An investigation of the properties of anti-angiogenesis drugs as mitochondrial inhibitors; A novel mechanism of action of a new class of anti-cancer chemotherapeutics".

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Report Date 20th January 2012

This report was written in partial fulfilment of a UROS bursary scheme (2011).

Abstract
Angiogenesis is the growth of new blood vessels from pre-existing vessels, and is a fundamental step in the transition of tumours from a pre-malignant to a malignant state, which requires an increased supply of oxygen and nutrients [1]. Angiogenesis is heavily dependent on energy in the form of a chemical called ATP [2] which is made in small sub-cellular organelles called mitochondria [3]. The chemical structure of several compounds that inhibit angiogenesis in cancer have structural similarities to compounds that Dr. Bates has already shown to inhibit mitochondrial function [4,5,6], some of which have been shown to protect against cancer in man [7]. Pig heart mitochondria were prepared [4,5] and incubated with a range of concentrations (0 – 100 µM) of various anti-angiogenesis drugs for 15 minutes at 37°C to allow for drug binding. Samples were assayed for mitochondrial complex I, II-III and IV enzyme activities [4,5]. Data obtained showed that several compounds were mitochondrial inhibitors, and suggest that development of anti-mitochondrial agents for use as anti-angiogenesis drugs in cancer would be extremely useful. This undergraduate research was financially supported by the Centre for Educational Research and Development (CERD) [8] as part of the "Student as Producer" and UROS projects [9].

Introduction
Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels, and is a normal and critical process in tissue growth and development, as well as in wound healing. However, angiogenesis is also a fundamental step in the transition of tumours from a pre-malignant to a malignant state, which requires the formation of a network of new blood vessels for supply of oxygen and nutrients to new tumour tissue. In normal tissues, the balance of pro-angiogenic and anti-angiogenic growth factors and proteins favours inhibition of angiogenesis, so that as new capillaries are needed, the balance of growth factors is adjusted to stimulate vascular growth. The major critical activator of angiogenesis is a pro-angiogenic growth factor known as vascular endothelial growth factor (VEGF), and the latest treatment for a number of different tumours is Avastin™, a drug that has been suggested to block VEGF. Avastin™ and a number of other drugs significantly inhibit angiogenesis in solid tumours, but the mechanism of action of these compounds is poorly understood. However, it is known that angiogenesis is heavily dependent on mitochondrial energy supply in the form of Adenosine 5' Triphosphate (ATP), which is needed for both the DNA and protein synthesis required for the laying down of new blood vessels. The chemical structure of several compounds that inhibit angiogenesis have structural similarity with compounds that Dr. Bates has already shown to inhibit mitochondrial function [4,5,6,].

The aims and objectives of this UROS project were focused on investigation of the effects of anti-angiogenesis drugs on mitochondrial function. The demonstration by laboratory experiments that known anti-angiogenesis drugs affect mitochondrial function directly would be extremely significant and publishable, as it would add significant weight to the hypotheses put forward by Dr. Bates in previous studies that many anti-cancer drugs have in fact a primary mechanisms of action at the mitochondria, and that many anti-cancer effects are in fact due to the direct inhibition of energy supply by these drugs, rather than their commonly accepted mechanisms of action, which were defined before the advent of the study of mitochondrial function and energy metabolism in cancer.

Process
This project made use a wide range of biochemical methods, many developed by Dr. Bates and published in the papers listed in the reference list below and many other publications by Dr. Bates and co-workers.

All chemicals used were of the highest grade available and were be from Merck Biosciences, Nottingham, UK, Sigma Chemical Company, Poole, UK, InVitrogen, Paisley, UK Tocris Bioscience, Bristol, UK, or New-Use Therapeutics Limited, U.K. A range of drugs that inhibit cancer cell angiogenesis were dissolved in 100% ethanol at a stock concentration of 10 mM. For all anti-angiogenesis ligands used, the diluent (ethanol) was never present at >1.0%. Mitochondria/anti-angiogenesis drug binding experiments. Freeze thawed porcine heart mitochondria were incubated with a range of concentrations (0 – 100 µM) of anti-angiogenesis ligands (or the diluent, ethanol) for 5 min at 37°C to allow for drug binding. Samples were be then pipetted off from the anti-angiogenic drug/mitochondria suspensions and added to separate assay plates containing all the assay

components needed for the assay of mitochondrial complex I activity, mitochondrial complex II–III activity or mitochondrial complex IV activity, as previously described [4,5,6,]. Mitochondrial complex I activity, complex II-III activity and complex IV activity were be measured spectrophotometrically as previously described [4,5,6,]. Protein concentration were be determined using a modified microplate Lowry assay with bovine serum albumin as a concentration standard (0 - 200 microgramsg/mL) as described previously [4,5,6].

Statistical analysis

All experiments were repeated n = 5 - 8 times. Statistical analysis was performed using Students' paired t-tests and ANOVA followed by Dunnett's multiple comparison tests for the complex I activity and complex II–III activity measurements. Significance was attributed when $P < 0.05$.

Results

Data obtained showed that several compounds were mitochondrial inhibitors, and suggest that development of anti-mitochondrial agents for use as anti-angiogenesis drugs in cancer would be extremely useful. An example of this is shown in Figure 1 below which details the inhibition of mitochondrial complex II-III activity by the anti-angiogenesis drug Tranilast. These data are completely novel and have been accepted for presentation at the British Undergraduate Research Conference (University of Warwick, 2012).

