Article

A Possible Candidate for the Fight against Alzheimer's Disease

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Introduction

Although there are examples of individuals living for a century and more with little decline in brain function, many others are not so fortunate and are afflicted with debilitating disorders like Alzheimer's disease (AD) even by the age of 65 years. It is projected that by 2050 there will be a 106 million people affected worldwide with this disease with 10% of the population affected in NZ.[1] Alzheimer's disease is a progressive neurodegenerative disorder resulting in cognitive, memory and behavioural impairments, and is the leading cause of dementia in humans. There is no currently known cure or treatment to reverse its pathology. The cognitive decline associated with AD has been proposed to result from overproduction of a *sticky peptide* amyloid-β (Aβ) in the brain. Excessive production of this peptide arises from an imbalance in the processing of the large parent amyloid precursor protein (APP) by proteases.

Although Alois Alzheimer made the connection between the combined presence of senile plaques and tangles in the brains of the deceased who had dementia in 1906, it took another 80 years before a clearer understanding of the genetic and biochemical complexities underlying the formation of these plaques and tangles began to emerge. Since the 1980s studies have shown that the plaques are primarily composed of Aβ,[2] and Aβ is neurotoxic.[3] Soluble aggregates of Aβ induce synaptic dysfunction leading to learning and memory deficits,[4,5] and eventually form insoluble plaques in the brain (Fig 1).[6]

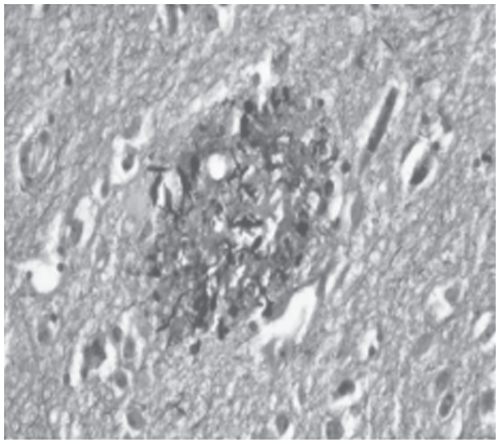


Fig 1. An insoluble amyloid-β (Aβ) plaque in a human brain histology section from an Alzheimer's patient (adapted from Selkoe (1999) in ref. 14 with permission from Macmillan Publishers Ltd.).

Risk Factors

While AD is age-related, environmental factors such as behaviour, consumption of a high calorie and fat diet, history of head trauma, and a sedentary lifestyle are also likely to increase the risk.[7] A recent study in an AD mouse model induced insulin resistance (as seen in humans with obesity and type 2 diabetes mellitus) by feeding the mice with 10% sucrose-sweetened water and also exacerbated memory deficits and increased insoluble Aβ deposition in the brain.[8] Low dietary folate intake and high intakes of lipids and metals such as copper and iron may also influence disease risk.[9] Controlling the consumption of sugar-sweetened beverages,[8] regular physical exercise, a strict dietary regimen, and maintaining a cognitively stimulating environment could be protective against the development of AD – and, as we are constantly reminded, for most other diseases![10]

Genetic factors that affect the precursor protein APP in particular, also increase the risk of AD, and causative mutations in APP have been discovered.[11] Subsequent studies also identified mutations within the genes presenilin-1 and -2 (PS-1, PS-2) to cause inherited AD.[12] It also seems likely that other genes will influence one's susceptibility to AD. One such example is apoliprotein E (ApoE), with individuals who produce the ApoE4 isoform being more likely to develop the disease.[13] The mutations within these genes are believed to be either directly or indirectly responsible for the increased production of Aβ by promoting an imbalance in APP processing.

Cleaving the Precursor Protein APP

A molecule of APP is cleaved at *one of two competing sites* by two distinct proteases, α- or β- secretase, releasing either a 612 amino acid (612aa) or a 596aa (Fig. 2) extracellular *N*-terminal product, respectively.[14] Following this first cleavage, another intramembrane cleavage follows downstream and is caused by another enzyme, the γ-secretase. When APP is cleaved by the α- and γ- secretases, an *N*-terminal fragment sAPPα is generated but, importantly, the amyloidogenic Aβ peptide cannot be produced. In healthy individuals there exists a fine balance between this pathway and the β-secretase pathway where Aβ can be produced. However, in AD patients, APP enters the β-secretase pathway more frequently and increases the production and concentration of Aβ whilst decreasing the production and concentration of sAPPα.[4,15]

