Interim Report to Breast Cancer Australia 01/01/09 – 30/06/09

Project Title: Preclinical development of a novel plant toxin, persin, as an anti-cancer agent

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Project overview:

Phytochemicals have provided an abundant and effective source of novel therapeutics for the treatment of cancer. This research centres upon the further preclinical characterisation of a novel plant toxin, persin, that has unique, *in vivo* actions in the mammary gland and exhibits potent cytotoxic synergy with the breast cancer therapeutic, tamoxifen in both ER-positive and ER-negative breast cancer cells. The latter is dependent upon expression of the proapoptotic protein, Bim. Bim is a sensor of cytoskeletal integrity, and there is evidence that persin acts as a microtubule-stabilising agent. Due to the unique structure of the compound, persin could represent a novel class of microtubule-targeting agent with potential specificity for cancers of the breast, and potentially other organs. Critical steps in the evaluation of this compound are to elucidate its mechanism of action through definition and characterisation of its intracellular targets, and to evaluate its *in vivo* effectiveness.

We hypothesize that persin is a molecule with significant potential as an anti-cancer therapeutic, that mediates its cytotoxicity in human cancer cells by direct interaction with microtubules and through modulating ceramide metabolism.

The overall aim of this project is to further develop and evaluate the therapeutic potential of persin as an anti-cancer agent. This will be achieved by addressing two specific aims:

1. define and characterise the intracellular targets of persin action
2. determine persin's *in vivo* efficacy

Breast cancer remains a major cause of cancer death amongst women in developed countries. Despite advances in detection and treatment which have improved survival, intrinsic or acquired therapeutic resistance remains a major obstacle to an effective cure, emphasising the *significant clinical need for the development of novel molecular therapeutics with increased selectivity and efficacy*. Our previous studies have emphasised persin's *significant potential as a novel therapeutic agent for the treatment of cancers of the breast, and potentially other organs*. They have also provide the rationale for the further preclinical development of this compound or more effective synthetic analogues, with the potential to act as an *innovative strategy to enhance and broaden therapeutic efficacy* and counteract refractory disease.

Progress to date:

Aim 1: Define and characterize the intracellular targets of persin action.

1.2. Persin as a novel modulator of ceramide metabolism. Our previous studies have demonstrated that persin treatment is associated with an increase in intracellular ceramide levels, and the latter is causal in persin's proapoptotic effects and its cytotoxic synergy with Tamoxifen (Fig. 1; Roberts *et al.*, 2007). The first step of *de novo* sphingolipid biosynthesis is the condensation of L-serine with palmitoyl coenzyme A (CoA), which is catalyzed by serine palmitoyltransferase (SPT) and blocked by the inhibitors, myriocin and L-cycloserine. Persin-induced apoptosis is attenuated in the presence of SPT-inhibitors, suggesting it may modulate ceramide production at this step (Fig. 1; Roberts *et al.*, 2007).

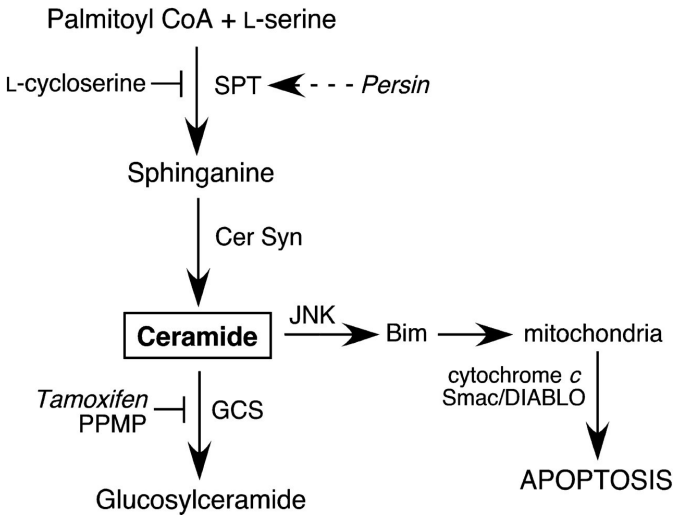

Fig. 1: A schematic representation of the putative interactions of persin with ceramide metabolism.

We have performed a series of experiments aimed at determining where persin acts in the *de novo* synthesis pathway. First, we investigated the effects of persin on SPT mRNA and protein expression by QT-PCR and immunoblotting, respectively. Fig. 2 demonstrates that no significant differences were observed in SPT levels following persin treatment compared to vehicle controls. To investigate whether persin directly modulates SPT activity, microsomal protein preparations were incubated with persin in the presence of [^{3}H]serine and an excess of palmitoyl CoA, then lipids extracted and ceramide species analysed by thin-layer chromatography and autoradiography, following standard methods (Holleran *et al.*, 1990). Addition of the ceramide synthase inhibitor, Fumonisin further facilitated determining where persin acts in this pathway. Preliminary data suggests that no change in SPT activity is observed following persin treatment. Further studies will adapt this protocol to look at the effects of persin directly on microsomes to improve efficacy and reproducibility of the results.

