X-linked Inhibitor of Apoptosis Protein Expression and Cisplatin Resistance in Ovarian Cancer

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Submitted: 1 Aug. 2007; Accepted: 16 Aug. 2007

Abstract
This study sought to investigate the apoptotic potential of Cisplatin and the changes in X-Linked Inhibitor of Apoptosis protein (XIAP) expression that occur with treatment. Following this analysis the study investigated means to relate this back to chemoresistance and whether cells with high levels of XIAP will be more resistant, and therefore more likely to be refractory to initially successful treatment, than other cell types. SKOV-3 and OVCAR-3 cells were used as models of chemoresistance and chemosensitivity respectively. Cisplatin was used over 24 hours at a 20µM dose and found to significantly increase apoptosis in both cell lines p<0.05. mRNA expression of XIAP was found to decrease under Cisplatin treatment in both cell lines p<0.05, while Caspase-3 expression increased in SKOV-3 only p<0.05. Western blot analysis of XIAP expression showed significant inhibition of XIAP protein in both cell lines however OVCAR-3 XIAP expression was reduced to below detectable levels p<0.05. This study has determined that expression of XIAP is an important factor in chemoresistance and the mechanisms of stabilization of this molecule is a potential key factor in the ovarian cancer cells ability to respond to Cisplatin induced apoptotic signalling.

Keywords: Apoptosis, XIAP, Ovarian Cancer, Cisplatin.

INTRODUCTION

So Human ovarian surface epithelial (hOSE) cancer is the most lethal gynecological cancer in the Western world and ranks fourth among the most common female cancers (Riman et al. 2002). One of the key problems of ovarian cancer is that most women, at diagnosis, present with advanced stage disease and therefore poor prognosis. The ability to rapidly accumulate resistance to both radiation and chemotherapy agents after initial diagnosis, and primary treatment, makes it one of the most lethal cancers in the world (Gibb et al. 1997).

Cisplatin is a key chemotherapeutic agent for ovarian cancer and the ability of ovarian cancer cells to become resistant to effective treatment dosages represents a significant hindrance to patient outcome (Gibb et al. 1997). The mechanisms of chemoresistance appear to be multifactorial and are generally thought of in terms of altered pharmacodynamics and gene expression (including multi-drug resistant genes), modified drug target, increased rate of DNA repair, decreased rates of drug induced DNA or macromolecule damage (LaCasse et al. 1998; Kartalou & Essigmann 2001). Cisplatin therapy is often combined with other drugs such as Taxol, Paclitaxel or Gemcitabine to decrease initial chemoresistance and increase functionality (Fraser et al. 2003). Resistance to Cisplatin therapy has recently been linked to high levels of the inhibitor of apoptosis (IAP) family of proteins, specifically the up-regulation of XIAP/IAP3 protein in ovarian cancer cells (Mansouri et al. 2003).

Apoptosis, the process of physiological cell death, activates cell death pathways in response to signalling from both intracellular and extracellular sources, and induces active
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fragmentation of DNA into 185 base pair multiples, with no inflammatory cytokine response from surrounding tissue (Kerr et al. 1972; Dharmarajan et al. 1994). Apoptotic signalling is used as a mechanism of ordered cell death by the body to remove cells with damaged genetic material, and as such has evolved into highly complex and multi-channeled pathways, to prevent accidental cell death from occurring (Schwartzman & Cidlowski 1993). Dysregulation of cell death pathways occur in cancer, autoimmune and immunodeficiency injuries and neuro-degenerative diseases (Deveraux & Reed 1999). Cancerous cells are able to prevent these messages of apoptosis from activating their intracellular pro-apoptotic pathways. IAPs are a family of anti-apoptotic proteins that are inappropriately activated by cancer cells for this purpose. Originally identified in baculovirus, where they function to keep the host cell alive while the virus replicates, they are characterized by a highly conserved, ~70 amino acid chain, termed the baculoviral inhibitory repeat (BIR) domain. The IAP family consists of 7 proteins; BRUCE, ML-ILP2, NAIP, HIAP-1, HIAP-2, Survivin and most importantly XIAP (Birkey Reffey et al. 2001). IAPs have been linked to the ability of ovarian cancer cells to prevent apoptotic signals from inducing cell death via multiple pathways (Deveraux & Reed 1999).