Is the C-terminal 16aa of sAPPα Neuroprotective?

sAPPα from the α-secretase pathway is identical in sequence to the *N*-terminal protein, sAPPβ (Fig 2) derived from the β-secretase pathway, with the exception of the C-terminus. sAPPβ is shorter by 16 amino acids. However, sAPPα is neuroprotective, is 10- to 100-fold more potent than sAPPβ in protecting neurons against Aβ mediated toxicity,[16-18] and it protects cells against oxidative stress resulting from glucose deprivation.[17,19] It has been demonstrated experimentally that the neuroprotection offered by sAPPα could be a result of its ability to signal an up regulation of the expression of neuroprotective genes.[20,21] While Aβ interferes with synaptic plasticity, neurite outgrowth, and elongation, *i.e.* axon/dendrite projections from a neuron, these pathogenic effects are prevented in

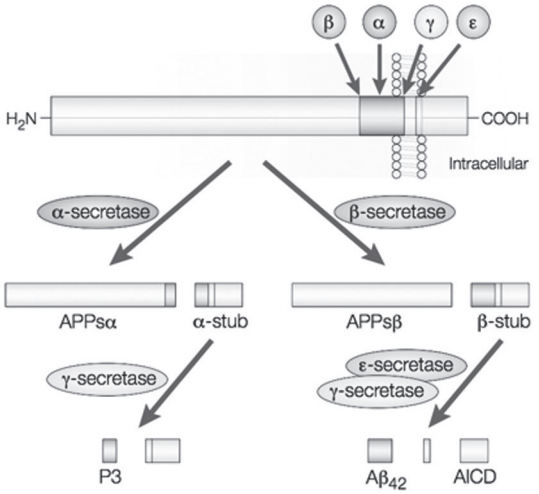


Fig 2. The two competing pathways driven by *α*- and *β*-secretases (adapted from LaFerla (2002) in ref. 14 with permission from Macmillan Publishers Ltd.).

the presence of sAPP*α*.[22] Even more exciting is *the observation made by our group that sAPPα alone plays an important role in the retention and restoration of spatial memory in rats*.[18]

A Specific Role for the C-terminal 16aa of sAPPα?

sAPP*α* is neuroprotective and plays important roles in preventing neurodegeneration and promoting memory. We believe that sAPP*α* may be as important as A*β* in the dynamics of the pathogenesis of AD. However, it carries 612 amino acids (aa – 612aa), and getting it across the blood brain barrier (BBB) poses a significant problem to any use as a therapeutic reagent. Could a region within sAPP*α* be a key to its neuroprotective function? A good place to start such a search is with the 16aa region present in sAPP*α* but absent in sAPP*β*, as this marks a very large change in effective function. Our study aims to test whether this region of sAPP*α* plays a critical role in its signalling function.

The C-terminal 16aa region of sAPP*α* is strongly hydrophilic in keeping with our proposal that it could be involved in ligand-receptor interactions, perhaps even the key to initiating sAPP*α* functions. Within this 16aa region there is also domain that could possibly bind heparin,[23] which could then play a crucial role in cell-substratum adhesion and neuroprotectivity. Indeed, preventing heparin binding causes a loss of both functions.[16] This region is a good candidate for association with the cell surface, preventing neurite retraction and conferring neuroprotection against A*β* toxicity. Additionally (or alternatively), the 16aa C-terminus could confer critical structural parameters on sAPP*α* crucial for its functions and thus have an indirect influence. Studies to test this hypothesis are to be carried out.

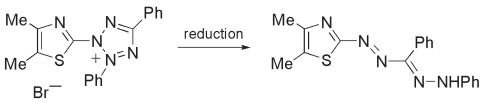
Experimental Strategy

A: Can the Neuroprotective Function of sAPPα be Validated?

We have produced recombinant sAPP*α* to circumvent the processing that occurs *in vivo*. Functional validation of native recombinant sAPP*α* produced in the laboratory was necessary before developing the sAPP*α* variants for testing the function of the C-terminal region. Two cell based assays namely, *cell viability* and *activation of a responsive promoter*, were chosen for this purpose.

