# Preclinical evaluation of Bisantrene alone and in combination with Decitabine for Acute Myeloid Leukemia

Joshua S Brzozowski[1,2], Nikita Panicker[1,2], Kasey Miller[1,2], Marinella Messina[3], Benjamin J Buckley[3], Michael J Kelso[3], Daniel Tillett[3], Anoop K Enjeti[4,5] and Heather C Murray[1,2] Nicole M Verrills[1,2]

1. School of Biomedical Sciences and Pharmacy, College of Health. Medicine & Wellbeing , University of Newcastle, NSW, Australia
2. Precision Medicine Program, Hunter Medical Research Institute, Newcastle, NSW, Australia
3. Race Oncology Limited, Sydney, NSW, Australia;
4. Calvary Mater Hospital, Newcastle, 2298, NSW, Australia
5. School of Medicine and Public Health, College of Health. Medicine & Wellbeing , University of Newcastle, NSW, Australia

Contact: nikki.verrills@newcastle.edu.au

## INTRODUCTION

Acute Myeloid Leukaemia (AML) is the most lethal form of leukaemia, carrying a 5-year survival rate of 24%. Anthracyclines (e.g daunorubicin and idarubicin) together with cytarabine comprise standard of care induction chemotherapy in acute myeloid leukemia (AML). Although 60% of patients achieve remission, the majority relapse within 1-2 years, with overall 5-year survival only 27%. Toxicity of induction therapy is a major barrier to treatment success, precluding many unfit and elderly patients. Hypomethylating agents (HMA; azacytidine, decitabine) provide a less toxic alternative and have improved treatment options for the unfit, however, most eventually acquire resistance resulting in relapse.

Bisantrene is an anthracene derivative originally developed as a less cardiotoxic chemotherapy alternative to anthracyclines. Clinical studies showed bisantrene is an effective AML salvage therapy, producing response rates up to 50% without accompanying cardiotoxicity (Rothman 2017 *Int J Cancer Res Ther*, **2**, 1-10). Bisantrene has also been reported to inhibit FTO, an RNA $N^6$-methyladenosine ($m^6A$) demethylase, that plays oncogenic roles in various cancers, including AML, and FTO inhibition sensitizes AML cells to HMAs (Su *et al., 2020 Cancer Cell* **38**, 79–96).

Therefore, we hypothesized that bisantrene may sensitize AML cells to HMAs, and thus provide an alternative chemotherapeutic option in combination with HMAs for AML therapy.

## AIMS

1. Test the efficacy of the bisantrene/decitabine combination *in vitro* and *in vivo*.
2. Investigate the mechanism of action of bisantrene and decitabine.

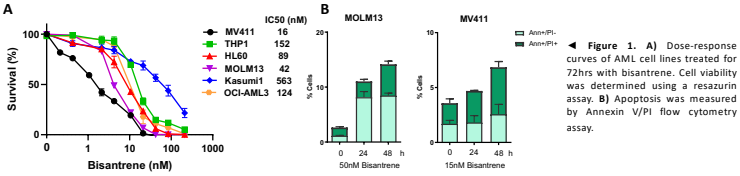
## METHODS

- The *in vitro* activity of bisantrene +/- decitabine was assessed in range of AML cell lines using resazurin assays and the combination effect determined using Bliss synergy analysis.
- The effect of bisantrene +/- decitabine on apoptosis was assessed by Annexin V/propidium iodide flow (PI) cytometry assays.
- The *ex vivo* sensitivity of primary AML patient bone marrow-derived mononuclear cells was determined using an ATP lite assay.
- The *in vivo* efficacy of bisantrene +/- decitabine was assessed in NSG mice engrafted with MOLM13-luc AML cells, or an AML patient derived xenograft (PDX-AML16, originally isolated from a 61yo female with AML M4 subtype, with normal cytogenetics, FLT3-ITD$^+$ mutant IDH2 (R140Q), NPM1 and WT1 (Lee *et al Haematologica* **100**, 914-926).
- The effect of bisantrene +/- decitabine on the proteome and phosphoproteome was investigated using label-free quantitative mass spectrometry and gene-set and ingenuity pathway analyses.