Down regulation of XIAP in chemoresistant cells has been shown to increase apoptosis through regulation of cell signalling pathways (Li et al. 2001; Hu et al. 2003). Analysis of chemoresistant cell signalling will provide insight into the mechanisms by which XIAP relates to ovarian cancer cell chemoresistance. Prevention of apoptosis by XIAP reflects a strong anti-apoptotic behavior pattern that gives an excellent target for gene expression, chemoresistance studies and potential clinical applications in modern chemotherapy treatments.

In the present study we examined the possible role for XIAP in the regulation of apoptosis in hOSE cancer cells and the possible involvement of this protein in Cisplatin-induced apoptosis in chemoresistant and chemosensitive cancer cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Two cell lines were used in this study, SKOV-3 and OVCAR-3. These cells represent a chemosensitive cell line (OVCAR-3) and a chemoresistant cell line (SKOV-3). OVCAR-3 cells were grown in RPMI 1640 with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10mM HEPES, and 1.0mM sodium pyruvate, 0.01mg/ml bovine insulin, 20% Fetal bovine serum and 1% Penicillin/ Streptomycin in a 5% CO₂ at 37°C. SKOV-3 cells were grown in McCoys 5a media (Invitrogen) with 1.5mM L-glutamine, 10% fetal bovine serum and 1% Penicillin/Streptomycin in a 5% CO₂ at 37°C. Both cell lines were grown in accordance to the American Type Culture Collection (ATCC) recommendations.

Cell Treatment

Cells were seeded at 25,000 cells/ml and grown for three days in 6-well plates (Sarstedt, South Australia). Medium was then removed and cells were treated with fresh medium (control) or 20μM Cisplatin (DBL, New South Wales) in fresh medium. After 24 hours cells were harvested for DNA, RNA and protein isolation and cells prepared for FACS analysis.

DNA 3' End Labeling

Total cellular DNA was extracted from cells as originally described (Gross-Bellard et al. 1973) (Roughton et al. 1999). DNA (1μg) was labeled with [³²P] ddATP (3000 Ci/mmol; Amersham Corporation, Arlington Heights, IL, USA) using terminal transferase (Roche, Mannheim, Germany) as described previously(Abdo et al. 2001) and separated on 2% agarose gels. Following electrophoresis, gels were dried in a slab gel drier without heat and exposed to autoradiography film (Kodak XAR-5; Eastman Kodak Company, Rochester, NY, USA). Low molecular weight (MW) DNA fractions (<15kb) were excised from the gel, mixed with 3ml scintillation fluid and counted in a -counter to provide a quantitative estimate of the degree of internucleosomal DNA cleavage among samples. Four groups (n = 4) were assessed for each variable under investigation.

Flow Cytometry Analysis

Control and treated cells were investigated for extent of apoptosis using Annexin-V-FLUOS (AVF) and Propidium Iodide staining. The cells were lifted off the culture plates using 1mL
and transferred to nitrocellulose membranes. μhomogenates was measured using the Bradford assay and processed with 0.1mM PMSF. Protein concentration of Triton X-100, 0.5% sodium deoxycholate, 0.1% (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% calcium chloride (CaCl2)), 20μM Hepes (pH 7.4), 140mM NaCl and 5mM TRITON X-100 were then centrifuged at 500 x g for five minutes. The supernatant was removed and 100 μL of PBS was added and pipetted up and down to wash the cells. The cells were then centrifuged at 500 x g for five minutes. The supernatant was removed and 100μL of labeling solution was added, the pellet re-suspended and the cells incubated in the dark for 10-15 minutes at 25°C. The labeling solution for the cells consisted of 1mL incubation buffer (10mM Hepes (pH 7.4), 140mM NaCl and 5mM EDTA, Gibco), and transferred to centrifuge tubes. 50μL of medium (with FBS) was added to inactivate the trypsin. Cells were centrifuged at 500 x g for five minutes. The supernatant was removed and one mL of PBS was added and pipetted up and down to wash the cells. The cells were then centrifuged at 500 x g for five minutes. The supernatant was removed and 100μL of labeling solution was added, the pellet re-suspended and the cells incubated in the dark for 10-15 minutes at 25°C. The labeling solution for the cells consisted of 1mL incubation buffer (10mM Hepes (pH 7.4), 140mM NaCl and 5mM calcium chloride (CaCl2)), 20μL Annexin-V-FLUOS labeling reagent (Roche Diagnostics), and 20μL PI solution (stock solution 50μg/mL in dH2O). Three separate controls of labeling solution were used to set parameters for analysis – a negative control with no PI or AVF, a control with AVF only, and a control with PI only. Missing volumes were made up with incubation buffer. After incubation with labeling solution, 0.6mL of incubation buffer was added and the cells were analysed by a Beckman Vantage Flow Cytometer at 488nm excitation, a 515nm band pass filter for fluorescein detection and a 600nm filter for PI detection. Three groups (n = 3) were analysed for each variable.