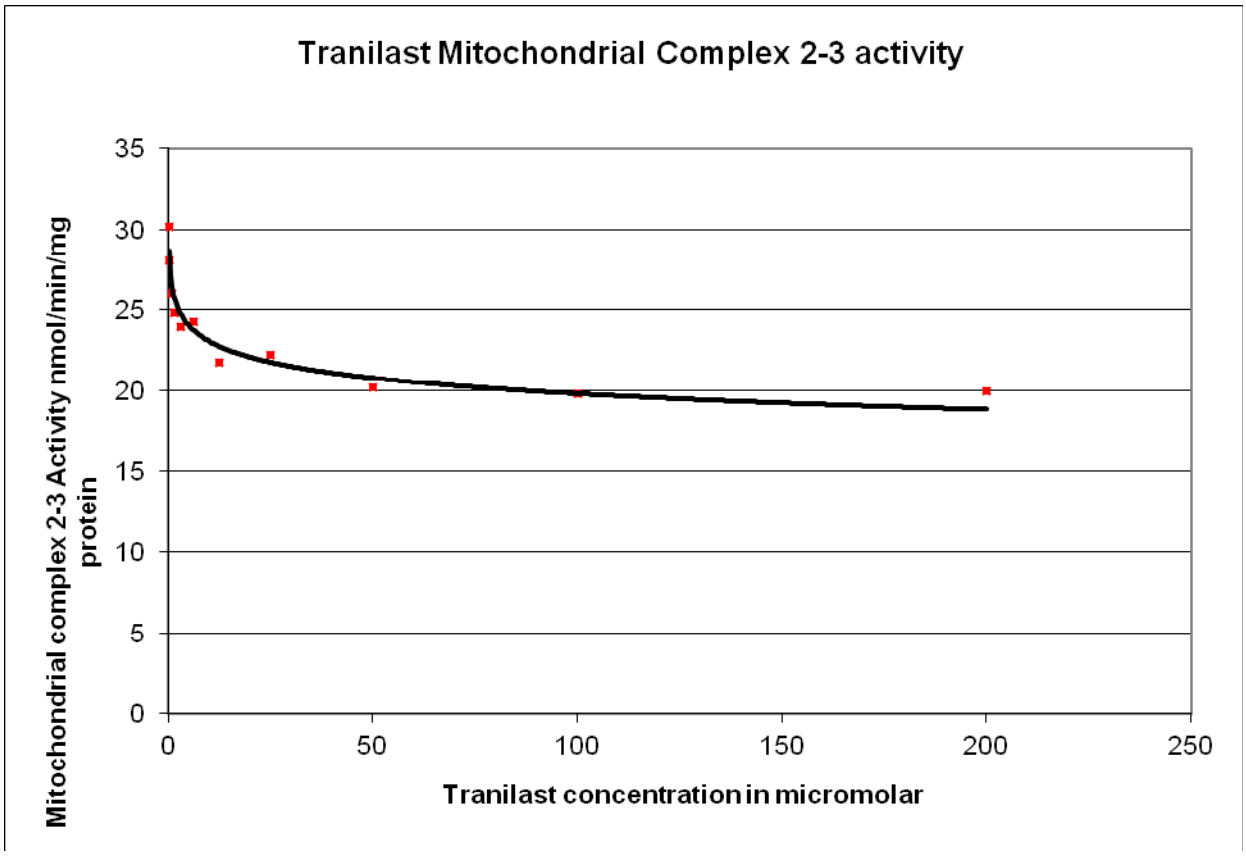

FIGURE 1 A graph showing the relationship between mitochondrial complex Ii-III enzymatic activity and the concentration of Tranilast, an anti-angiogenic drug used in cancer chemotherapy. The graph clearly shows a concentration dependent inhibition of mitochondrial complex II-III activity by Tranilast and suggests that mitochondrially mediated apoptotic cell death would be initiated in cancer cells by this (and possibly other) anti-angiogenic drugs.

Summary

The data we obtained confirm the initial main hypothesis of this project, as we have shown that compounds with supposedly established mechanisms of action as anti-angiogenic compounds are inhibitors of mitochondrial enzyme activity. This has major implications for the design of novel anti-cancer drugs as the established mechanisms of action of anti-angiogenic drugs may in fact be due to inhibition of mitochondrial energy metabolism rather than their effects on cytoskeletal proteins. These data are likely to strongly influence the decisions made by pharmaceutical companies in their medicinal chemistry projects, as they strongly suggest that major efforts should be made to design molecules with the ability to bind to and inhibit mitochondrial enzymes, rather than inhibit cytoskeletal protein synthesis and assembly**.**

Conclusions

The major conclusion of the project is that anti-angiogenic drugs such as Tranilast have potent anti-mitochondrial activities, which may be the major route by which these compounds initiate cancer cell death by type 2 (mitochondrial) apoptosis/programmed cell death

This work has been accepted to be presented at the British Conference for undergraduate research (2012) at the University of Warwick on the 19th-20th March 2012 (see http://www.bcur.org/conference/ for details).

Future Work

Dr. Bates is continuing the work detailed in this report in collaboration with the two UROS students (Sophie G. Cowper and Tammy E .Wiltshire), his 3rd year Biomedical Sciences 3rd year B.Sc. project students and an Erasmus student from Holland. This work will be written up for publication in an internationally competitive scientific Journal with an impact of at least 2.5 [8].

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Acknowledgments
We are extremely grateful for the help and support of Dr. Julian Bartrup (Technical Services Manager, School of Life Sciences) and Mrs. Angela Murtagh and Mrs. Beverley (Biomedical Sciences Technicians, School of Life Sciences) whose help has been invaluable in facilitating the research detailed in this report.

We are also grateful for the support of Professor Mike Neary and the Centre for Educational Research and Development, University of Lincoln, (CERD) [9] as part of the “Student as Producer” and UROS projects [10].