Cell viability assay — The cell viability assay (often referred to as the MTT [**1**, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in the literature) is a colorimetric assay used to compare the viability of cells subjected to different treatments, from their oxidant/anti-oxidant status. MTT is a yellow coloured compound that is reduced by cellular mitochondrial dehydrogenases to purple coloured crystals of formazan (**2**; Scheme 1). The assay depends on measuring the health of the cells by their ability to produce **2**.

Scheme 1



1, MTT, yellow — **2**, formazan, purple

Our experimental strategy involved pre-incubating neuroblastoma cells (SHSY5Y - a model-neural cell line) and African green monkey kidney fibroblast-like cells (COS-7 - a commonly used mammalian cell line) with sAPP*α* and determining in what way they reacted to the stress of glucose deprivation. The aim was to confirm that our laboratory-produced recombinant sAPP*α* could protect cells from the adverse effects of hypoglycaemic damage (oxidative stress).

NFκB promoter activation assay — The second assay used luciferase as a reporter gene and was based on the activation of the NFκB promoter (nuclear factor kappa-light-chain-enhancer of activated B cells). This promoter encompasses a major family of transcriptional factors involved in the regulation of the transcription and expression of neuroprotective genes. Previous studies[17,24] had shown that sAPP*α* can somehow activate this family of transcription factors. The COS-7 cells were used because they have a functioning NFκB promoter that can be further stimulated by sAPP*α*. They were transiently transfected with the NFκB-dependent luciferase reporter plasmid (pNFκB-Luc) and, after cell lysis, luciferase activity was assayed *in vitro*. The light emitted was then measured in a luminometer.

B: Generation of sAPPα Variants

Substitution and deletion variants of sAPP*α* within its C-terminal region were designed to test our hypothesis that the 16aa C-terminal region, present in sAPP*α* but absent in sAPP*β*, plays an important role in its function. These variants have been prepared and now are available for testing either to mimic or interfere with the function observed in recombinant sAPP*α* with the natural sequence. In addition, chemically synthesized peptides (by Professor Margaret Brimble, Auckland University) corresponding to the 16aa region are to be tested at the same time.

As changes had to be introduced at the C-terminal end of the sAPP*α* gene, site-directed mutagenesis was per-

formed using reverse mutagenic primers to introduce variations within sAPP*α*. Following amplification by the polymerase chain reaction (PCR) to incorporate the mutations, the gene products were then cloned into a special vector (the mutated sAPP*α* gene was inserted into a small DNA molecule, termed the vector, which then replicates within bacteria). Following confirmation that the introduced sequences were correct, the different vectors were then integrated into the genomes of cells of a mammalian cell line—the process of transfection for the creation of stable cell lines. The human embryonic kidney cell line (HEK293) has been used for this purpose as it can be easily transfected with the specialized vector to give stable cell lines. After transfection, the cells were grown in media containing the antibiotic geneticin. The transfecting vector has a gene resistant to geneticin thereby ensuring that only stably transfected cells survive. Individual colonies that survived 2-3 weeks of treatment with geneticin were selected for the establishment of permanent stable cell lines. These stable cell lines produced the variant sAPP*α* proteins.

We designed and generated five variants of sAPP*α* within the unique C-terminal 16aa of sAPP*α* (see Fig. 6a). One targeted two consecutive histidines by substituting them with neutral alanines. These consecutive histidine moieties could be crucial to sAPP*α*'s ability to behave like a receptor and provide a hydrophilic face for interaction with other proteins. Two deletion variants removed i) five amino acids (VHHQK) by inserting a stop codon after a glutamic acid codon in the gene (see also Fig. 6a) - a region implicated in heparin binding[23] - and, ii) ten amino acids by inserting a stop codon after an arginine codon in the gene. The remaining two variants of sAPP*α* are based upon the claim that the terminal lysine (the 612th aa of sAPP*α*), plays a role in the trafficking of full length APP for cleavage.[25] Tests will be made to determine if sAPP*α* function is impaired by substituting the lysine with either an alanine (charge and size difference) or a valine (charge difference but similar size).

Experimental Results

A: Functional Validation of sAPPα

The development and validation of assays that could reliably demonstrate the protective functions of sAPP*α* produced as a recombinant protein in the laboratory were necessary before the development of the variants themselves. Both the cell viability assay and the promoter activation assay have demonstrated that recombinant sAPP*α* could behave as a protective compound and gave us confidence to develop the sAPP*α* variants.