### Western Blot Analysis

Cells were homogenized in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1mM PMSF) Protein concentration of homogenates was measured using the Bradford assay and 30μg resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Following transfer, membranes were stained with Ponceau-S-stain to check for efficient transfer and uniform gel loading. Membranes were blocked in 5% non-fat milk in Tris-buffered saline/0.1% Tween-20 (TBST) for 30 minutes at 37°C. Membranes were then separately probed with antibodies against XIAP (1:250; Transduction laboratories, # 610762) and AIF (1:500; Santa Cruz, # Sc-9416) in 3% nonfat milk in TBST overnight at 4°C. Following primary incubation membranes were washed in TBST and incubated with (XIAP) anti-mouse horseradish peroxidase (HRP) (1:10,000; Dako) and (AIF) anti-goat HRP (1:10,000; Santa Cruz) in TBST for 1h at room temperature. Protein signals were detected by enhanced chemiluminescence (Supersignal West Pico ECL substrate, Pierce, Rockford, IL, USA) and exposure to autoradiographic film (Kodak XAR-5). Non-specific staining was checked by the omission of the primary antibody.

### Table 1: Primers and Annealing Temperature

<table>
<thead>
<tr>
<th></th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
<th>Product Size</th>
<th>Annealing temperature (°C)</th>
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<tbody>
<tr>
<td>AIF</td>
<td>5’-GAT CAC GCT GTG GTG AGT GG-3’</td>
<td>5’-TCT TGT GCA GTT GCT TTT GC-3’</td>
<td>179 bp</td>
<td>61</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5’-AAA GGA TCC TTA ATA AAG GTA TCC ATG GAG AAC ACT-3’</td>
<td>5’-AAA GAA TTC CAT CAC GCA TCA ATT CCA CAA TTT CTT-3’</td>
<td>322 bp</td>
<td>55</td>
</tr>
<tr>
<td>XIAP</td>
<td>5’-GGG GTT CAG TTT CAA GGA CA-3’</td>
<td>5’-CGC CTT AGC TGC TCT TCA GT-3’</td>
<td>183 bp</td>
<td>56</td>
</tr>
<tr>
<td>L19</td>
<td>5’-CTG AAG GTC AAA GGG AAT GTG-3’</td>
<td>5’-GGA CAG AGT CTT CAT GAT CTC-3’</td>
<td>194 bp</td>
<td>52</td>
</tr>
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** Figure 1:** DNA autoradiograph for 3’ End Labeling. The degree of internucleosomal DNA fragmentation present in the cell lines at 24 hours post treatment. A) as a 3’ end labeling gel autoradiograph exhibiting DNA laddering. B) as quantitated levels of low molecular weight DNA fragmentation between chemoresistant and chemosensitive cell lines. *SKOV-3 Control vs. SKOV-3 Cisplatin p=0.004. ** OVCAR-3 Control vs. OVCAR-3 Treated p=0.006. Values represent the mean for each group ± SEM.
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Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using RNA-Bee (Tel-Test Inc., Texas, USA) according to manufacturer’s instructions. RNA was treated with DNase using DNAfree (Ambion) to remove contaminating genomic DNA then 1 µg reverse transcribed using MMLV (Promega) and random primers according to manufacturer’s instructions. The resultant cDNA was purified using the MoBio Ultraclean kit.

