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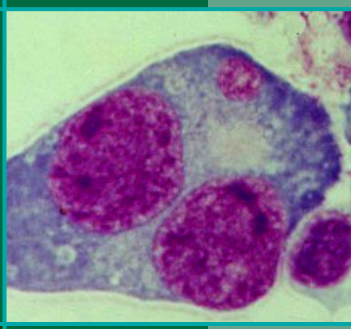
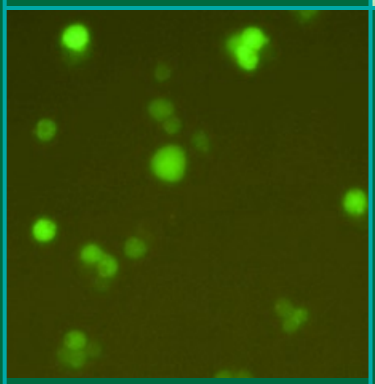
Optimisation of Cylindrospermopsin Toxicity Assay





Research Report 66

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Optimisation of Cylindrospermopsin Toxicity Assay

Suzanne Froscio, Emma Cannon and Andrew Humpage

Research Report No 66

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Research Report 66: Optimisation of Cylindrospermopsin Toxicity Assay
ISBN 18766 1692X

FOREWORD

Research Report Title: Optimisation of Cylindrospermopsin Toxicity Assay

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CRC for Water Quality and Treatment Project No. 2.0.1.2.2.3 – Optimisation of Cylindrospermopsin Toxicity Assay.

EXECUTIVE SUMMARY

The aim of this project was to optimise a rapid assay for cylindrospermopsin (CYN) using cells that express a green fluorescent protein (GFP).

The assay utilises CYN's inhibitory effects on protein synthesis as a diagnostic endpoint for detection of the toxin. Cell-lines expressing GFP were created in order to provide a continuous measure of protein synthesis in the cells. The GFP signal in Vero-GFP cells was demonstrated to respond to the inhibitory effects of CYN within 4 hrs of exposure.

However, while the standard assay format allowed for rapid detection of CYN, the Vero-GFP cells were noted to be relatively insensitive to CYN when compared to the responses of known protein synthesis inhibitors cycloheximide and emetine.

Results in this study indicated that limited sensitivity to CYN was likely to be due to poor uptake of the toxin by the Vero-GFP cells rather than its potency at the site of action. A number of strategies were trialed in order to improve sensitivity. Greatest improvement to the assay was made following incubation of the Vero-GFP cells in growth medium of lower than normal composition (0.75X to 0.65X). This modified media was demonstrated to both increase GFP production by the cells and increase susceptibility to CYN, possibly as a result of a stress response of the cells to the reduced media composition.

The improved GFP assay allowed detection of ≥ 1 μM CYN following 4 hr incubation of the cells with the toxin. This provides a more rapid and sensitive response than previously achieved using cytotoxic endpoints. The sensitivity of the assay requires concentration of raw or processed water samples prior to testing to enable toxin detection. In other applications, the Vero-GFP cells should also provide a useful research tool to further investigate mechanisms of CYN toxicity.

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ABBREVIATIONS

ATCC	American Type Culture Collection
AWQC	Australian Water Quality Centre
CHEX	Cycloheximide
CYN	Cylindrospermopsin
EV	Electronic volume
GFP	Green fluorescent protein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
SSC	Side scatter
Vero-GFP	Vero cells transfected to produce GFP

1 INTRODUCTION

1.1 Aim

The aim of this project was to optimise a rapid assay for cylindrospermopsin (CYN) using cells that express a GFP reporter protein.

1.2 Background

The cyanobacterial toxin cylindrospermopsin (CYN) is a cytotoxin capable of inducing toxic effects in a range of different cell types. Although cytotoxicity can be used as an endpoint to detect CYN in unknown samples, the nature of the toxicity is protracted requiring at least 18 hr incubation for detection in primary hepatocytes (Froscio *et al.*, 2003a; Runnegar *et al.*, 1994) and 24-48 hr incubation or longer for detection in less susceptible cell lines (Bain *et al.*, 2007; Chong *et al.*, 2002; Neumann *et al.*, 2007).

CYN is also known to be a potent inhibitor of protein synthesis (Froscio *et al.*, 2001; Terao *et al.*, 1994), and a more rapid and diagnostic assay can be achieved using this endpoint. In primary hepatocytes it has been shown that CYN-induced effects on protein synthesis can be detected within a few hrs of toxin exposure (4 hr), well prior to the onset of cytotoxicity at 18 hrs (Froscio *et al.*, 2003b). One drawback is that the cell-based assay for determining protein synthesis (using radiolabeled precursors) requires extensive processing of samples and is therefore unsuitable for screening purposes.

In CRC for Water Quality and Treatment Research Report 60 'Screening Assays for Water-borne Toxins' we established Vero cell lines that stably express green fluorescent protein (GFP) to provide an ongoing measurement of protein synthesis in the cells. The Vero-GFP cells generated express a destabilised form of GFP, and the short half life allows for rapid detection of changes in protein synthesis as determined by GFP levels. A number of researchers have similarly utilised luminescent or fluorescent reporter proteins in bacteria as a rapid method for screening samples for inhibitors of prokaryotic protein synthesis or general antibacterial properties (Collins *et al.*, 1998; Lampinen *et al.*, 1995). Mammalian cell cultures stably expressing GFP have also been used in a variety of screening assays, although generally for cytotoxic endpoints (Chen *et al.*, 2005; Steff *et al.*, 2001).

Preliminary data obtained in Research Report 60 indicated that CYN could be rapidly detected by GFP assay, reducing GFP levels within 4 hrs of exposure of the Vero-GFP cells, however concentrations of CYN detected this assay were lower than expected based on previous research work.

