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The cytotoxic effect of Glycerophospholipid: Cholesterol Acyltransferase (GCAT) on mammalian and fish cell lines *in vitro*

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Abstract

Glycerophospholipid: cholesterol acyltransferase (GCAT) is an enzyme produced by the bacteria *Aeromonas salmonicida*. *A. salmonicida* is the causative agents of the fish disease furunculosis and GCAT is an important enzyme when considering the virulence and progression of this disease.
This study aims to show how the cytotoxic affects of the GCAT enzyme can vary between mammalian and fish cells. Both cloned and crude native forms of GCAT were exposed to mammalian and fish cell lines and observed for signs of damage over a 24 hour period. The cloned GCAT extraction was exposed to both fish and mammalian erythrocytes, while the crude native form was exposed to just mammalian erythrocytes to check for cytolytic activity.
This study shows that GCAT produced from an *A. salmonicida* A-layer mutant is capable of lysing mammalian erythrocytes, as well as Chinese Hamster Ovary (CHOs) cell lines and lysis of Goldfish Skin (GFSK) cell lines. However once a cloned, purer, sample of GCAT was produced by transformation of the GCAT gene into *E. coli,* the GCAT product was incapable of lysing CHO cell lines but retained its ability to lyse fish cells in the form of Rainbow Trout Gonad (RTG) cell lines. The purified GCAT also demonstrated low levels of haemolysis with both mammalian and fish erythrocytes.

1. Introduction

Aeromonas salmonicida is a species of gram negative bacterium that contain five subspecies; ssp. *salmonicida*, ssp. *achromogenes*, ssp. *masoucida*, ssp. *smithia* and ssp. p*ectinolytica*. None of these are considered to be any pathogenic threat to humans as they are incapable of growth at human body temperatures (Reith *et al*, 2008). The main difference between the subspecies of *A. salmonicida* is the presence of different LPS and surface proteins on the bacterial A-Layer. This layer is thought to be very important to the virulence of *A. salmonicida* and has been shown to protect the bacteria from bactericidal activity in serum and provide protection against phagocytes (Lund *et al*, 2008).

A. salmonicida spp *salmonicida* is responsible for the fish disease known as typical furunculosis. The other four subspecies are still capable of inducing furunculosis but it is referred to as atypical furunculosis *(*Magnadóttir *et al*, 2002).
The disease presents with haemorrhagic areas around the pectoral fins and mouth, also ulcerative lesions on the dorsal and ventral surfaces (*Santos et al.,* 2005). Furunculosis, both typical and atypical, was endemic in areas with intensively produced fish stocks, such as Iceland and Scotland. The disease caused huge loss of fish stock before mass vaccination programmes were introduced.

GCAT itself has been known to be lethal to salmonid species of fish since as early as 1981 (Titball & Munn 1981). The gene encoding for GCAT (specifically in *Aeromonas salmonicida* spp *salmonicida*) was sequenced in 1996 by Nerland. It consisted of 1005bp which coded for a total of 335 amino acids, an 18 amino acid signal peptide (responsible for directing the post-translational modification of the polypeptide) and a 317 amino acid mature polypeptide. The 317 amino acid mature polypeptide had a molecular mass of 38kDa. However, free non-dimerised GCAT has a molecular weight of 26kDa (Lee and Ellis, 1990), this implies that the 317aa GCAT is simply a proform and must require cleaving to become fully active.

GCAT can exist in a number of forms in the natural environment. That is to say that GCAT can be a monomer, a dimer, a polypeptide aggregate or a single polypeptide complexed to LPS. In 2006, Eggset *et al* measured that around 10% of haemolytic activity of GCAT was due to a complexed form, 35-40% activity due to aggregated GCAT and the other 50% activity to free GCAT.

GCAT also bears large similarities to a human enzyme known as lecithin-cholesterol acyltransferase (LCAT). There are a few differences between the enzymes, mainly over the variety of compounds that the enzymes can use as acyl donors and receptors. Another important difference is that the disulphide bridge in LCAT has been shown to be essential for enzymatic function, whereas in GCAT removal of the bridge appears only to reduce the enzymes ability to withstand adverse conditions, not on its ability to carry out the reaction (Qu *et al,* 1993).

This study is being carried out to test whether or not it would be possible to use the GCAT enzyme as a treatment for cancer. Biological components from microbes have been used for a long time; most notable are antibiotics which have saved millions of lives since Penicillin had its medicinal properties recognised in the early 1930s. Other more recent developments include using Streptokinase from *Strepococci* in the treatments of blood clots in myocardial infarction and pulmonary embolism. More

importantly, GCAT would not be the first application of a microbial product to anti-cancer treatments. Lots of chemotherapy drugs come from microbial sources, these include Doxorubicin from *Streptomyces peucetius*, and Bleomycin from *Streptomyces verticillus*.

Currently there is a lot of work on a compound known as borrelidin (Figure 1). Borrelidin is a polykeytide of *Streptomyces parvulus*, it has anti-malarial (Otoguro *et al*, 2003), anti-viral and antibacterial activity (Lumb *et al,* 1965). Importantly it is also a proven anti-angiogenesis compound (Wakabayashi *et al*, 1997). Angiogenesis has been shown to be vital to both continued tumour growth and eventual metastasis; further to this, solid tumours beyond $2mm^3$ have been shown to require angiogenesis for continued growth (Ferrara and Kerbel, 2005). Angiogenesis inhibition is already a target of some chemotherapy drugs such as paclitaxel, sunitinib and sorafenib and so angiogenesis is established as an effective cancer treatment.

Rather than using borrelidin produced directly from *S. parvulus*, work is being done on total biochemical synthesis of the molecule. In 2006 a set of borrelidin analogues were created by Wilkinson *et al*, using pre-cursor directed biosynthesis. They discovered that to retain the anti-cancer properties of the compound a nitrile group at C12 was crucial. In 2008 Krishna *et al*, published a method for creating the main framework of borrelidin (C3-C17) including the above mention nitrile group.