Primers and conditions used in the present study are shown in table 1. For all transcripts, quantitative RT-PCR was conducted in 10 µl reactions using 1X iQ SYBR green supermix (BioRad), 0.5 µM primers and 1 µl cDNA in the Corbett Rotor-gene 3000 (Corbett Research, Sydney). Amplification was checked by melt curve analysis and electrophoresis in 2% agarose/ethidium bromide for product size. Threshold cycle values for cDNA samples were compared against a standard curve obtained by amplification of 10-fold dilutions of corresponding PCR product. For each cDNA sample, XIAP, Caspase 3, and AIF mRNA levels were normalized by dividing by the corresponding value for L19 mRNA (housekeeping gene). Standards and samples were run in duplicate and mean values per cDNA sample determined before normalization to the L19 housekeeping gene.

Statistical analysis

All data is represented as mean and standard error of the mean (SEM). Differences between group means were assessed using Student’s t-test where a probability less than 0.05 were deemed significant. All statistics were run on Gen-Stat version 7.0 while graphs were plotted using Microsoft Excel X for PC. Two Tailed t-Tests (unpaired, unequal variance) were performed to analyze significance between groups.

RESULTS

Detection of DNA Fragmentation Using 3’-End Labeling

Internucleosomal fragmentation indicative of apoptosis was evident in SKOV-3 and OVCAR-3 cell lines (n = 4) following 20 µM dose of Cisplatin treatment for 24 hours compared to control (Figure 1). Cisplatin significantly (p<0.001) increased DNA fragmentation in both cell lines but the degree of DNA fragmentation was much greater in the OVCAR-3 cell line compared to SKOV-3.

Flow Cytometry Analysis

Density plots show results of flow cytometry using Annexin-V-FLUOS and Propidium Iodide analysis. The lower left quadrant of the graphs...
XIAP expression in ovarian cancer represents the number of live cells in the population. The lower right quadrant represents the apoptotic cells. The upper left quadrant represents the necrotic cells, and the upper right represents the late apoptotic and/or necrotic cells. The region quantified was the lower right apoptotic region. The density plots and graph for the OVCAR-3 and SKOV-3 cell lines (Figure 2) show that there is a definite shift in the cell population to the lower right quadrant in the treated groups, which is indicative of an increase in apoptosis in the cell lines. A significant difference was seen between the SKOV-3 control and treated groups (p=0.0001).

Expression of XIAP, Caspase-3 and AIF mRNA was analyzed in OVCAR-3 and SKOV-3 cells as shown in Figure 3. All results were compared to expression of the L19 housekeeping gene. The levels of expression of XIAP mRNA was significantly (p<0.05) decreased in both cell lines. Interestingly, Caspase-3 mRNA levels increased significantly (p<0.05) only in SKOV-3 treated cells. The mRNA levels of AIF were significantly reduced in both cell lines (p<0.05).

Western Blot analysis for apoptotic markers

The XIAP protein corresponding to a molecular weight of 54kDa was found to be down-regulated between control and treatment groups in the SKOV-3 cell line, and below detectable levels in the OVCAR-3 cell line (Figure 4). The differences between the groups were significantly different (p<0.01). AIF protein corresponding to a molecular weight of 57kDa was found not to significantly change in both cell lines over control and treatment groups.

mRNA analysis for XIAP, Caspase 3 and Apoptosis inducing factor (AIF)

Figure 3: Quantitative Real Time PCR for XIAP, Caspase 3 and AIF. The standardised, quantitated levels of XIAP (A), Caspase-3 (B) and AIF (C) expression between SKOV-3 and OVCAR-3 cell lines are shown in graphs. Values represent the mean for each group ± SEM.

Figure A - * SKOV-3 Control vs. SKOV-3 Cisplatin p=0.037, ** OVCAR-3 Control vs. OVCAR-3 Cisplatin p=0.002.
Figure B - *SKOV-3 Control vs. SKOV-3 Cisplatin p=0.022.
Figure C - * SKOV-3 Control vs. SKOV-3 Cisplatin p=0.029, ** OVCAR-3 Control vs. OVCAR-3 Cisplatin p=0.012.