Cell viability assay — Using the cell viability assay, we aimed to demonstrate that recombinant sAPP*α* applied in the culture media could protect cells against the adverse effects of hypoglycaemic damage. As noted above, this assay measures the anti-oxidant status of the cell as an indicator of viability. The time required for glucose deprivation to affect significantly the viability of cells is between 48-72 h (Fig. 3). We demonstrated for the first time that exposure of cells to sAPP*α* for just 2 h was sufficient to protect cells against the effects of glucose deprivation (Fig 4a). This suggests that sAPP*α* behaves like a switch, which can induce changes to the cell physiology when cells are subjected to stress, perhaps by the expression of protective genes and proteins. We carried out this assay with sAPP*β* that lacks the C-terminal amino acids as well. Interestingly here, the sAPP*β* had a *toxic* rather than a protective effect on cells. Intriguingly, in the different COS-7 non-neural cell line, sAPP*α* was biphasic in titrations of over a range of concentrations and actually toxic at higher concentrations (10 nM) (Fig. 4b).

NFκB promoter activation assay — COS-7 cells have endogenous NFκB promoter activity as shown by the expression of the luciferase from the NFκB promoter (see Fig 5), but they have potential for further enhancement by treating the cells with sAPP*α*.[17] An increased emission of light over that from the untreated sample will occur if the

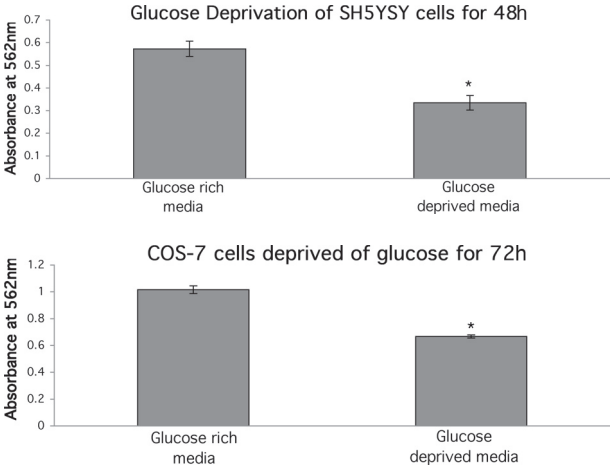


Fig 3. Cell viability in response to deprivation of glucose in SHSY5Y (upper) and in COS-7 (lower); * indicates significance at p<0.05 (student's two-tailed T-test) between cells with and without glucose; Y-axis = absorbance values.

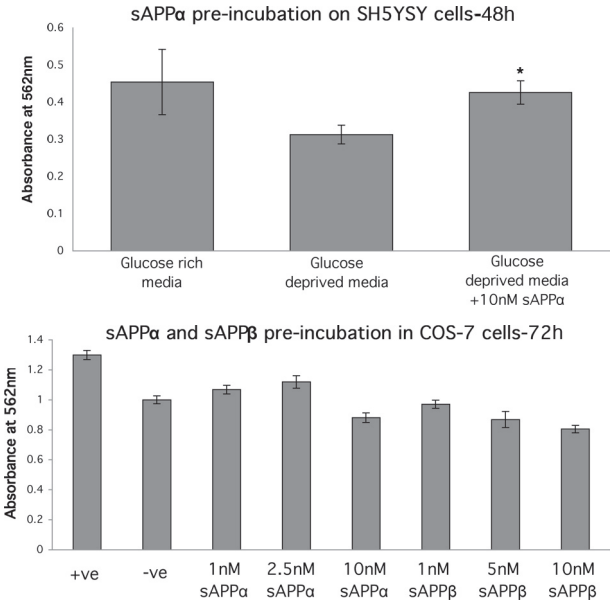


Fig 4. Upper: sAPP*α* protection of glucose deprived cells; * indicates significance at p<0.05 (student's two-tailed T-test) between samples treated with 0.1 x PBS and 10 nM sAPP*α*. Lower: sAPP*α* protection and sAPP*β* sensitization to glucose deprived cells;. +ve, glucose rich media; -ve, zero glucose media treated with 0.1 x PBS; data are normalized to the -ve control.

NFκB promoter is more activated as more of the luciferase will then be expressed. Treating transfected COS-7 cells with sAPP*α* demonstrated sAPP*α*'s ability and sAPPα's inability to signal the downstream activation of NFκB-dependent transcription (Fig. 5); the positive control used was lipopolysaccharide (LPS).