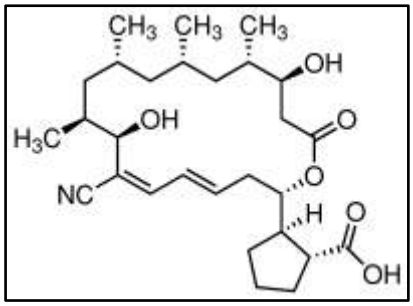

Figure 1. This shows the chemical structure of the molecule borrelidin. Picture from www.microbialscreening.com

The above example shows that microbes have been used for a very long time to augment human health, so the fact that GCAT is a microbial product is no barrier to it becoming a treatment for cancer.

2. Materials and Method

2.1 Obtaining concentrated crude GCAT sample

Cultures of MT004 (an A-layer mutant, fully missing the A-layer) were grown on a TSA medium. It was evident that the organism growing was *A. salmonicida* by the trademark brown rings that occur when *Aeromonas* species are grown on a tryptophan rich medium.

After 48hours incubation 70ml of TSB was inoculated with 4 colonies of *A. salmonicida* from the TSA plate. The flask culture was placed in a shaken water bath at 22^{o}C and a 20ml sample taken from the flask after 72 hours, centrifuged to remove cells and the supernatant stored at -20^{o}C until used.

To concentrate the sample, supernatants were placed in a membrane concentrator (Millipore Miniplus 9061, 15kda) until it had concentrated down 200 times.

2.2 Obtaining concentrated cloned GCAT sample

The gene for GCAT was transformed into a DH1 *E. coli* strain. The gene was carried on plasmid PACYC184, clone P9. 1.5ml of *E. coli* was centrifuged for two minutes at 7000 rpm in a microcentrifuge. The supernatant was removed and the cells were resuspended in 200µL of ice cold $CaCl_2$. This was left on ice for 20 minutes and then centrifuged again in the same fashion. The supernatant was again removed and the pellet was resuspended in 100µL of ice cold $CaCl_2$. The resuspended cells were then transferred to another microcentrifuge tube containing 10µL of transforming plasmid. A plasmid containing genes for both GCAT and kanamycin resistance was used. This was mixed and left on ice for 10 minutes. After 10 minutes the microcentrifuge tube was removed from the ice for five minutes, and then placed back on ice for 2o minutes. To heat shock the cells into up taking the plasmid they were placed in a 42^{o}C water bath for exactly two minutes. Then 0.9ml of LB broth was added to the microcentrifuge tube and left in a 37^{o}C water bath for one hour.

Three LB plates containing kanamycin were streaked with 10µL, 50µL and 100µL of transformed *E. coli*. One plate was spread with 100µL of untransformed *E. coli* as a control. They were then left for 48 hours to grow.

Unfortunately in this case, colonies grew on the control as well as the test plate so they could not be used. I was provided with two 5ml vials of pre-prepared cloned GCAT obtained by the same method. These were concentrated in a membrane concentrator by 5 times.

2.3 Sub-culturing cell lines

All the cells used were as monolayers set in a 24 well plate. For the Chinese Hamster Ovary (CHOs) cell lines each well was set to a concentration of between 8 x 10^5 and 1 x 10^6 cells per well. The growth medium used was HAMs F-12 (Sigma-Aldrich®) + 10% FBS (Fetal Bovine Serum).

For the Rainbow Trout Gonad cell lines (RTGs), each well was set at a concentration of 1 x 10^6 cells per well. The growth medium used was MEM (Minimum Essential Media, from GIBCO®) + NEAA (Non-Essential Amino Acids) + 10% FBS.

For the Goldfish Skin (GFSK) cell lines each well was set at a concentration of 3 x 10^5 cells per well. The growth medium used was L-15 + L-Glu + L-amino acids + 10% FBS.

They were all incubated for a 24 hour period and allowed to become confluent and cover at least 80% of the well floor before being exposed to either cloned or crude GCAT preparations.

2.4 Exposing cell lines to crude GCAT (GFSK, CHO)

The old culture medium was removed and the cells were covered in 200µL of fresh medium. Three wells had 2µL of GCAT added; two more wells had no GCAT added but had 2µL of DPBS added as a control.

2.5 Exposing cell lines (RTG and CHO) to cloned GCAT and further controls

As with the crude GCAT, the cells were prepared by removal of the old culture medium and addition of 200µL fresh medium to each well. For the CHO cells, one well was exposed to 50µL GCAT, one was exposed to 100µL GCAT, one well to 5µL and one well to 10µL of concentrated *E. coli* supernatant (100x concentrated) and the last well was exposed to 100µL unconcentrated LB broth (*E. coli* growth medium).

The RTG cells were exposed to the same conditions but they also has two extra wells that were not exposed to GCAT and acted as controls.
To ensure all preparations were free from bacterial contamination, they were all passed through a .22µM filter.

2.6 Exposing crude GCAT to mammalian erythrocytes

A dilution factor was set up so that the first well in a 96 well plate contained 1µL of crude GCAT in 50µL PBS. The dilution factor halved the concentration of GCAT in every successive well for 10 wells. To each well 2µL of whole horse blood was added. Two wells contained no GCAT, and had 2µL of PBS added to function as a control.

2.7 Exposing cloned GCAT to mammalian and fish erythrocytes

Three wells containing 45µL PBS and 5oµL cloned GCAT were set up. To each of these wells 5µL horse erythrocytes was added. Two wells containing only 95µL PBS were set up as a control. The exposure to fish erythrocytes was carried out by the same method, Tilapia erythrocytes were used. 50µL of GCAT solution was used as the solution was only five times concentrated.

3. Results

3.1 Effect of crude native GCAT on horse erythrocytes

Crude native GCAT lysed horse erythrocytes. Over the course of one hour the lowest concentration that was able to cause complete lysis of 2µL horse erythrocytes was 0.011µL GCAT in 50µL PBS. Wells with lower concentrations of GCAT did display evidence of lysis but it was not complete. This was evident by a very fine red ring at the bottom of the well. In the wells that did undergo complete lysis this red ring had disappeared and the PBS had turned a darker shade of pink than those with incomplete lysis. The erythrocytes in cloned PBS showed no signs of lysis.