## RESULTS

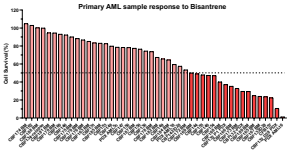
### 1. Bisantrene shows single agent activity in AML

- **Bisantrene inhibits growth (Fig 1A) and induces apoptosis (Fig 1B) of AML cell lines.**



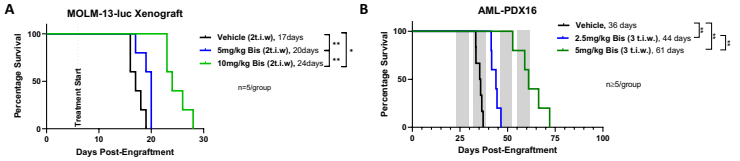

◄ **Figure 1. A)** Dose-response curves of AML cell lines treated for 72hrs with bisantrene. Cell viability was determined using a resazurin assay. **B)** Apoptosis was measured by Annexin V/PI flow cytometry assay.

- **Bisantrene displays variable single agent efficacy against primary AML bone marrow derived mononuclear cells *ex vivo* (Fig 2).**
- **Correlation with mutation status revealed NPM1$^{mut}$ AML were more sensitive to bisantrene than NPM1$^{WT}$ *ex vivo* (p<0.05). KRAS, ASXL1 and TET2 mutant AMLs showed a trend towards higher bisantrene sensitivity.**




► **Figure 2. *Ex vivo* response of human AML patient samples to bisantrene .** Primary AML blasts or cells isolated from AML PDXs, were treated with 1 uM bisantrene for 24hrs. Cell viability was determined using an ATPlite assay. Cell survival relative to untreated cells. Samples displaying 50% or lower survival in response to 1uM Bisantrene are shown in red (13/40 primary AMLs; 17/46 including PDXs).

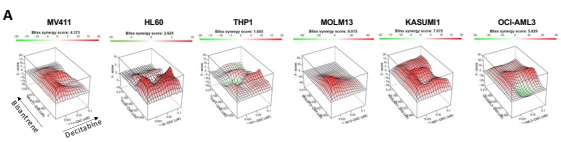
- **Bisantrene produces a dose-dependent increase in survival of mice engrafted with AML cell line or patient-derived xenografts (Fig. 3).**




▲ **Figure 3. Bisantrene increases survival of mice engrafted with AML. A)** NSG mice were engrafted with MOLM-13-luc cells and leukaemia burden monitored by bioluminescence imaging. Bisantrene was administered three times per week at 5mg/kg or 10mg/kg for up to 3 weeks. **B) .** PDX-AML16 cells were engrafted into NSG mice and once human CD45+ cells reached ~1% in the peripheral blood, mice were randomised and treated with vehicle control, 2.5mg/kg or 5mg/kg i.v. bisantrene (Bis) 3 t.i.w. as indicated (grey shading). Mice were sacrificed when the percentage of human CD45+ cells in the peripheral blood reached 25% (determined by flow cytometry). Kaplan-Meier survival curve. n≥5 mice/group. Median survival in days shown. **p<0.01.

### 2. Bisantrene enhances the effects of decitabine *in vitro*

- **AML cells pre-treated with decitabine for 1 day, followed by decitabine plus bisantrene for 3 days, induced synergistic cytotoxicity at multiple doses for all cells (Fig. 4A).**
- **MOLM13 cells displayed the highest overall synergy (Fig. 4B).**



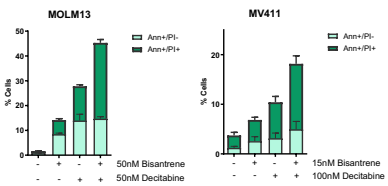

B

| Cell Line | Overall Synergy Score | Most Synergistic Area Score |
|---|---|---|
| MV411 | 4.37 | 9.51 |
| HL60 | 2.62 | 6.16 |
| THP1 | 1.60 | 6.7 |
| MOLM13 | 8.02 | 23.61 |
| KASUMI1 | 7.67 | 10.58 |
| OCI-AML3 | 5.83 | 10.98 |

Values >10 are considered synergistic (red).