The aim of this project is to optimise the GFP assay for CYN detection. To do this, an understanding of the behaviour of the Vero-GFP cells and GFP output is required. CYN effects will be compared to other protein synthesis inhibitors.

Specifically the aims were to:

- Confirm that the sensitivity of the cells to CYN has not altered following stable transfection of cells with GFP. The cytotoxic response of Vero and Vero-GFP cell lines will be compared following exposure to CYN and other protein synthesis inhibitors.
- Enhance expression of GFP in the Vero-GFP cell lines.
- Assist delivery of CYN to the Vero-GFP cells. Determine whether toxin uptake is likely to be a problem for the assay and whether this can be overcome using standard delivery techniques such as use of cationic lipid transfer.

2 MATERIALS AND METHODS

2.1 Purified Cylindrospermopsin

Purified cylindrospermopsin (CYN) was obtained from the Australian Water Quality Centre (AWQC), prepared as detailed in Humpage *et al.,* (2005).

2.2 Reagents

The selection agent G-418 Sulfate was obtained from Promega Corporation (Madison, WI, USA). Kanamycin, ampicillin, cycloheximide and emetine were obtained from Sigma-Aldrich (MO, USA). The pTurboGFP-dest1 vector was obtained from Evrogen (Moscow). A 50X amino acid solution was obtained from Sigma-Aldrich (MO, USA).

2.3 Cell Culture

The Vero (African Green Monkey Kidney, ATCC #CCL-81) cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were grown in 1X Medium 199 with Earle's salts, supplemented with 0.6 mM L-Glutamine, 0.04 mg/mL gentamycin, 20 mM sodium bicarbonate, 0.0005% (w/v) phenol red and 6% new-born calf serum (GIBCO), 37°C, 5% CO_2. Vero-GFP clones were also maintained in Vero growth medium. GFP-clones were exposed to G-418 (600 µg/mL, 7 day monolayer exposure) on resuscitation of stocks from liquid nitrogen.

2.4 Generation of Vero-GFP clones

2.4.1 Plasmid Propagation

The pTurboGFP-dest1 vector was obtained from Evrogen (Moscow). The plasmid was propagated in *E. coli* DH5-α competent cells (Invitrogen) and plasmid DNA isolated from transformants using UltraClean™ Endotoxin-Free Plasmid extraction kit (MOBIO Laboratories, CA, USA). The plasmid was linearized with *Apa*I prior to transfection experiments.

2.4.2 Transfection

Vero cells (1 x 10^6 cells/plate) were seeded on 100 mm plates and grown overnight to 40% confluency. Transfection of plasmid (10 or 20 µg) was performed using a calcium phosphate precipitate (Profection® Mammalian Transfection system (Promega). Growth medium was replaced at 24 hr. At 48 hrs cells were split by trypsin digest, and dispensed into growth medium supplemented with G-418 (600 µg/mL) for selection. GFP expression in clones was screened by fluorescence microscopy. PCR analysis of cell extracts confirmed the presence of the destabilised GFP sequence. Vero-GFP clone designated VeroGFP LDE11 was used in these experiments.

2.5 Cytotoxicity assays

2.5.1 MTS Assay

Cells were seeded into 96-well flat bottom cell culture plates (Sarstedt, Australia) at a density of 1 x10^4 cells/well. Following incubation at 37ºC, 5% CO_2 overnight (20-24 hrs), cells were treated with CYN (0.1-100 µM), CHEX (0.001-100 µM) or emetine (0.001-100 µM) until assay. Cell proliferation was then measured by MTS assay. The CellTitre 96® Aqueous One Solution Reagent from Promega Corporation (Madison, WI, USA) was used that contains both the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and electron coupling reagent PES (phenaxine ethosulfate). The MTS Reagent was diluted 1/10 in cell culture growth medium. For assay, media were removed from microplate wells and 100 µL of the diluted MTS Reagent added. Plates were incubated for 2 hrs at 37 C, 5% CO_2. The absorbance was read at 490 nm on a Perkin Elmer VICTOR^3 (Wellesley, MA, USA) plate reader. The MTS reagent was added to the cells 22 hrs post treatment, and readings carried out at 24 hrs post toxin treatment.

2.5.2 GFP Assay – Standard

Vero-GFP cells were seeded into 96-well flat bottom cell culture plates (Sarstedt, Australia) at a density of 1 x10^4 cells/well. Following incubation at 37ºC, 5% CO_2 overnight (20-24 hrs) to produce a monolayer, cells were treated with CYN (0.1-100 µM), CHEX (0.1-100 µM) or emetine (0.1-100 µM). At the time of assay (4 hrs or 24 hrs), medium was removed from wells and cells were trypsinised by adding 50 µL of 0.25% trypsin-EDTA solution per well. Plates were incubated at 37°C for approx 10 mins, then 200 µL of fresh medium was added. Vero-GFP cell suspensions were analysed for GFP fluorescence on a Cell Lab Quanta^{TM} SC flow cytometer with MultiPlatform Loader (Beckman Coulter Inc, CA, USA). Excitation was provided by the 488 nm laser and GFP was detected in the FL1 region (525 /20 nm bandpass filter). For each sample 3000 cells were collected on the side scatter vs electronic volume plot and analysed for GFP fluorescence in the FL1 channel. Designation of non-GFP and GFP regions were based on the autofluorescence of untransfected Vero cells. A sample of Vero cells were run in parallel with each assay to confirm regions.

2.5.3 GFP Assay – Modified

The Vero growth medium was diluted with sterile Milli Q water to a final strength of 0.75X, 0.65X or 0.5X. Vero-GFP cells (1 x 10^4 cells/well) were seeded into 96-well plates in the reduced strength media. Following incubation overnight (20-24 hrs) to produce a monolayer, cells were treated with 0.1-100 µM CYN (prepared in the same reduced strength media) for 4 hrs. Samples were processed for GFP assay by flow cytometry as detailed above (Section 2.5.2).