3.2 Effect of crude native GCAT on CHOs

Exposure of CHOs to the crude GCAT preparation causes lysis and severe damage to cells (Plate 1.). This damage is self evident when compared to the control well (Plate 2). The cells are a lot smaller, have less well defined membranes, appear a lot more granulated and are less confluent to the bottom of the well. All these changes were observed over a period of 24 hours during which time the cells were incubated at 36^{o}C.

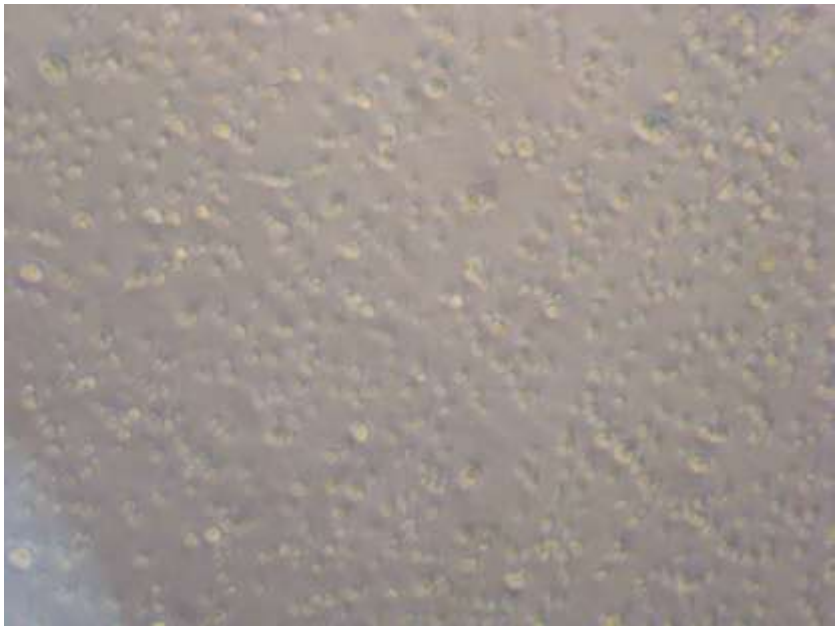

Plate 1. CHO cells 24 hours after exposure to crude GCAT preparation
These cells display a much lower density, implying large levels of cell death, they are much less well defined, are quite granulated and show little, if any sign of ongoing mitosis

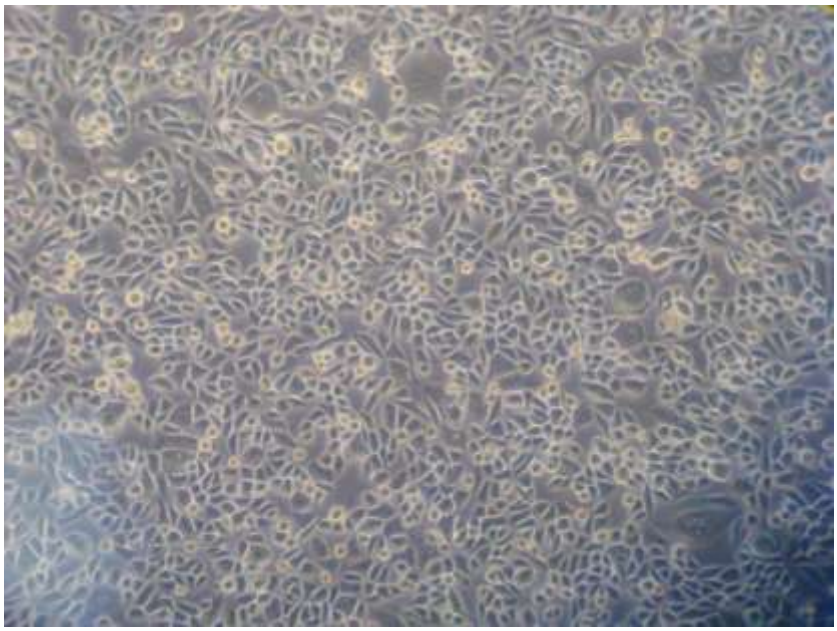

Plate 2. Control CHO cells after 24 hours
Healthy control CHOs. Notice the density, well defined membranes and signs of ongoing mitosis marked out by the highly refractive cells.

3.3 Effect of crude native GCAT on GFSK cells

The effect on GFSK cell line was much the same as the effect on CHOs. The cells showed signs of severe stress, with a much lower cell density, poorly defined membranes and appearing more granulated. The GFSK control is shown in Plate 3; the GFSK 24 hours post GCAT exposure is shown in Plate 4. The wells were incubated at 20^{o}C for the 24 hours between exposure and results.

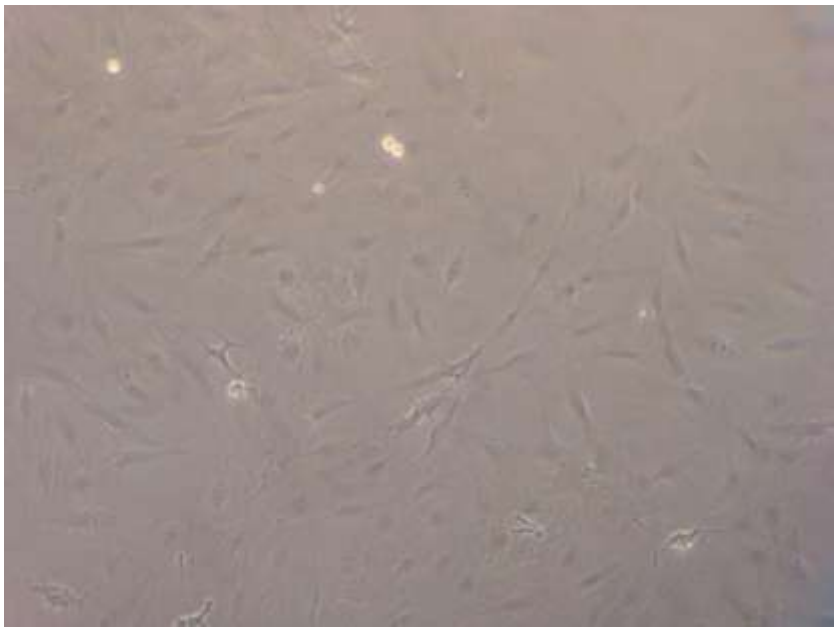

Plate 3. Control GFSK cells after 24 hours
Healthy GFSK cells. Large, well defined cells at an appropriate density. There is evidence of ongoing mitosis with the highly refractive cells.