▲ **Figure 4: Bliss Synergy Analysis.** Cells were pre-treated with decitabine for 24hrs, then bisantrene plus decitabine for 72hr at indicated doses. **A)** 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. **B)** Table of Bliss scores for the average across all doses, and the most synergistic 2x2 area. Values >10 are considered synergistic (red); below -10 antagonistic; and between-10 to 10 are additive.

- **Combined decitabine and bisantrene induces more apoptosis than either drug alone (Fig. 5).**




► **Figure 5. Decitabine and bisantrene induce apoptosis in AML cells.** Cells were pre-treated with decitabine (DAC) for 1 day, then Bisantrene plus Decitabine for 2 days at indicated doses Apoptosis was measured by Annexin V/PI flow cytometry assay.

### 3. Bisantrene enhances the efficacy of decitabine *in vivo*

- **The effect of combining bisantrene with two different decitabine dosing regimens was tested in the MOLM13 cell line mouse model (Fig. 6).**
- **Administration of either 0.5 mg/kg decitabine for 5 days only; or 0.2 mg/kg decitabine 5 days/ week for 3 weeks, reduced leukaemic burden and enhanced the survival alone.**
- **The combination of bisantrene and decitabine showed significantly higher efficacy than either drug alone in both experiments.**

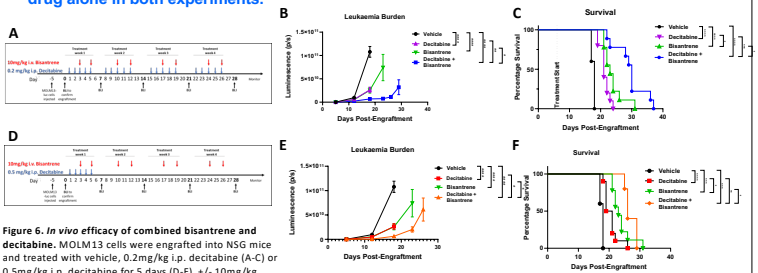
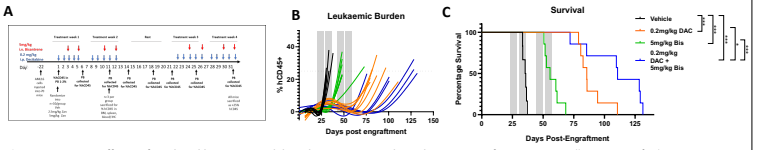



**Figure 6. *In vivo* efficacy of combined bisantrene and decitabine.** MOLM13 cells were engrafted into NSG mice and treated with vehicle, 0.2mg/kg i.p. decitabine (A-C) or 0.5mg/kg i.p. decitabine for 5 days (D-F), +/- 10mg/kg i.v. bisantrene, as indicated. Leukaemia burden was detected by bioluminescence imaging (BLI) at indicated intervals. A,D) Study design. B,E) Quantitation of BLI; mean +/- SEM. C,F) Kaplan-Meier survival curve. n=10 mice/group. C) Median survival for vehicle =18; decitabine = 21; bisantrene = 23; Combined drugs = 30 days. F) Median survival for vehicle =18; decitabine = 20; bisantrene = 23; Combined drugs = 26 days ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

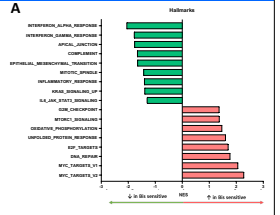
- **The combination of bisantrene and decitabine also showed significantly higher efficacy than either drug alone in the PDX-AML16 mouse model (Fig. 7).**