DMEM media was obtained from Gibco (MD, US). Lipofectamine 2000 was obtained from Invitrogen (CA, USA). FugeneHD was obtained from Roche (Basel, Switzerland).

2.6 *In vitro* Translation Reactions in Cell-free Extract

Translation reactions were carried out in rabbit reticulocyte lysate obtained from Promega Corporation (WI, USA). For generation of concentration-response curves, translation reactions (25 µL) were treated with CYN (0.01-1 µM), cycloheximide (0.001-30 µM) or emetine (0.001-30 µM). Protein synthesis was initiated with 20 µg/mL luciferase mRNA template. Samples were incubated for 90 mins at 30ºC. The amount of luciferase formed was quantified in a white 96-well microplate (PerkinElmer, MA, USA). Samples were diluted 100 fold in lysis buffer (25 mM tris-phosphate, 2mM DTT, 2 mM 1,2-diaminocyclohexane N,N,N'N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) containing 1mg/mL BSA and then 2.5 µL added to 60 µL of BrightGlo reagent (Promega, WI, USA). Luminescence of samples was read (10 sec read time) on a Victor^3 1420 luminescence microplate reader (Perkin Elmer, MA, USA).

3 RESULTS

3.1 Characterisation of Vero-GFP cells

3.1.1 Decay curve for destabilised GFP

Production of GFP by the Vero-GFP cells was visualised by fluorescence microscopy (Figure 1A). The half-life of the GFP was determined to be 2.2 hrs following treatment with the protein synthesis inhibitor emetine (Figure 1B), indicating that the destabilised protein has a rapid turnover rate as expected.

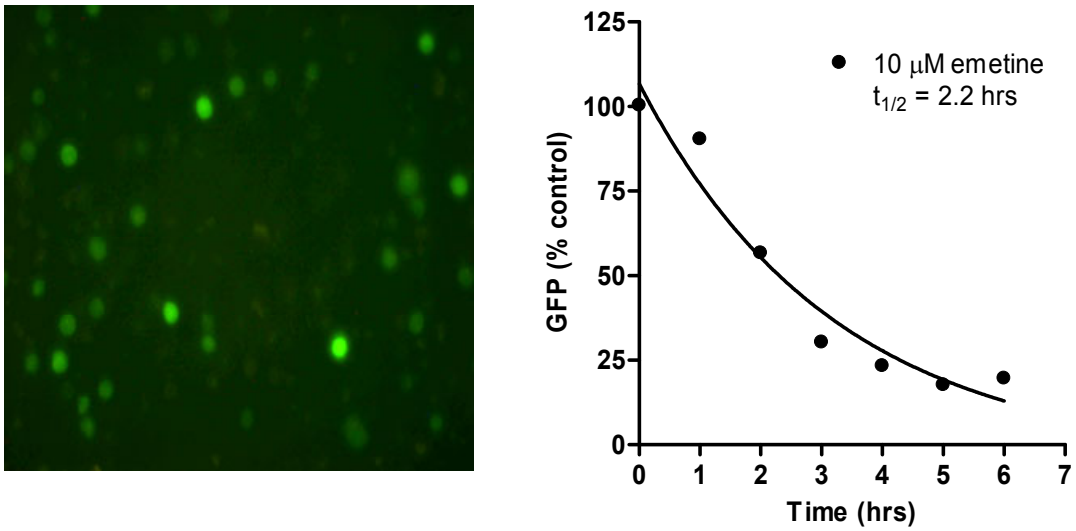
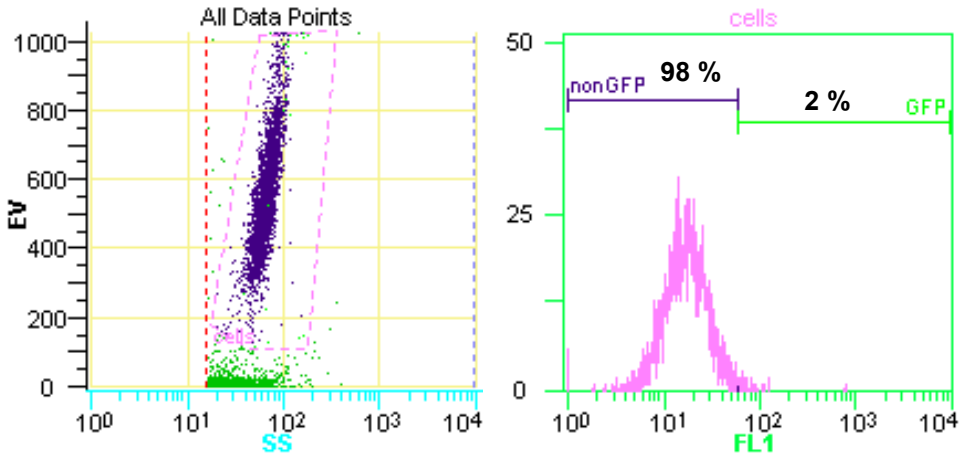


Figure 1 A. Vero-GFP cells expressing the pTurboGFP-dest1 vector. Cells were visualised by fluorescence microscopy with WB excitation. B. Half-life of the GFP signal is 2.2 hrs. Vero-GFP cells were treated with 10 μM emetine for 1-6 hrs and GFP fluorescence was determined by flow cytometry. Data are mean ± SE of triplicates and modelled to exponential decay curve.