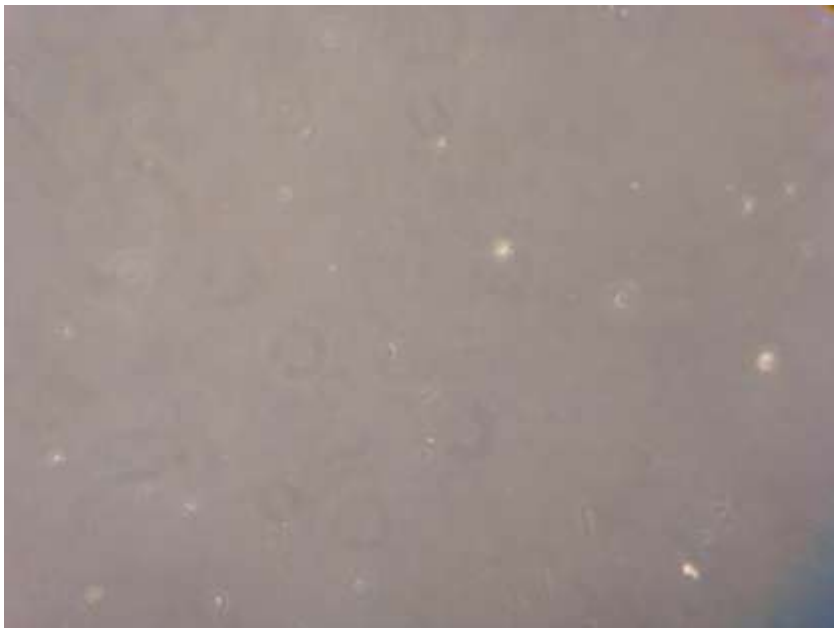

Plate 4. GFSK cells 24 hours after exposure to crude GCAT preparations
Cells are very small and misshaped making them very difficult to see. There is a much lower density of cells present, again suggesting a high level of lysis. There is however more evidence of ongoing mitosis as there is a relatively large number of highly refractive cells

3.4 Effect of cloned GCAT on horse and Tilapia erythrocytes

Purified GCAT concentrate had a limited lysing effect on both horse and tilapia erythrocytes. After one hour of exposure complete lysis had still not occurred. Tilapia blood showed greater levels of lysis within one hour than the horse blood, but lysis was observed in both cases. The level of lysis was determined relatively by the

shade of pink that the PBS became. The results indicated that there was a low level of ongoing lysis. The lowest concentration of GCAT that was capable of producing any lysis within the time frame was not determined.

3.5 Effect of cloned GCAT on CHO cells

Exposure of CHO cells to the cloned GCAT preparation had no discernible effect (Plate 5) on the cells in comparison to control wells. CHO cells were also exposed to other controls in the form of 10µL 50 times concentrated *E. coli* supernatant (Plate 6) and 100µL unconcentrated LB broth (Plate 7). Both of these controls resulted in noticeable, severe damage of the CHOs

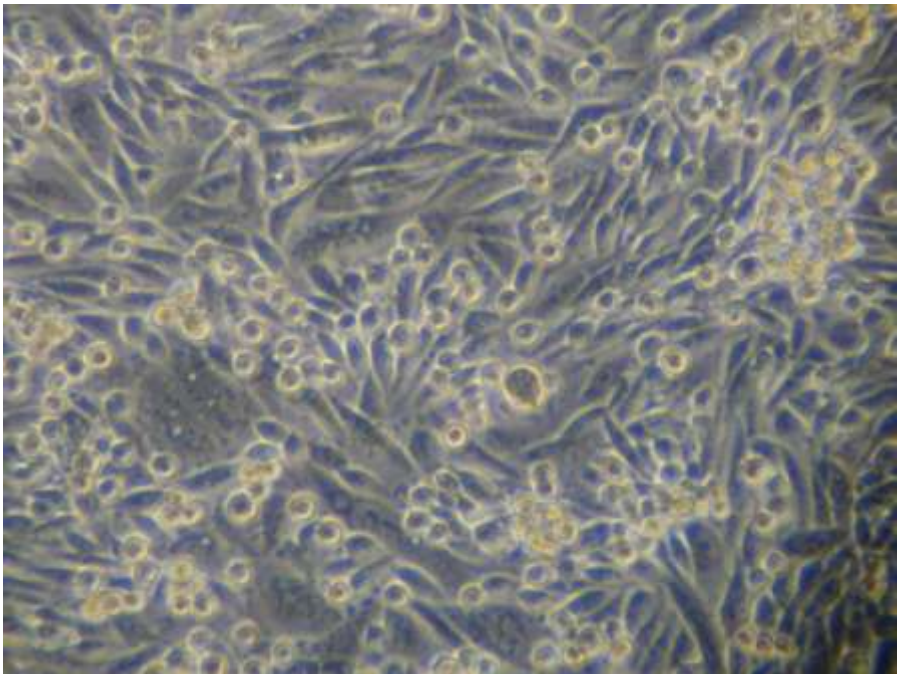

Plate 5. CHOs 24 hours after exposure to 100µL of purified GCAT extract
The cells all appear healthy. They are at a healthy density; cells are well formed and well defined with signs of ongoing mitosis.