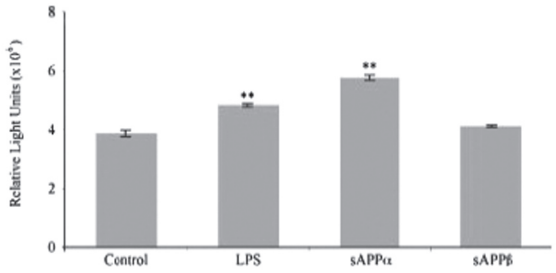


Fig 5. Activation of NFκB promoter by sAPP*α* but not by sAPP*β*; PBS (control), LPS (+ve control), sAPP*α* (2.5 nM) or sAPP*β* (2.5 nM); results are the average of four separate experiments following normalization; error bars show standard deviation.

B: Generation of sAPPα Variants

By designing appropriate primers and employing PCR, the desired sAPP*α* mutant genes were generated (Fig. 6) and then cloned into the specific integration vector for the production of stable cell lines. Positive colonies were confirmed diagnostically by a restriction enzyme digest using the *Bam*H1 enzyme - a 1.3 kb band along with a 6.3 kb band indicates a positive clone (Fig. 6 lower) and the correct modifications were confirmed by sequencing.

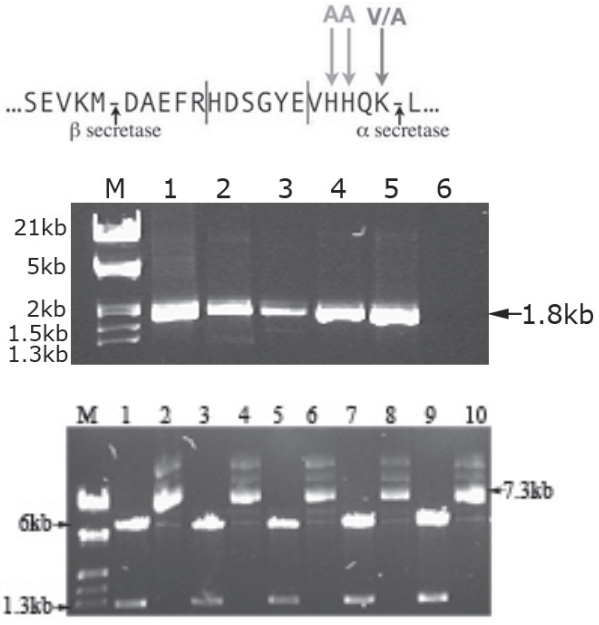


Fig 6. (Upper) Deletion and substitution variants of sAPP*α*.; vertical lines after arginine and glutamic acid represents insertion of stop codon. ***(Centre)*** synthesis of mutant sAPP*α* genes by mutagenic primers and PCR; **M**-Marker – **Lanes 1-5**: H609&610A, 1-607, 1-601, K612V, K612A, – **Lane 6**: -ve control. ***(Lower)*** Diagnostic restriction digest for successful clones –.the 1.3 kb band confirms the presence of insert; **M**-Marker – **odd lanes**:-digested H609 & 610A, 1-607, 1-601, K612V & K612A; .– even lanes: undigested H609 & 610A, 1-607, 1-601, K612V & K612A.

C: Expression of sAPPα Variants by Stably Transfected HEK293 Cells

HEK293 cells were transfected with the recombinant DNA to create stable cell lines that produced the recombinant sAPP*α* protein variants. The experimental design allowed the protein to be secreted directly into the culture media.[17] The presence of heparin binding domains within the sAPP*α* and sAPP*β* has allowed the development of a one-step protein purification strategy.[17]

Cells are usually maintained in serum enriched media. However, serum free media can be substituted during protein harvest to ensure that only the required protein and a small number of other proteins are present, ensuring a simple purification process. To confirm that the required protein was being produced and secreted into the media by the HEK293 cells, an immunoblot for each of the variants was carried out using an antibody that recognized the *N*-terminal of the protein variants. The immunoblot demonstrated that the HEK293 cells had been successfully transfected and were producing and secreting the required sAPP*α* variants into their media (Fig 7). Each of these is to be tested for neuroprotective functions and compared with the native sAPP*α* and sAPP*β*.