3.1.2 GFP Production in Cell Culture

Flow cytometry profiles of Vero-GFP cells showed that the cells consisted of two populations in the FL1 channel, designated non-GFP and GFP regions (Figure 2B). The non-GFP population corresponded with the autofluorescence of untransfected Vero cells (Figure 2A). During normal cell culture conditions the Vero-GFP cells always displayed two peaks. The percentage of cells in the non-GFP/GFP regions was consistent within each experiment. However, GFP production was noted to reduce over successive passages of the cell culture. In Figure 2, 77% of the Vero-GFP cells analysed have detectable GFP signal in the FL1 channel. These cells were at passage number 4. A decrease in cellular GFP signal was then observed with further culture of the cells (Figure 3). By passage number 15 – 20 (approximately 2 months in culture) approximately 65% of the cells have a detectable GFP signal. This apparent reduction in GFP production did not alter the response of toxins when the data was expressed as % control GFP levels. Cell lines were discarded if the percentage of GFP decreased below 50%, however latter work indicates that this may not be necessary (See section 3.3.3).

A. Vero



B. Vero-GFP

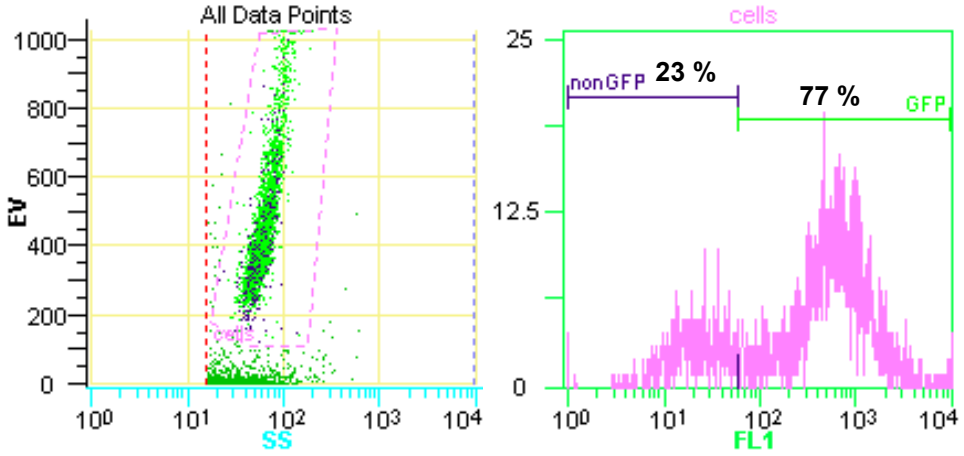


Figure 2 Flow cytometry profiles for A. Vero, and B. Vero-GFP cells. Cells were collected in the SS vs EV scatter plots. The gated cell population was then analysed for GFP in the FL1 histogram. The percentage of cells in the non-GFP and GFP regions was determined.

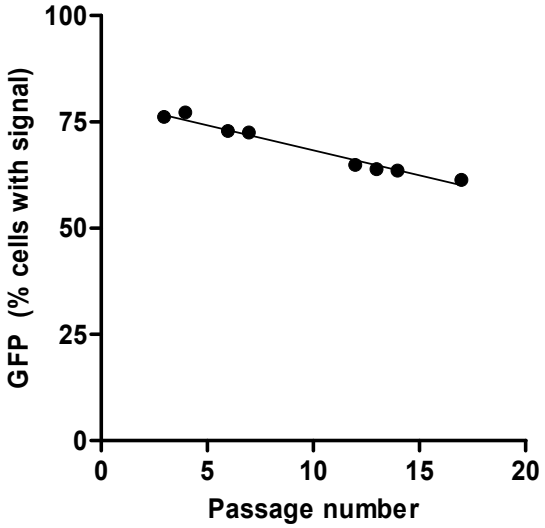


Figure 3 Decrease in GFP signal with increasing passage number. Vero-GFP cells were analysed by flow cytometry. Data are mean ± SE of 3-6 replicates.

3.1.3 Comparison of Vero and Vero-GFP Cell Sensitivity to Toxins

To assess whether the transfected Vero-GFP cells had undergone changes in sensitivity that could impact on the development of the GFP assay, the cytotoxic responses of Vero and Vero-GFP cells were compared following toxin treatments.

Vero and Vero-GFP cells were exposed to CYN (0.1-100 µM), CHEX (0.01-100 µM) or emetine (0.01-100 µM). Following 24 hrs incubation, cytotoxicity assessment was carried out by MTS assay. The cytotoxicity results show that similar concentration-response curves for CYN, CHEX or emetine were obtained in Vero-GFP cells compared to the parent Vero cells (Figure 4). Some enhanced sensitivity to the toxins was noted with the Vero-GFP cells. For example, while treatment with 100 µM CYN reduced Vero cell viability to 46 % ± 2.3 %, the viability of Vero-GFP cells was further reduced to 21 ± 2.0%. IC_{50} determinations are reported for CYN (Vero = 7.6 µM; and Vero-GFP = 6.0 µM) and emetine treatments (Vero = 1.4 µM; Vero-GFP = 0.28 µM). CHEX did not reduced viability below 50% in either of the cell lines.

The results demonstrate that Vero-GFP cells remain sensitive to CYN, CHEX and emetine. The concentration range 0.1-100 µM was shown to be effective for each of the toxins and will be used for evaluation of the GFP assay.