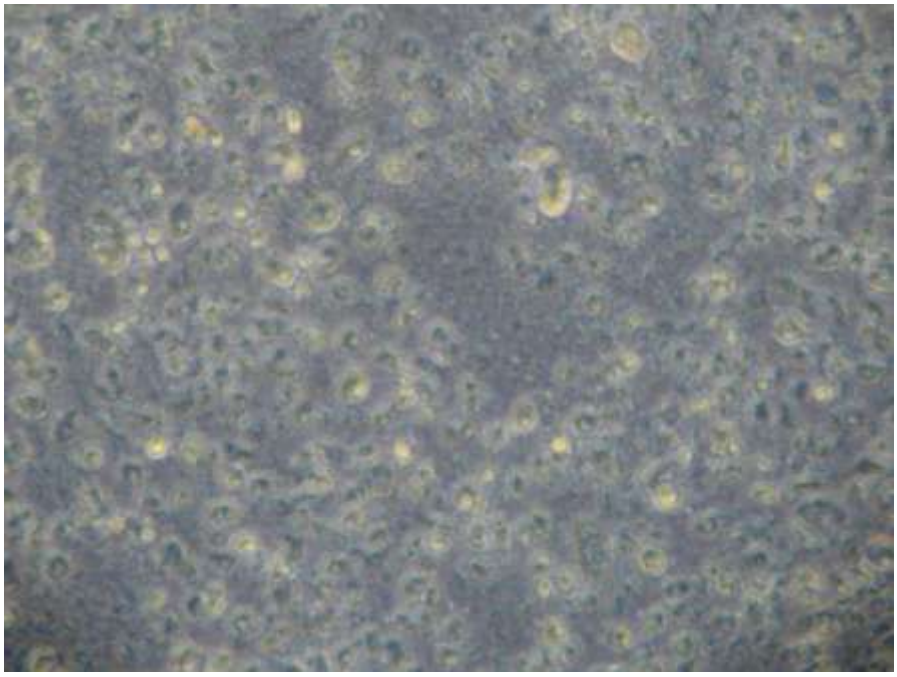

Plate 6. CHOs 24 hours after exposure to 10µL of concentrated *E. coli* supernatant
The cells all appear to have shrunk, are much less well defined but appear to be at a similar density to the control wells. This implies there has been no, or limited, lysis of the CHO cells. There is still some evidence of ongoing lysis although it is less widespread than in the control well.

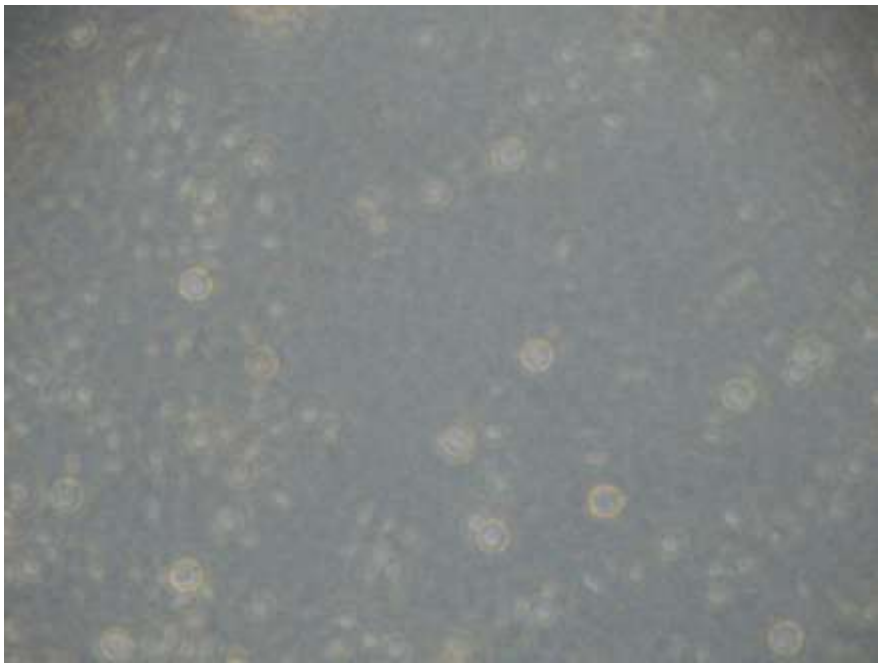

Plate 7. CHOs 24 hours after exposure to 100µL of unconcentrated LB broth
As with the CHOs that were exposed to *E. coli* supernatant, the cells appear smaller and less defined. These cells however appear at a lower density than the controls implying that some lysis has occurred. There is no evidence of ongoing mitosis, the brighter cells in this plate are simply out of focus.

3.6 Effect of cloned GCAT on RTG cells

Exposure of RTG cells to purified and filtered GCAT lead to severe damage to all the cells in comparison to the control cells (Plate 8). The cells were exposed to two different concentrations of GCAT, 50µL (Plate 9) and 100µL (Plate 10). The difference between the two concentrations is evident to see after 24 hours, the well containing 100µL of GCAT displays cells that are much less well defined, rounded and bunched up and show no signs of mitosis. The 50µL GCAT well still shows signs of extensive damage.

The RTG cells, like the CHOs, were exposed to concentrated *E. coli* (Plate 11) and unconcentrated LB broth (Plate 12) as controls. Unlike the CHOs however the RTGs were much more resilient to these compounds and showed no signs of adverse reactions to their inductions.