DISCUSSION

The objective of this study was to investigate the relationship between X-linked Inhibitor of Apoptosis Protein (XIAP) expression and chemoresistance in ovarian cancer cells.
Treatment with Cisplatin and other synergistic drugs are most effective against ovarian cancer, but rarely curative with very short remission periods, and subsequent treatment being less effective as the cancer cells develop resistance (Gibb et al. 1997).

**XIAP** is a ubiquitously expressed protein that controls anti-apoptotic processes within cells, by binding to, and preventing, cellular apoptotic pathways from inducing apoptotic processing (Bratton et al. 2002). **XIAP** is a target for studies on chemoresistance in ovarian cancer cells, as the control of pro-apoptotic pathways, and its altered expression with treatment, could be a major factor in the anti-apoptotic behavior exhibited by chemoresistant cells. **XIAP** up-regulation, in response to treatment, has been found to result in an inactivity of cellular apoptosis pathways to induce apoptotic processing (Mansouri et al. 2003). The expression of **XIAP** is tightly regulated in cells but altered expression levels between chemosensitive and chemoresistant cell lines is thought to contribute to their classification (Li et al. 2001).

The major finding of this study was, following treatment, there was a down-regulation of **XIAP** in both chemoresistant and chemosensitive cells. A corresponding increase in apoptosis, although evident, was not equivalently demonstrated. The observed decrease in **XIAP** content, following treatment, indicates that regulation of **XIAP** plays a major role in the ability of a cell to prevent extrinsic apoptotic signalling activity.

**XIAP** prevents activation of **Caspase-3**, the end point effector caspase used by most mitochondrial apoptosis pathways (Asselin et al. 2001). The regulation of **XIAP** signalling was similar between the two cell groups and was demonstrated by a significant down-regulation of **XIAP** mRNA expression in both chemoresistant and chemosensitive cells. The expression of **XIAP** protein levels in the two cells lines was also significantly reduced following Cisplatin treatment. The difference in down-regulation between resistant and sensitive cell types was clearly discernable with the chemoresistant cells showing a slight decrease in protein content versus chemosensitive cells which showed an overall decrease to below detectable levels, suggesting that down-regulation of **XIAP** within the chemosensitive cells is greater than in the chemoresistant cells.

Cisplatin induced apoptosis and Cisplatin resistance are both multifactorial events (Kartalou & Essigmann 2001). **XIAP** has been indicated to have a regulating effect in the cellular response to Cisplatin (Asselin et al. 2001). The induction of apoptosis by Cisplatin is through genotoxic stress and activation of signaling molecules such as **Fas-L** (Mansouri et al. 2003). More importantly Cisplatin has been shown to form adducts with DNA, resulting in **p53** activation, which causes increased DNA repair or apoptotic inducement by activation of the intrinsic mitochondrial apoptosis pathways (Yazlovitskaya et al. 2001).

3’-End labeling was used to quantitate the levels of apoptosis within the ovarian cell lines following treatment with a 20µM dose of Cisplatin for 24 hours. Results indicated a significant increase in cellular apoptosis following treatment compared to the respective controls. The chemoresistant cell lines displayed a lower level of apoptosis than that of the chemosensitive. While both treatment groups exhibited an increase in Cisplatin induced apoptosis, the response of chemosensitive cells was five-fold greater than that of the chemoresistant cells. Apoptosis was also identifiable using flow cytometry analysis of cells tagged with both Annexin-V-FLUOS and Propidium Iodide. Both cells lines confer with the 3’-End labeling data and show an increase in apoptosis after treatment with Cisplatin. SKOV-3 cell line showed a significant increase in apoptosis in the treated group compared to the control group. OVCAR-3 did not show a significant change amongst control and cisplatin groups, however results show a trend towards significance and also mimic results seen in 3’-End labeling.