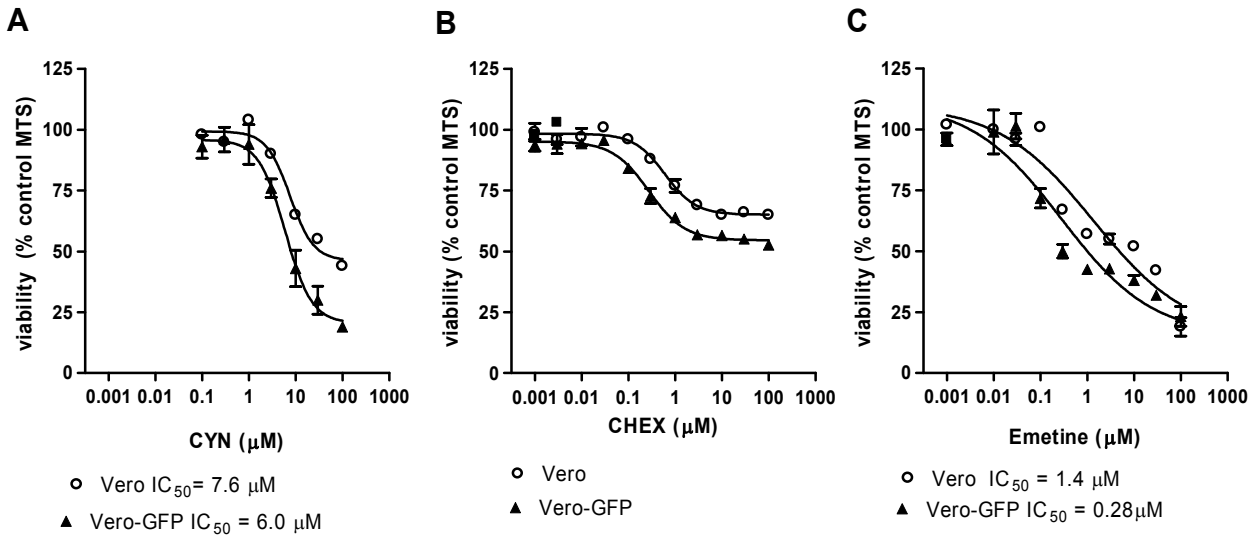


Figure 4 Viability of Vero and Vero-GFP cells following 24 hrs exposure to (A) CYN, (B) CHEX or (C) emetine. Data are mean ± SE of n = 3-4 independent experiments. Data were analysed by sigmoid concentration response (variable slope).

3.2 Evaluation of the GFP Assay for Detection of CYN and other Protein Synthesis Inhibitors

3.2.1 GFP Assay Results

The GFP assay was evaluated for detection of CYN and the protein synthesis inhibitors CHEX and emetine. Vero-GFP cells were exposed to the inhibitors (0.1- 100 µM) for 4 hrs or 24 hrs. An example of changes in GFP flow cytometry profiles following 4 hrs CYN treatment is given in Figure 5 illustrating progressive shift of the GFP peak with increasing concentrations of toxin. Results in Figure 6 showed that each of the inhibitors tested reduced cellular GFP, although their responses differed. Refer also to Table 1 for IC_{50} values. As shown in Figure 6A, CYN induced a concentration-dependent reduction in GFP signal at 4 hrs ($IC_{50,}$ 16 µM) with further reduction of GFP, or increased sensitivity, at 24 hrs (IC_{50} 2.7 µM). In contrast, the GFP concentration-response curves produced by both CHEX (Figure 6B) and emetine (Figure 6C) had similar potency at the two time points tested as reflected in IC_{50} values. The data were analysed by one-way ANOVA to determine the limit of toxin detection (Table 1). Concentrations of ≥ 10 µM CYN could be detected 4 hrs and ≥ 3 µM CYN at 24 hrs ($p<0.01$ from control). Emetine was at least 10 fold more sensitive with detection of 0.3 µM of this inhibitor at both 4 hrs and 24 hrs ($p<0.01$ from control).

3.2.2 Comparison GFP Assay Results to Reticulocyte Lysate Results

The relative potency of protein synthesis inhibitors CYN, CHEX and emetine was also compared in reticulocyte lysate, a cell-free assay for protein synthesis. The data were fitted to sigmoid dose response (variable slope), and determined that CYN had an IC_{50} = 131 nM (95% CI 114-151nM), similar to CHEX IC_{50}= 141 nM (95% CI 100-200 nM) and more potent than emetine IC_{50} = 543 nM (95% CI 271-1085 nM). The characteristic steep slope of -3.3 was obtained for CYN, while CHEX and emetine slopes were -1.8 and -1.1 respectively.

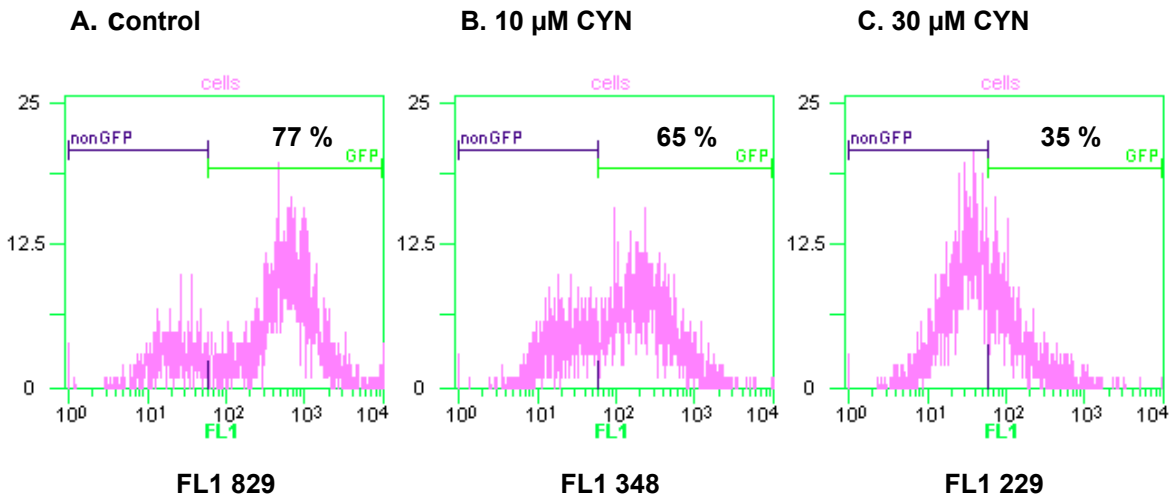


Figure 5 Changes in Vero-GFP flow cytometry profiles following toxin treatment. (A) Untreated cells (B) 10 µM CYN for 4 hrs and (C). 30 µM CYN for 4 hrs. The percentage of cells with a GFP signal is indicated. The FL1 GFP peak values are indicated below each histogram.