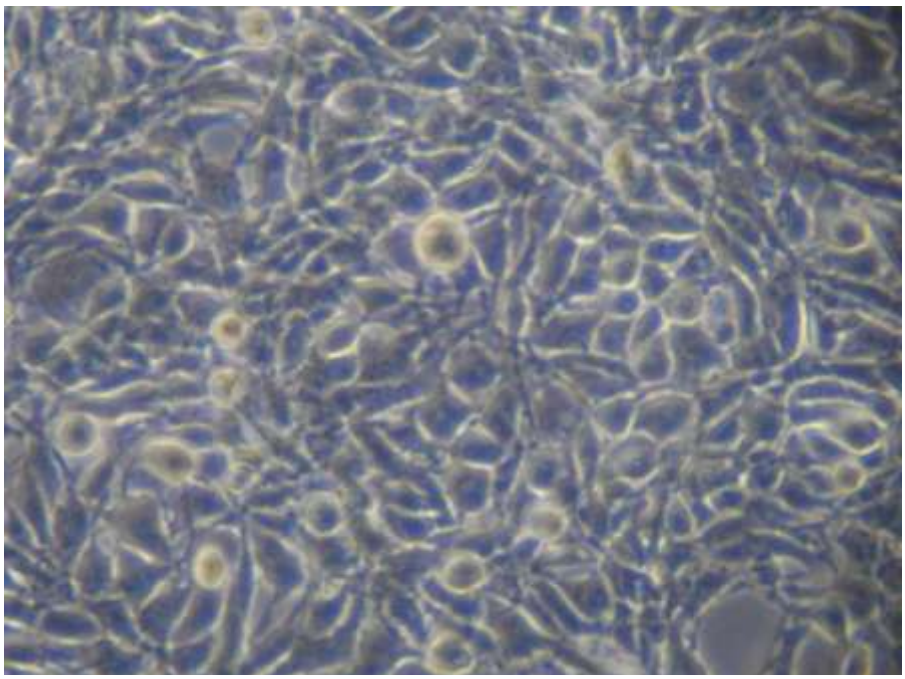

Plate 8. RTG control cells after 24 hours
These control cells all appear healthy. They are at an appropriate density; cells are all well formed with distinct borders and signs of ongoing mitosis.

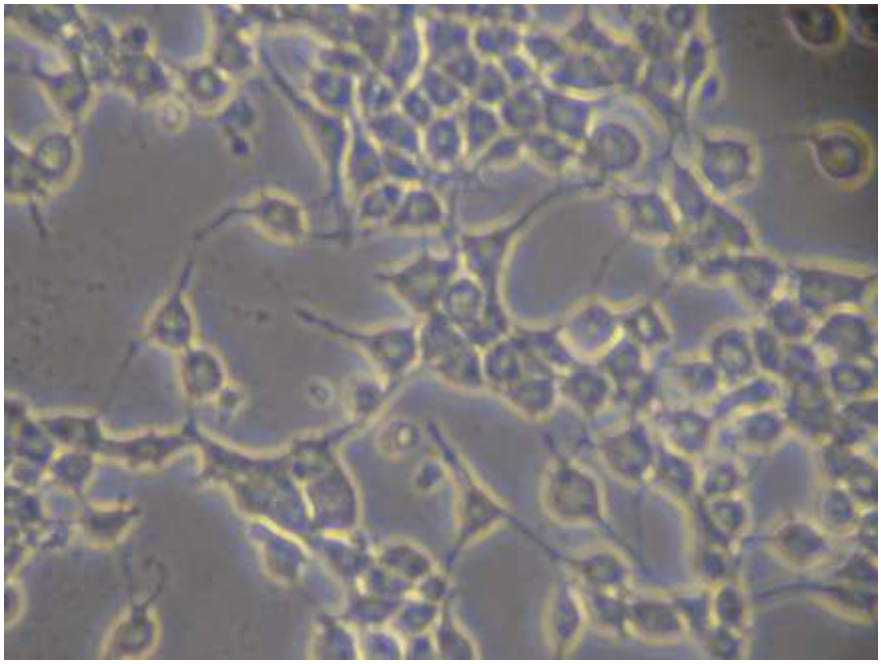

Plate 9. RTG cells 24 hours after exposure to 50µL of cloned, filtered GCAT
The cells show signs of heavy damage to most cells, however there are still some healthy cells present. They are at a much lower density than the control cells and have less well defined and improperly formed membranes. There is also no sign of ongoing mitosis

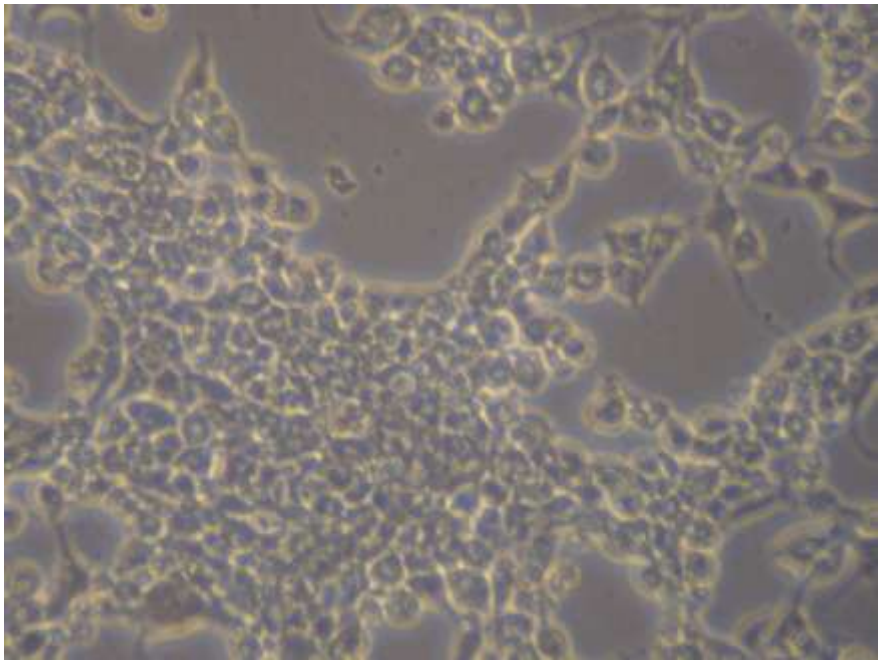

Plate 10. RTG cells 24 hours after exposure to 100µL of cloned, filtered GCAT
The cell shows signs of extensive damage to every cell in the culture. They are all shrunken, rounded and granular. They are at a lower density than the control wells, but at a higher density than the 50µL GCAT plate but have no viable cells.