▲ **Figure 7. *In vivo* efficacy of combined bisantrene and decitabine in a patient derived AML xenograft.** PDX-AML16 cells were engrafted into NSG mice and once human CD45+ cells reached ~1% in the peripheral blood, mice were randomised and treated with vehicle control, 0.2mg/kg i.p. decitabine (DAC) 5 t.i.w, 5mg/kg i.v. bisantrene (Bis) 2 t.i.w., or both drugs, for 4 weeks as indicated (grey shading). Mice were sacrificed when the percentage of human CD45+ cells in the peripheral blood reached 25% (determined by flow cytometry). **A)** Study design. **B)** Leukaemic burden over time for individual animals. **C)** Kaplan-Meier survival curve. n=7 mice/group. Median survival for vehicle = 36; decitabine = 86; bisantrene = 56; Combined decitabine + bisantrene = 111 days. ***p<0.001; *p<0.05.

### 4. Key cellular pathways targeted by combined bisantrene and decitabine

- **Differential proteome and gene set enrichment analysis was used to compare the three most bisantrene-sensitive versus least sensitive cell lines (Fig 1A). Increased expression of MYC targets, G2M checkpoint, MTORC and E2F targets were associated with bisantrene sensitivity (Fig. 8A).**
- **Phosphoproteomic analysis of MOLM13 cells treated with decitabine +/- bisantrene identified inhibition of MYC, MTOR and RhoA signalling & activation of DNA damage repair and the UPR (Fig. 8B)**
- **Inhibition of MYC and MTOR signalling are likely key drivers of the anti-leukaemic effect of combined decitabine and bisantrene in AML.**




B

| Pathway | DAC | Bis | DAC + Bis |
|---|---|---|---|
| mTOR Signaling | -1.41 | 0.49 | 2.20 |
| Regulation of eIF4 and p70S6K Signaling | | 0.36 | -2.02 |
| RHOA Signaling | | | -1.72 |
| MMC Mediated Apoptosis Signaling | | | -1.40 |
| HIF1a Signaling | | | -0.29 |
| Cell Cycle Control of Chromosomal Replication | -0.92 | -1.67 | 0.90 |
| Role of CHK Proteins in Cell Cycle Checkpoint Control | 3.08 | -3.26 | 1.12 |
| Role of BRCA1 in DNA Damage Response | 2.17 | -0.90 | 2.10 |
| BER (Base Excision Repair) Pathway | | -0.72 | 2.10 |
| DNA Methylation and Transcriptional Repression Signaling | 3.91 | 0.29 | 2.22 |
| Sirtuin Signaling Pathway | 1.85 | 0.73 | 2.32 |
| p53 Signaling | 1.82 | -0.21 | 2.56 |
| Spliceosomal Cycle | 1.75 | -0.20 | 2.98 |
| PPARa/RXRa Activation | 1.23 | 0.27 | 3.09 |
| ATM Signaling | 2.70 | 1.23 | 3.30 |
| Unfolded protein response (UPR) | | | 3.34 |

► **Figure 8: Proteome/Phosphoproteome Pathway Analysis.** A) Proteome analysis of the three most and least bisantrene-sensitive AML cell lines was performed by quantitative mass spectrometry. Differentially expressed proteins were analysed by GSEA and significantly enriched pathways identified. NES, normalised enrichment scores. **B)** Phosphoproteome analysis of MOLM13 cells treated with 50nM decitabine (DAC) 48hr, 50nM bisantrene (Bis) 24hr, or the combination of both drugs. Phosphopeptides significantly enriched with treatments were analysed by IPA to identify enriched pathways. The predicted activation score indicates pathway inhibition (blue) or activation (red).

## Conclusion: Combining bisantrene and decitabine is a potential therapeutic strategy for AML therapy

**Acknowledgements:**
We thank Prof Richard Lock and Dr Charles de Bock, Children's Cancer Institute Australia, for providing AML-PDXs and the MOLM13-luc cells, respectively.

**Funding:**
RACE ONCOLOGY
NHMRC
cancer institute NSW