective at slowing the growth of kidney cancers cells at drug concentrations similar to those obtained when using low, frequent dosing.

Have you obtained IP protection for these new discoveries?

Yes. The synergistic combinations identified in this study have important clinical relevance in the treatment of kidney cancer. We have submitted a patent application covering these discoveries, which if granted, will provide IP protection until 2042.

What is the market potential of this discovery?

As outlined at the 2021 Race Annual General Meeting, kidney cancer has significant commercial potential with the existing drug market estimated to exceed US$2 billion per year. In addition, ccRCC is considered an orphan indication by the FDA and EMA offering the potential for an Orphan Drug Designation (if granted), provides market exclusivity for 7 years in the USA and 10 years in the EU irrespective of patent protection status.

When can Race investors expect the next update?

We are currently evaluating the best kidney cancer animal models in order to identify the most effective drug combination(s) to take into the clinic. This work is well advanced, and we expect to be able to update our shareholders on the results in CY Q3 2022.

Materials and Methods

Drugs

Zantrene (bisantrene dihydrochloride) and DAC51 were reconstituted in dimethylsulphoxide (DMSO) at 20mM. Everolimus (RAD001), sunitinib, sorafenib, pazopanib, lenvatinib, cabozantinib (BMS-907351) were reconstituted in (DMSO) at 17.2mM (lenvatinib), and 100mM (all others). All other drugs were stored at -20°C (all others) and aliquoted to reduce the number of freeze-thaw cycles.

Cell Culture

Human renal cell lines were cultured in a humidified chamber at 37°C with 5% CO_2 in the cell culture medias listed in Table 6.

Table 6. Cell culture media and supplements for renal cell lines.

Cell Line(s)	Base Media	Supplements
769-P, 786-O	RPMI-1640 (with GlutaMAX)	10% foetal bovine serum (FBS), 20mM HEPES, 1mM sodium pyruvate
Caki-1, Caki-2	McCoy's 5A	10% FBS, 2mM L-glutamine
A-498, A-704, ACHN, HEK293	Minimum Essential Medium (MEM)	1 x non-essential amino acid solution, 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate
KMRC-1, RCC4 EV, RCC4 VHL	DMEM (high glucose)	10% FBS, 2mM L-glutamine, 20mM HEPES Additional supplementation for RCC4 EV and RCC4 VHL: 0.5mg/mL G418
HK-2	Keratinocyte Serum-Free Media (KSFM)	Recombinant human EGF, bovine pituitary extract (supplied as kit with base media), 2mM L-glutamine

Cytotoxicity Assays

Cell viability was determined using a resazurin metabolic activity assay. Cells were seeded in duplicate wells of 96-well microtitre plates at 1 x 10^3 cells/well (*786-O, RCC4 EV, RCC4 VHL, KMRC-1*), 3 x 10^3 cells/well (*Caki-1, Caki-2, HK-2, 769-P, A-498, HEK293*) or 5 x 10^3 cells/well (*A-704, ACHN*) and cultured for 24h. Drugs were diluted in media and added to wells, and cells cultured for a further 72h. Viability was determined using the fluorogenic viability dye resazurin (Ex 544nm, Em 590nm; 0.6mM resazurin, 78µM methylene blue, 1mM potassium hexacyanoferrate (III), 1mM potassium hexacyanoferrate (II) trihydrate (Sigma Aldrich), dissolved in PBS)[12].

Resazurin is metabolised into the red-fluorescent resorufin by metabolically active cells. Fluorescence was measured 5hrs post-addition of resazurin solution (1:10, v/v) at 544nm excitation/590nm emission on a FLUOstar OPTIMA plate reader (BMG LabTechnologies). Graphpad Prism 9 software was used to generate graphs.

Drug IC_{50} values were determined by cubic spline-lowess regression analysis using *Prism 9*. At least three independent replicates were performed for each cell line and each drug combination, and data is represented as mean ± standard error of the mean (SEM).

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Clonogenicity Assays

Clonogenicity assays were used to determine the colony-forming ability of cells treated with Zantrene. Cells were seeded into 6-well plates at 150 cells/well (*786-O*), 1000 cells/well (*RCC4 EV, RCC4 VHL, A-498*) or 2000 cells/well (*HK-2, A-704, 769-P, Caki-1, Caki-2, KMRC-1, ACHN, HEK293*) and allowed to adhere for 24h. Zantrene was diluted in media and added to wells, and cells were cultured for 96h. Drug-containing media was removed and replaced with fresh media (without drug) and cultured for an additional 96h to allow the formation of cell colonies. At the endpoint, media was removed from wells and cells washed with cold PBS twice.

Cells were fixed with ice-cold methanol for 10 minutes on ice followed immediately by staining with crystal violet solution (0.5% crystal violet, 25% methanol in PBS) at room temperature. Excess crystal violet solution was washed away, and pictures of plates captured on a *ChemiDoc MP Imaging System* (Bio-Rad).

Images were analysed using the *ColonyArea* plugin[13] for *ImageJ*[14] and the percent area of the well filled by colonies determined and presented as % of untreated cells using *Prism 9*. Four independent replicates were performed for each cell line and data is represented as mean ± SEM.

Synergy Analysis

For combination drug treatments, three different synergy analyses have been conducted, including the fraction product method of Webb[10] and the BLISS synergy method[11] using *SynergyFinder 2.0* software[15].

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About Associate Professor Nikki Verrills

After completing her PhD in 2005 on chemotherapy resistance in childhood leukaemia, Associate Professor Verrills was awarded a Peter Doherty Postdoctoral Fellowship from the National Health and Medical Research Council in 2006. In the same year, she was the inaugural recipient of a Hunter Medical Research Foundation grant for young cancer researchers. Since then, she has established an innovative research lab at the University of Newcastle studying the differences between cancer cells that respond well to drug treatments and those that do not.

Professor Verrills is currently supported by a fellowship from the Australian Research Council and project funding from the National Health and Medical Research Council. She has published over 60 journal articles with an H-index of 24.

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

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