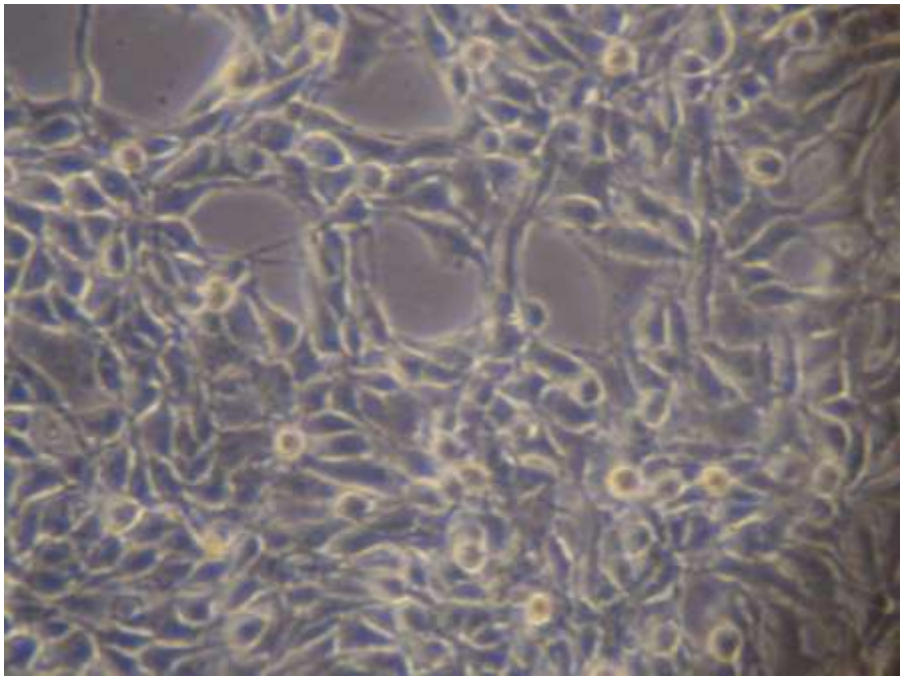

Plate 11. RTG cells 24 hours after exposure to 10µL concentrated *E. coli* supernatant
These cells, in contrast to CHOs, appear healthy. They are at an appropriate density; cells are all well formed with distinct borders and signs of ongoing mitosis

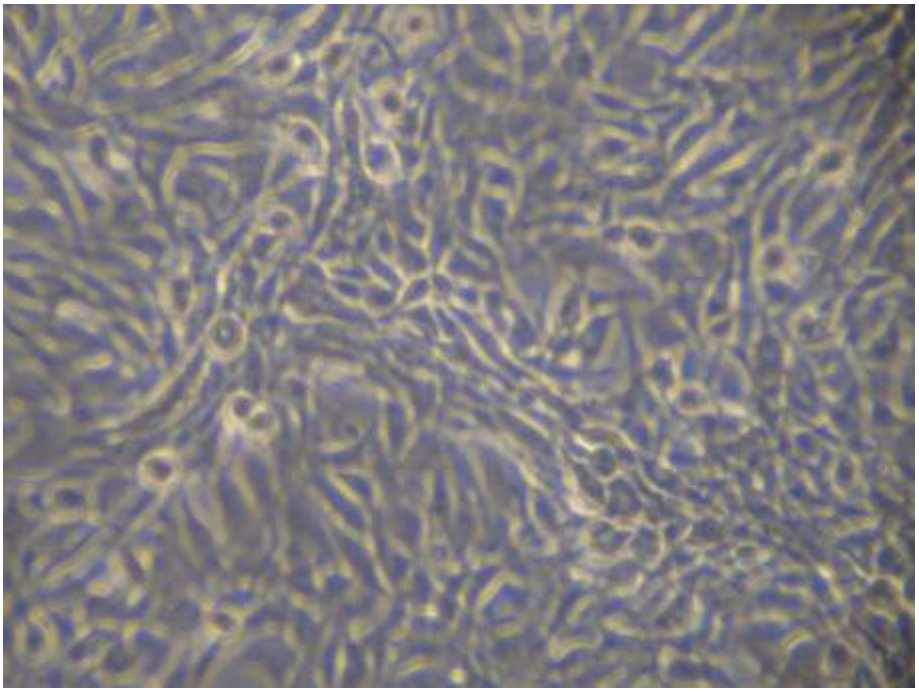

Plate 12. RTG cells 24 hours after exposure to 100µL of unconcentrated LB broth
These cells all appear healthy, all cells are similar in appearance to both the control and the *E. coli* wells.

4. Discussion

4.1 Effect of crude GCAT on GFSK and CHO cells

The crude GCAT preparation effectively lysed GFSK and CHO cells, as well as horse erythrocytes. When the genome of *A. salmonicida* was sequenced in 2008, by Reith *et al* they found genes for 15 different secreted enzymes (including one pseudogene, rendered inert by a frame shift mutation). These secreted enzymes include three other proteases, a serene protease and two different metalloproteases. Two different amylases, two different extracellular nucleases and a further two phospholipases were found. Any of these enzymes could explain why the crude GCAT preparation was able to lyse mammalian cells as well as fish cells.

As well as the secretable enzymes described above, *A. salmonicida* is capable of producing and secreting three different toxins (two more have been rendered pseudogenes by frame shift and an insertion mutation). These toxins are Aerolysin, Haemolysin and RTX-Cytotoxin. Aerolysin is known to have an effect on mammalian cells (Abrami *et al*, 1998, Nelson *et al,* 2002). Haemolysin will have an effect on erythrocytes and explains the lysis of whole horse blood. RTX-Cytotoxin is also known to have a toxic effect on mammalian cells. Broadly they fall into two categories, haemolysins and leukotoxins, both of which are capable off interacting with, and harming mammalian cells (Lally *et al,* 1997, Lally *et al*, 1999).

Further work would have to be done to discover which of these, if any, had the greatest cytolytic effect on the mammalian cells. The lysing effect of the fish cells could also have been performed by any of the above enzymes, but the GCAT enzyme has been established as the major lethal toxin in *A. salmonicida* since 1981 (Titball and Munn, 1981), so it is reasonable to assume that this is the major influence in the cell death.

4.2 Effect of cloned CGAT on RTG and CHO cells

The cloned GCAT was able to lyse the RTG fish cells but was unable to lyse, or at all affect, the CHO mammalian cells. This was the expected result for both cell types. As purified GCAT was not used, it cannot be said conclusively that it was the CGAT that lysed the RTG cells, however as was stated in **4.1** it has long been known that GCAT is the major lethal toxin involved. All that can be definitively concluded is that whatever was responsible for the lysing of the fish cells is unable to lyse the mammalian cells under the same conditions.