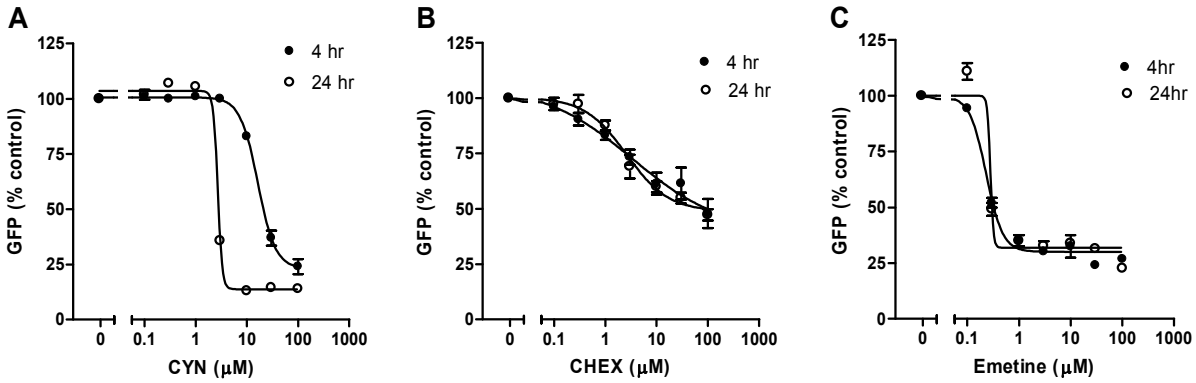


Figure 6 The GFP assay for detection of (A) CYN, (B) CHEX or (C) emetine. Vero-GFP cells were incubated in the presence of toxins for 4 hrs or 24 hrs, and the change in GFP levels from the control was determined by flow cytometry. Data are mean ± SE of ≥ 3 independent experiments. Data were modelled to a sigmoid dose-response curve (variable slope).

Table 1 Summary of IC_{50} values and limit of detection (lod) for CYN, CHEX and emetine by GFP assay carried out at 4 hrs or 24 hrs. IC_{50} values were determined from sigmoid dose-response curves. Lod was determined by one-way ANOVA as the lowest concentration tested to significantly reduce GFP levels from the control at each time point. $p < 0.01$ for all lod determinations.

	CYN		**CHEX**		**emetine**	
	IC $_{50}$	**lod**	**IC_{50}**	**lod**	**IC_{50}**	**lod**
4 hrs	16 μM	10 μM	3.3 μM	1 μM	0.23 μM	0.3 μM
24 hrs	2.7 μM	3 μM	2.6 μM	1 μM	0.27 μM	0.3 μM

lod = limit of detection.

3.3 GFP Assay Improvements

Given the results obtained in Section 3.2, further experiments were designed to improve the GFP assay. Work aimed to increase both cellular GFP output and uptake of CYN.

3.3.1 Trial of Media Modifications to Increase GFP Output

As flow cytometry demonstrated that a portion of the Vero-GFP cell population did not produce a detectable GFP signal over background levels (Refer to Section 3.1.2), a number of modifications to the growth medium were trialled with the aim of improving the GFP production by the cells. This included; (1) supplementation of the medium with amino acids to provide additional precursors to the highly expressed GFP protein, (2) Use of DMEM medium that contains higher levels of glucose and vitamins as an additional energy source, and (3) reduction of serum levels from 7% to 2% in the growth medium. It has been suggested that reduced serum levels can lead to unbalanced program of growth, reducing cell division while maintaining protein synthesis.

Of the methods trialled, amino acid supplementation was the most promising for enhancing GFP production, with a 5X amino acid stock increasing the percentage of fluorescent cells in the population up to 10% above control levels (data not shown). However, it was also noted that the amino acid supplements resulted in toxic effects seen as a reduction in cell numbers. Long term exposure (1 week) to lower concentrations of amino acids was trialled to avoid any toxic responses, but did not enhance the % fluorescent cells in the population. No change to cellular GFP levels was observed following use of DMEM media or with reduction in serum levels in normal media.

3.3.2 Trial of Lipid Assisted Delivery of CYN

Given that CYN is a potent inhibitor of protein synthesis in the cell-free assay (Section 3.2.2), yet of low potency in the cell-based GFP assay (Section 3.2.1) it was considered that uptake of CYN into the Vero-GFP cells may be limited. The lipids reagents Lipofectamine 2000 and Fugene 6 were trialled in an attempt to assist the delivery of CYN into the cells. Reagents were mixed with CYN before incubation with Vero-GFP cells, however the lipids were not observed to influence CYN entry as measured by the GFP assay response (data not shown).

3.3.3 Use of Low Composition Media

Incubation of Vero-GFP cells in media diluted with MilliQ water to < 1X composition was observed to increase the GFP levels and increase susceptibility to CYN. Flow cytometry data shows the response of Vero-GFP cells when incubated in a range of media compositions (0.875X to 0.375X) for 4 hrs or 24 hrs (Figure 7). As shown in Figure 7A, GFP production increased following 24 hrs incubation in all media with < 1X composition. This was not seen at 4 hrs. Cells numbers doubled over 24 hrs in the 1X medium (Figure 7B), while cells incubated in all other media of < 1X composition had similar numbers at 4 hrs and 24 hrs. A trend for decreasing cell size, as determined by EV, was seen with reduced media compositions (Figure 7C) while internal cell complexity increased as indicated by SS (Figure 7D). Both EV and SS parameters showed similar responses at 4 hrs and 24 hrs.