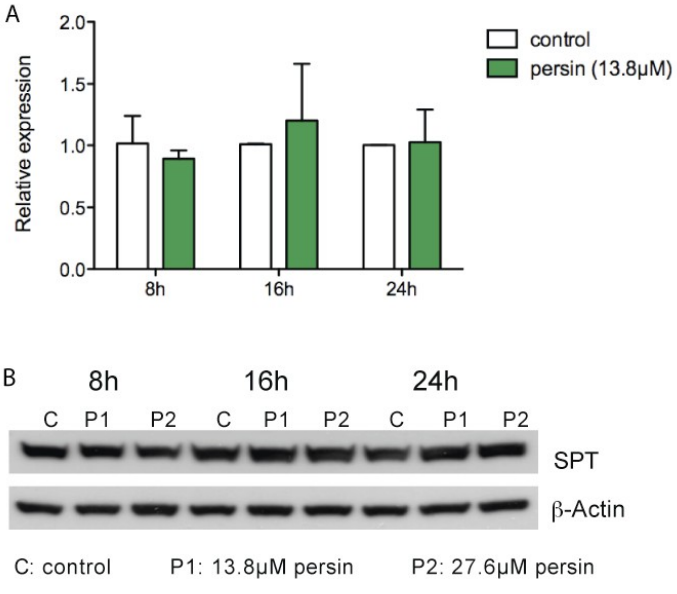

Fig. 2: Levels of SPT mRNA (A) and protein (B) are unchanged following treatment with persin compared to control in MCF-7 breast cancer cells.

If it is established that persin has no effect on SPT expression or activity, we will determine its effects on upstream components of ceramide metabolism such as the activity of the

desaturase enzyme, stearoyl-CoA desaturase 1 (SCD1) which mediates the desaturation of palmitoyl CoA in colloaboration with AI Biden.

1.2. Upstream initiators of persin action – the role of ER stress. Recent studies have highlighted the role of the endoplasmic reticulum in the initiation of a pathologically and therapeutically-relevant apoptotic program. Imbalances in endoplasmic reticulum homeostasis through the accumulation of misfolded proteins, leads to the generation of an endoplasmic reticulum-mediated stress (ER-stress) response and the triggering of an intrinsic apoptotic pathway, mediated, in part, through the generation of ceramide. Thus, the generation of an ER-stress response could represent an upstream initiating event in persin-induced cytotoxicity. ER-stress induced apoptosis is mediated by activation of caspase-4. To further determine the role of ER-stress as an initiator of persin-induced apoptosis, we determined the cytotoxic effects of persin in the presence of the caspase-4-specific inhibitor, z-YVAD-fmk. Figure 3 demonstrates that persin-induced apoptosis is ablated in the presence of z-YVAD-fmk suggesting that it is dependent on the generation of an ER-stress response.

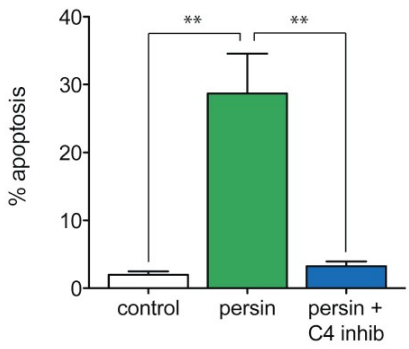

Fig. 3: Persin-induced apoptosis is ablated in the presence of an inhibitor of the ER-stress-specific caspase-4, z-YVAD-fmk.

We also confirmed and extended our preliminary data on the effects of persin on markers of ER-stress – CHOP and XBP-1. Figure 4 shows a significant induction of CHOP expression following treatment with either persin or the known ER-stress inducer, tunicamycin compared to controls. A similar activation of another ER-stress marker, XBP-1 was also observed following persin or tunicamycin treatment (Figure 5).

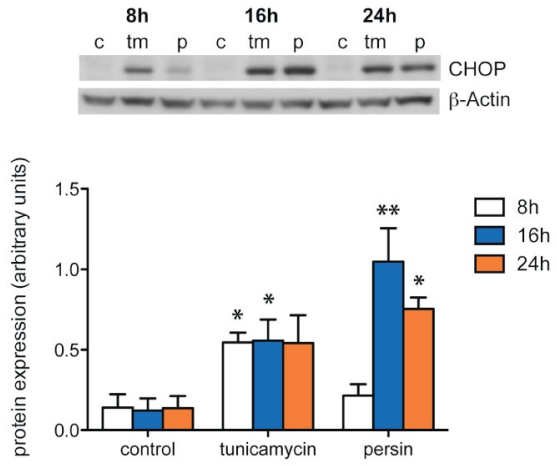

Fig. 4: Significant induction of the ER-stress marker, CHOP following treatment with persin or the known ER-stress inducer, tunicamycin in MCF-7 breast cancer cells.