Chemoresistance and **XIAP** regulation are both multifactorial processes. The ability of these two processes to be altered by cancer cells will provide a determining factor in chemoresistance levels in tumour cells. **Caspase-3** mRNA expression increased in response to Cisplatin treatment, although only the chemoresistant cells (SKOV-3) showed a significant alteration to **Caspase-3** gene expression. Interestingly, the chemoresistant cells showed a distinct increase in **Caspase-3** gene expression, and yet there was no comparable increase in apoptosis in these cell lines. **Caspase-3** is effectively blocked by **XIAP**, by binding to, and preventing activation of
Caspase-3 and its main activator Caspase-9. The increase in Caspase-3 activity seen in chemoresistant cells could be attributed to the activation of the Fas/Fas-L pathway by Cisplatin, resulting in mediation of the Caspase-8/Caspase-3 cell death pathway. Cisplatin could also affect cells through non caspase dependant apoptosis, AIF signalling levels were found to be downregulated in treatment groups for both cell lines however there was no detectable change in protein expression indicating that Cisplatin works through caspase activation.

The increase in apoptosis in the chemoresistant cells is not reflected by a proportional increase in Caspase-3 levels, yet the marginal increase in apoptosis seen in the chemoresistant cells is followed by an almost 3 fold increase in Caspase-3 levels. It is possible that XIAP in the chemoresistant cells is being stabilized, most likely by Akt, and as a result a higher activity for Caspase-3 binding than that of the chemosensitive cells. As a compensatory mechanism Caspase-3 levels would have to increase substantially to attenuate even a small increase in apoptotic activity. Caspase independent cell death was also investigated looking at Apoptosis Inducing Factor (AIF) and while a significant decrease in mRNA signalling was shown there were no significant changes in overall protein expression in either cell line (data not shown). Chemosensitive and chemoresistant cells exhibit different phenotypes and this may be due to presence or absence of XIAP stabilization. Nevertheless, chemoresistance is a multifactorial problem, and XIAP is only one of the factors. XIAP confers its ability to resist Cisplatin induced therapy by preventing activation of the Caspase-3, Apaf-1/Caspase-9 and Fas/Fas-L cell death pathways.

Down-regulation of XIAP occurs after release of Smac/DIABLO or Omi/HtrA2 from the mitochondria in response to apoptosis signalling(MacFarlane et al. 2002). Smac and Omi bind to, and prevent, XIAP from targeting Caspase-3, resulting in apoptosis. The significant difference in response to Cisplatin induced down-regulation of XIAP can be explained in two ways. Firstly the chemoresistant cells used in this study contained a p53 null mutation, resulting in a lower degree of pro-apoptotic signalling in response to DNA damage. Secondly the stabilization of XIAP in chemoresistant cells by Akt and via IAP-IAP binding, can prevent Cisplatin induced down-regulation of XIAP (Fraser et al. 2003). Akt competitively binds to the same sites on the XIAP protein that Smac and Omi use to induce down-regulation. Treatment with cytotoxic drugs and radiation has been shown to increase levels of Akt providing another measure of anti-apoptotic behavior and ensuring the ability of XIAP to prevent caspase pathway activation (Dan et al. 2004). Recent papers have suggested that chemoresistant ability of XIAP is due to stabilization of XIAP along its E3 ubiquitination site(Dan et al. 2004). Stabilization prevents degradation of XIAP by Smac or other similar XIAP binding molecules. Degradation of Smac and Omi have also been shown indicating that cytoprotective IAP’s can inhibit apoptosis through the neutralization of IAP antagonists, rather than by directly inhibiting caspases(Wilkinson et al. 2004). Finally Survivin, another inhibitor of apoptosis protein, has been shown to bind with XIAP in response to cell death stimulus. The resultant complex is resistant to proteasomal/ubiquitination destruction and can still inhibit apoptosis (Dohi et al. 2004). Stabilization of XIAP in these ways prevents activation of Caspase-3 and high levels of cytosolic XIAP expression with an inability for the cell to down-regulate expression levels of XIAP without first inhibiting the stabilization factor, such as Akt.

Acknowledgement

The authors would like to thank Mr Greg Cozens and Dr Kathy Heel-Miller from CMCA, The University of Western Australia, for their technical help.

References


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