The response of CYN in the GFP assay was then analysed when media of <1X composition were used for the assay. Cells were incubated 0.75X or 0.65X media overnight and then exposed to 0.1-100 µM CYN for 4 hrs in the same reduced medium. In both media tested, Vero-GFP cells showed increased susceptibility to CYN (4 hr exposure), improving sensitivity at least 3 fold over 1X medium (Figure 8A). Expression of the data as % mean FL1 peak also improved sensitivity and allowed CYN concentrations ≥1 µM CYN can be detected following 4 hr exposure (Figure 8B). The IC_{50} values and lod obtained in each of the media are summarised in Table 2.

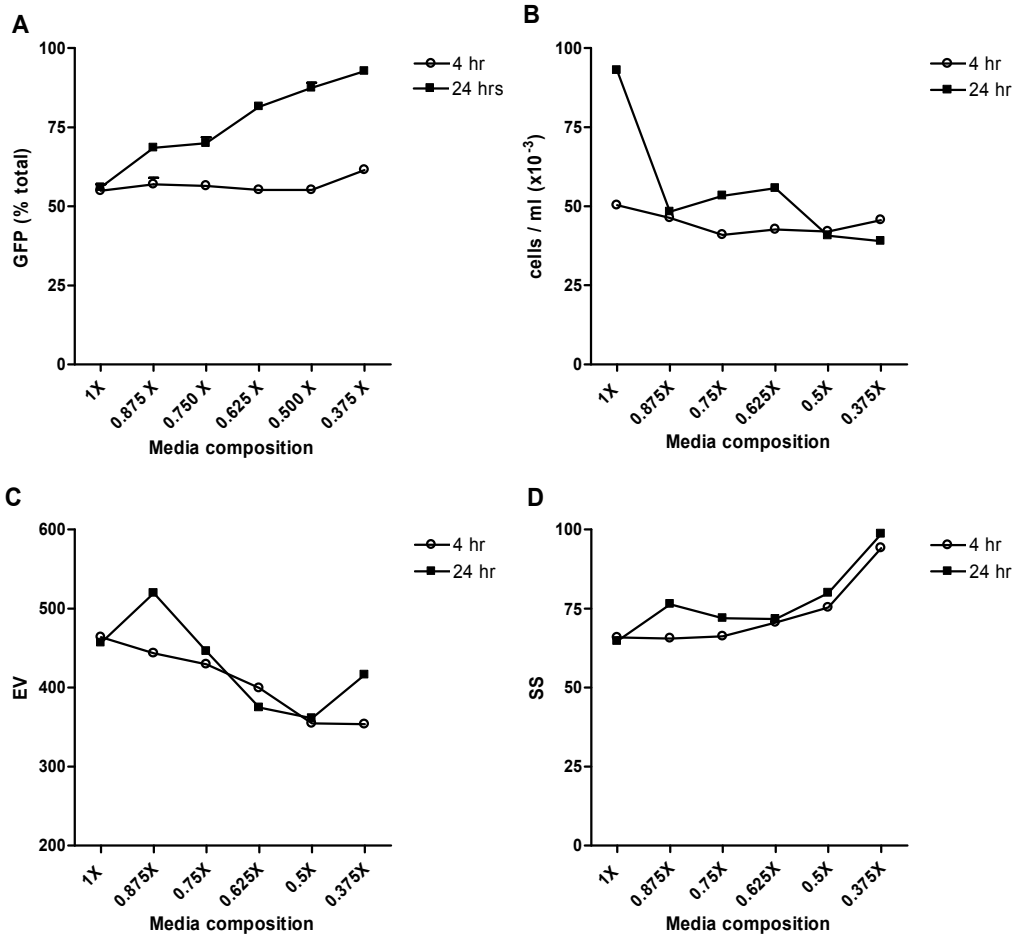


Figure 7 Effect of reduced media composition on Vero-GFP cells. A. GFP production; B. cell number; C. cell size as measured by electronic volume (EV); and D. internal cell complexity as measured by side scatter (SS). Vero-GFP cells were incubated in various media for 4 hrs or 24 hrs and analysed by flow cytometry. Data are mean ±SE of triplicates.

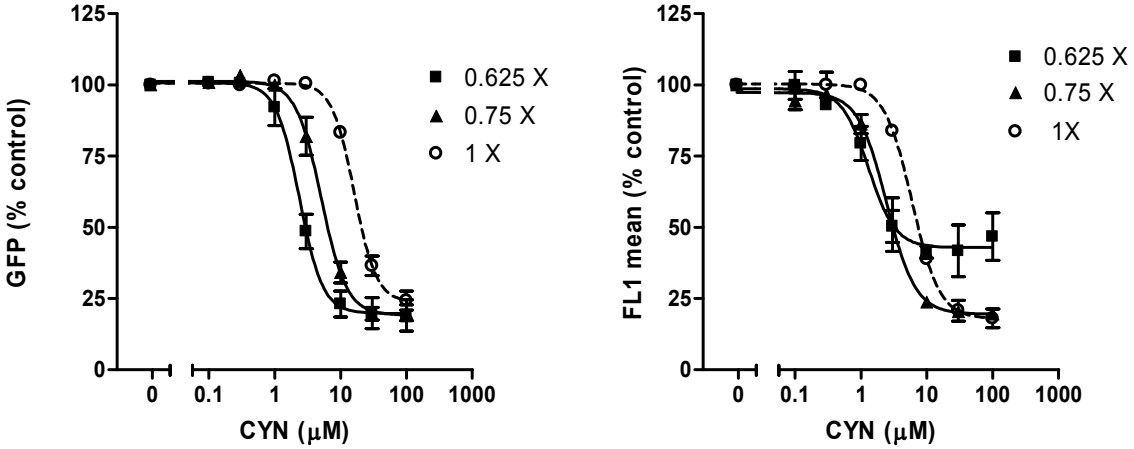


Figure 8 Increased sensitivity to CYN occurs when Vero-GFP cells are incubated in low composition media. Cells were incubated in 0.75X or 0.65X media overnight prior to exposure to CYN for 4 hrs. CYN response in 1X media is shown for comparison. Data analysed as either (A) GFP region (% control) or (B). Mean FL1 peak (% control). Data are mean ± SE of 3 independent experiments.