To further establish the role of ER stress in the execution of persin-induced apoptosis, we determined the effects of knocking down CHOP using specific siRNA on persin's cytotoxicity. Figure 6A shows

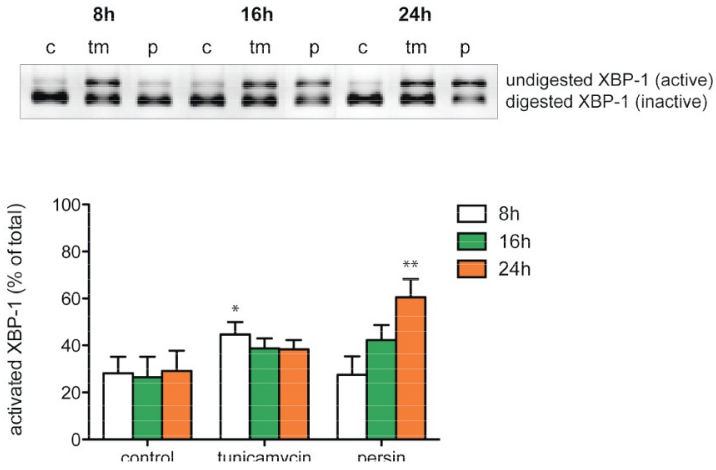

Fig. 5: Significant activation of the ER-stress marker, XBP-1 following treatment with persin or tunicamycin.

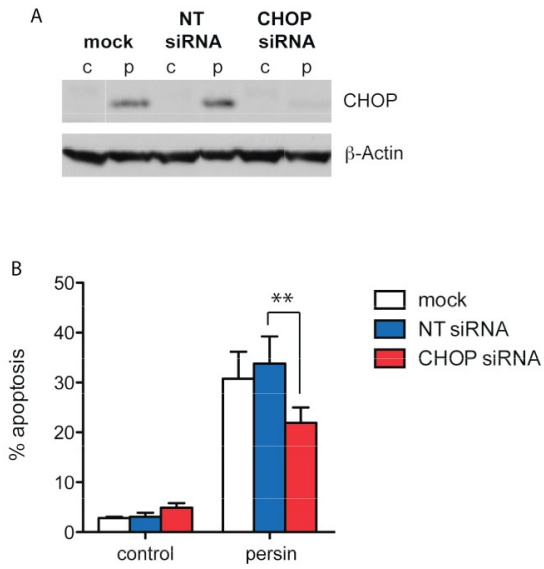

Fig. 6: Specific knockdown of CHOP using siRNA (A) attenuates persin-induced apoptosis in MCF-7 cells (B).

that siRNA successfully attenuated persin-induced CHOP expression compared to mock transfected or non-targetting (NT) controls. Knockdown of CHOP resulted in a significant attenuation of persin-induced apoptosis (Fig. 6B). However, these data also suggest that other, CHOP-independent pathways may also contribute to persin's cytotoxicity in MCF-7 cells.

1.3. Determination of tissue specificity.

The rationale for using human breast cancer cell lines in our analysis of the *in vitro* cytotoxicity of persin was primarily based on its apparent specificity for the mammary gland in a lactating mouse model. However, determining the tissue specificity of persin is vital for therapeutic relevance. To further address this, we examined the *in vitro* anti-cancer activity of persin on a range of different cancer cell types using the (3-[4,5'dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) MTT reduction assay. Table 1 shows that in addition to a range of breast cancer cell lines, ovarian and prostate cancer cells are also responsive to persin. Interestingly, response to persin appears to be independent of estrogen receptor (ER) and p53 status.

Table 1: Response of cancer cell lines to persin.

Tissue	Cell line	ER status	p53 status	IC_{50} (mM)
Breast	MCF-7	+	wt	22.61 ± 1.98
	T47D	+	mut	29.78 ± 2.71
	MDA-MB-468	-	mut	32.66 ± 3.48
	MDA-MB-157	-	mut	17.32 ± 2.60
	SkBr3	-	mut	24.51 ± 1.04
	Hs578T	-	mut	33.99 ± 2.50
	MDA-MB-231	-	mut	NR
	MCF-10A (n)		wt	NR
Ovarian	OVCAR3	n/a	mut	30.39 ± 3.53
	IGROV-1	n/a	mut	22.25 ± 2.32
Prostate	PC-3	AR -	mut	35.71 ± 1.44
	LNCaP	AR +	wt	24.56 ± 1.38

Presentations:

Poster Presentation – Shelley EJ, Biden TJ, Laybutt DR, Sutherland RL & Butt AJ. Proapoptotic actions of the plant toxin persin are mediated by induction of endoplasmic reticulum stress in human breast cancer cells. Australian Society for Medical Research NSW Scientific Meeting, 1 June, 2009.

References:

Holleran WM, Williams ML, Gao WN, Elias PM (1990). Serine-palmitoyl transferase activity in cultured human keratinocytes. J Lipid Res **31:** 1655-61.

Roberts CG, Gurisik E, Biden TJ, Sutherland RL, Butt AJ (2007). Synergistic cytotoxicity between tamoxifen and the plant toxin persin in human breast cancer cells is dependent on Bim expression and mediated by modulation of ceramide metabolism. Mol Cancer Ther **6:** 2777-85.