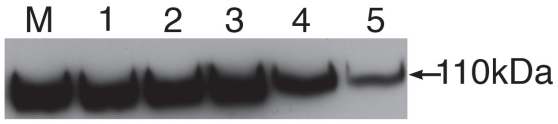


Fig 7. Immunoblot to detect sAPP*α* variants in culture; **lane 1**: H609&610A; **lane 2**: 1-607 sAPP*α*; **lane 3:** 1-601 sAPP*α*; **lane 4:** 1-601 sAPP*α*; **lane 5:** K612V: **lane 6**: K612A.

Discussion

AD is beginning to impact on health budgets both here and abroad and this will only intensify over time. There is no effective therapeutic strategy currently to reverse the pathology of AD, and in NZ even the best available drugs, acetylcholinesterase inhibitors, remain to be funded by Pharmac.

In common with other neurodegenerative disorders, AD has abnormal accumulation of aggregated proteins (amyloidosis). Hence, a therapeutic strategy that alleviates the pathology of AD might also be applicable to other neurodegenerative disorders such as Parkinson's and Huntington's diseases.

Our assays show that laboratory produced sAPP*α* is protective and/or beneficial to cells of both neural and non-neural cell lines. This implies that the effect is likely to be general. Furthermore, we have demonstrated sAPP*α*'s ability to influence and promote the transcription of protective genes indirectly, by showing that it can activate the NFκB family of transcription factors.

A quite brief pre-exposure of cells to sAPP*α* is sufficient to protect them from the adverse effects of glucose deprivation. However, while low concentrations of sAPP*α* are protective, higher concentrations become toxic as previously demonstrated by us;[18] low concentrations of sAPP*α* restored memory mechanisms in rats, but higher concentrations (10 nM) were counterproductive and even

toxic. This implies that there exists a delicate balance in the proteolytic processing of APP and that any imbalance results in detrimentally higher concentrations of one product over the other.

Since sAPPα can be applied in the culture media, the studies strongly suggest both neuroblastoma and COS-7 cells have a sAPPα-specific surface receptor(s) that have yet to be identified and characterized. We are currently directing our work toward this.

The only difference between sAPPα and sAPPβ is the 16aa at the C-terminus. Yet, sAPPα is almost 10- to 100-fold more potent than sAPPβ, as we have indicated and reported also by others.[16,19] We have suggested that this region of the protein could be responsible for the activity of sAPPα. To test this hypothesis deletion and substitution variants of sAPPα have been generated with the changes within the C-terminal end of sAPPα. We are now poised to assess whether the variants mimic or interfere with the function of wild type sAPPα. In addition, we have prepared a synthetic 16aa peptide that corresponds to the C-terminal end of sAPPα and its activity will be tested independently to see if it acts as a mimic or a competitor. A previous study[20] suggested a protective function for a 10aa peptide spanning the β-secretase junction and we aim to see if this finding can be replicated. Should the 16aa peptide itself prove be a potent neuroprotector, then it has the potential to be utilized for a viable therapeutic strategy.

Acknowledgements

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Science Scene continued

Astronomy's Year

2009 is the International Year of Astronomy. It was chosen because it is the 400th anniversary of the first use of an astronomical telescope by Galileo Galilei.

The aim of the organising groups, the International Astronomical Union and the United Nations, Educational, Scientific and Cultural Organisation, is a global celebration of astronomy and its contributions to society and culture.

John Hearnshaw from the University of Canterbury is participating in the cosmic diary blogs as part of the celebrations. You can follow his blog at http://cosmicdiary.org/blogs/john_hearnshaw/

Part of the celebrations is to encourage worldwide interest in astronomy using the theme; "the universe, yours to discover." Many events have been planned and educational resources are available through the website; *www.astronomy2009.org*

To find out about events happening in New Zealand visit the website; *www.astronomy2009.org.nz*

Getting the measure of things

New Zealand's Virtual Institute of Metrology in Chemistry (VIMC) is finding demand increasing for information and help on chemical and biological measurements.

The website is *http://msl.irl.cri.nz/si-units/chemical/index.html* Here you can find information on reference materials, proficiency tests and chemical standards programmes, as well as other useful references.

Head of the VIMC is Dr Laly Samuel at Industrial Research Limited. She was part of a group that set up the National Metrology Institute in Japan in 2001. She says, "the institute is helping laboratories to ensure New Zealand's routine analysis is up to international level."

Many of the requests to the VIMC involve uncertainty around test results that require traceability back to a referenced measurement.

"The VIMC is an intermediate and cost effective solution but there is a clear need to establish a fully accredited facility in New Zealand," says Dr Samuel.