Table 2 Summary of IC_{50} values and limit of detection for CYN by GFP assay carried out in reduced media. Lod was determined as the lowest concentration tested to significantly reduce GFP levels from the control at each time point. $p < 0.01$ for all lod determinations.

	CYN, 0.625X Media		**CYN, 0.75X Media**		**CYN, 1 X Media**	
	IC_{50}	**lod**	**IC_{50}**	**lod**	**IC_{50}**	**lod**
4 hrs GFP	2.4 μM	3 μM	5.1 μM	3 μM	16 μM	10 μM
4 hrs FL1	1.3 μM	1 μM	2.5 μM	1 μM	5.9 μM	3 μM

4 DISCUSSION

This project demonstrates that Vero-GFP cells expressing a GFP reporter protein can be utilised as a rapid cell-based assay for detection of the cyanobacterial toxin CYN. This GFP assay is based on the toxin's inhibitory effects on protein synthesis to provide a diagnostic endpoint for detection of the toxin. The assay responded to CYN, and other known inhibitors CHEX and emetine, allowing detection of the toxins after 4 hr exposure.

While rapid detection of the inhibitors was obtained, CYN showed relatively low sensitivity when compared to the responses of CHEX and emetine. Higher concentrations of CYN (10-30 fold) than the other toxins was required to inhibit the GFP signal at 4 hrs. Improved sensitivity for CYN detection was observed at 24 hrs, but only with concentrations of CYN similar to those expected to produce a cytotoxic response by MTS assay at the same time-point. This was somewhat unexpected since CYN is known to be a potent inhibitor of protein synthesis in the cell-free assay (Froscio *et al.*, 2001; Neumann *et al.*, 2007; Terao *et al.*, 1994), and sub toxic concentrations of CYN had previously been observed to rapidly inhibit protein synthesis in primary hepatocyte culture (Froscio *et al.*, 2003a). The low cellular potency of CYN and progressive effects over 4-24 hrs in the GFP assay suggests uptake of toxin into the Vero-GFP cell line may be limited. To date only one study in the literature has evaluated possible mechanisms of CYN uptake, suggesting that the toxin may enter cells by passive diffusion, with some additional involvement of the bile acid transport system facilitating transport into primary hepatocytes (Chong *et al.*, 2002).

To support the theory that cellular transport is limited for CYN, the reticulocyte lysate protein synthesis assay was also utilised to assess effects of CYN, CHEX and emetine in the absence of the cell membrane and compare results to those obtained in the cell-based GFP assay. Results confirmed that CYN is a potent protein synthesis inhibitor in the lysate, with similar potency to CHEX and approximately 5 fold greater potency than emetine. The high potency of emetine in the GFP assay coupled with lesser effects in the cell-free assay is consistent with this toxin being actively transported into the cells. Intracellular concentration of emetine has previously been shown in the HeLa cell line (Grollman, 1968). Given similar effective concentrations of CYN and CHEX in the cell-free assay, the reduced effectiveness of CYN in the cells could be explained by limited cellular entry of CYN. If both inhibitors cross the cell membrane via passive diffusion the smaller size of CHEX (molecular weight 281 Daltons) may favour its transport over CYN (415 Daltons).

Cationic lipids were trialled to assist the transport of CYN across the lipid membrane but were unsuccessful in improving GFP assay sensitivity to CYN and not pursued further. The most promising results came with use of 'low composition' media. Incubation of cells in media of < 1X normal composition increased sensitivity to CYN, and also had the advantage of improving GFP production of the Vero-GFP cells. To provide an explanation for these results, the flow cytometric profiles of the cells were compared in various media compositions. It was identified that the cells were unable to proliferate in the reduced media (no cell loss apparent). The increase in GFP levels did not occur immediately, and was only evident following 24 hr incubation, suggesting the induction of a stress response by the cells that increases overall protein production including GFP. Further, the Vero-GFP cell size was reduced and internal complexity increased at all time points tested. It is suggested these results could occur if the permeability of the cell membrane was altered by reduced osmolarity of surrounding media. As intracellular ions are lost to the media, the cells may reduce their size in order to maintain the internal osmolarity. The altered membrane permeability may be sufficient to allow CYN to enter. The extent of the changes was noted to be dependent on the media composition. Further optimisation of the data by reporting responses as changes in the mean FL1 peak value also aided in improving assay sensitivity. Overall, a 10 fold improvement in assay sensitivity was able to be made by use of media of 0.65X or 0.75X the normal composition. Vero-GFP cells were first incubated overnight in this reduced media to induce the cellular response, followed by 4 hr exposure to the inhibitors. Concentrations of ≥ 1 µM CYN could be detected following 4 hr incubation with the toxin. Compared to use of cytotoxic endpoint (MTS) this has improved both turn around time and sensitivity. However, although sensitivity has been improved, for testing of raw or processed waters for CYN a pre-concentration step will be required. The proposed guideline level for CYN in drinking water is 1µg/L (Humpage and Falconer, 2002, 2003) and the limit of CYN detection at 4 hrs by GFP assay is equivalent to 416 µg/L (1 µM).

While allowing for rapid detection of CYN and other protein synthesis inhibitors, the Vero-GFP cells should also provide a useful research tool to further investigate mechanisms of CYN toxicity. As CYN-induced effects on GFP can be rapidly detected, the cells can be used to monitor for toxin uptake. The effects on GFP levels can be multiplexed with other cellular toxicity endpoints to provide a more comprehensive picture of CYN effects.

5 ACKNOWLEDGEMENTS

We would like to Thank Melody Lau for completing GFP assay results in the absence of Emma Cannon.

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