4.3 Effect of concentrated *E. coli* supernatant and unconcentrated LB broth on RTG and CHO cells

The CHO cells were lysed upon exposure to both concentrated *E.coli* supernatant and normal LB broth. The RTG cells were not harmed by either of the two preparations.

The fact that the CHOs were unharmed by the cloned GCAT preparations, while suffering severe damage when combined with concentrated *E. coli* supernatant suggests an anomalous result. This is because the only difference between the two preparations should have been the presence or absence of GCAT.

The one difference between the two preparations (excluding the presence or

absence of GCAT) was the amount of times the preparations were concentrated. Both the *E. coli* supernatant with GCAT and *E. coli* supernatant without GCAT were left overnight in a membrane concentrator; however they only concentrated down five times and 50 times respectively. From this, one can hypothesise that there will be a compound (compound X) in *E. coli* supernatant which has cytotoxic properties to CHO cells and is too large to be filtered out by the membrane concentrator (therefore, >15kDa). X was not at a high enough concentration in the 5 times concentrated, GCAT positive preparation and so could not have an appreciable effect on the CHO cells within 24 hours. When X was 50 times concentrated it was at a high enough concentration to have a visible effect within 24 hours.

The problem with this hypothesis is that only 10µL of the 50 times concentrated, GCAT negative *E. coli* supernatant was added, as opposed to 100µL of the five times concentrate. This means that in theory the same amount of X was added to each well. However in the GCAT negative *E. coli* supernatant, that lysed the CHO cells, the total volume of the well would have been 210µL, compared to 300µL in the GCAT positive well, so X would have been at a higher overall concentration.

Whether or not so named, X, was responsible would need further experimentation. The RTG cells were not lysed by just *E. coli* supernatant, but they were lysed by the cloned GCAT preparation. The RTG cells were also unaffected by addition of unconcentrated LB broth, while the CHO cells were heavily damaged by the addition of LB broth. This implies the presence of an endotoxin (most likely an LPS or a derivative of) that is removed by the membrane concentrator. This endotoxin would have an action against CHO cells but either have no, or reduced, effect on the RTG cells.

4.4 Conclusion and Validity of Results

The conclusions for the study are that concentrated *A. salmonicida* supernatant is capable of lysing mammalian cells and fish cells alike. Also *E. coli* with the transformed GCAT gene is capable of only lysing fish cells, and has no discernible effect on mammalian cells after a 24 hour period.

The aim of this study was to assess the feasibility of applying the GCAT enzyme as an anti-cancer treatment. For GCAT to be able to successfully differentiate between mammalian and fish cells, there must be a cellular marker, or cellular makeup, that GCAT recognises that is not present in mammalian cells but is present in fish cells (it is assumed to be present in fish cells as *A. salmonicida* is a fish pathogen and so its extracellular products must be able to positively identify fish cells).

The difference that is recognised by GCAT could be the percentage of fatty acids in the cell membrane. Mammalian cell membranes have around a 30% fatty acid make up, while fish cells typically are made up of 60% fatty acids (Gilpin, 2009, personal communication). If this is the cellular property recognised by GCAT then, in theory, a neoplastic cell that acquires a mutation that leads to an increase in the fatty acid content of the cell membrane, it could be selectively targeted and destroyed by the GCAT, along with all the progeny of the aforementioned cell.

It is already known that modification of the cell membrane content can increase the

susceptibility of cells to autolysis. In 1980, Yoo *et al*, found that an increase in fatty acid membrane composition in hepatoma cells led to increased rates of complement mediated cytolysis. The increase in lysis due to a change in osmotic potential of the cell was ruled out by a control. They also found that the degree to which susceptibility increases is relative to the change in lipid content of the cell membrane. In 1982 another paper by Yoo *et al*, demonstrated that an increase in fatty acid composition of hepatoma cells makes them more susceptible to the action of natural killer cells. They found that specific lysis of the fat enriched cells increased by 60% relative to the control ($P = <0.01$). Both of these papers by Yoo *et al* used rat hepatoma cells, which are a mammalian cell line so the results may carry over to other mammals. Both experiments were also carried out *in vitro* so it cannot be applied to normal biology as a certainty.

In 1987 Burns and Spector, carrier out an *in vivo* experiment on the affect of altering membrane content of L1210 leukaemia cells in carrying mice. They found that the increase in membrane fatty acid content was accompanied by an increase in susceptibility to Adriamycin and hyperthermia treatments. These experiments show that an increase in the fatty acid content of cell membranes can be used as a target for therapeutic intervention. However none of the papers show that the cancer cells could achieve this increase in fatty acid content without either being grown in a fat rich environment or having the host fed on a fat rich diet.

There are however a few papers that show the importance of the enzyme fatty acid synthase to the survival and proliferation of a range of cancers (Pizer *et al,* 1996*,* Pizer *et al*, 1996, Kuhajda, 2002, to name just three). Although it is not stated in the papers, it could be speculated that if a cancer cell were to acquire a mutation that increased the efficiency of fatty acid synthase then that cell would inherit a significant proliferative advantage and could be preferentially targeted by the GCAT enzyme based upon the associated increase in the fatty acid composition of the cell membrane.

4.5 Targets for further work

As only cloned GCAT was used, I would like to repeat the experiment using purified GCAT enzyme. A method for producing purified GCAT using fast protein liquid ion-exchange chromatography was outlined by Lee and Ellis, 1990. This would remove the possibility of any interference from extracellular products of *E. coli*. Further to this work I would like to test a greater range of mammalian cells to test that the lack of degradation of CHO cells is not due to a special resistance property of CHO cells.
If all mammalian cell lines proved to be immune to GCAT then I would attempt to subculture a mix of human and fish cell lines together, then expose them to purified GCAT and observe if GCAT would preferentially break down the fish cells.
If this proved successful then I would attempt to find a cancer cell line with raised phospholipid content, with which GCAT could react. Once this cell line was found, and it was established that GCAT could lyse the mutated mammalian cells, a subculture of healthy and mutated (with the raised phospholipid content) would be raised and exposed to GCAT to see if GCAT could preferentially lyse the mutated cells while not acting upon the healthy cells.

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