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Award Number: W81XWH-06-1-0496

TITLE: Killing Breast Cancer Cells through Activation of the Apoptosome

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REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2008	Annual	8 May 2007 – 7 May 2008

4. TITLE AND SUBTITLE

Killing Breast Cancer Cells through Activation of the Apoptosome

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0496

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Duke University
Durham, NC 27708

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cells die in response to chemotherapeutic agents by activating a mitochondrial cell death pathway. This pathway leads to activation of proteases known as caspases through the release of cytochrome c from mitochodria. Once released, cytochrome c engages a cytoplasmic receptor known as Apaf-1, which oligomerizes and activates caspase 9. We demonstrated that breast cancer cells have apoptosomes which are hypersensitive to cytochrome c. Thus we have proposed to develop cytoplasmic variants of cytochrome c and/or small molecule cytochrome c mimetics to activate the apoptosome in breast cancer cells.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	7	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

In response to chemotherapeutics, breast cancer cells initiate a cell death program culminating in the release of cytochrome c from mitochondria, binding of cytochrome c to a protein known as Apaf-1, and consequent Apaf-1-induced activation of a cell death protease known as caspase 9. We had reported previously that breast cancer cells were more sensitive to cytochrome c-induced apoptosis than their normal counterparts. Based on these findings, we wished to develop cytochrome c mimetic therapeutics for the treatment of chemoresistant breast tumors. We proposed both to modify cytochrome c itself as a potential therapeutic and screen for small molecules that might mimic cytochrome c.

Our original statement of work contained the following aims:

Task 1: Construction of cytochrome c and heme lyase variants allowing the production of cytoplasmically active cytochrome c (months 1-6)

Task 2: Testing of cytoplasmic cytochrome c/heme lyase in tissue culture and mice (months 12-20)

Task 3: Identification and testing of small molecule post-mitochondrial caspase activators (months 21-36)

As detailed in last year's report, we began with **Task 3** and isolated several potentially interesting small molecules that promoted cell death. Unfortunately, these compounds turned out to kill cells in an Apaf-1-independent manner. While these may be of interest to pursue, they are not likely to be breast cancer selective in their killing. We have now prepared additional cytosolic lysates and have introduced a secondary screen using Apaf-1 null lysates (in which compounds of interest should not induce caspase activation).

In an alternative approach, we have also developed a novel single cell screen for regulators of caspase activity using the near-infrared caspase activity indicator (the caspase-3 IRDye, available from LI-COR Biosciences Inc) and the Xenopus oocyte system. Injection of these large cells is technically simple and thousands of cells can be injected in 4-5 hours. As shown in Fig. 1, oocytes injected with this indicator and cytochrome c very rapidly cleave the substrate to produce an infrared signal detected by the LI-COR Odyssey reader. This signal is quite robust and enduring; caspase activation could be detected as little as 3 minutes after injection and signal persisted for 24 hours or more (Fig. 2). Similar results were obtained when we injected the pro-apoptotic Bcl-2 family member, t-Bid. Moreover, the signal was inhibited by co-injection of oocytes

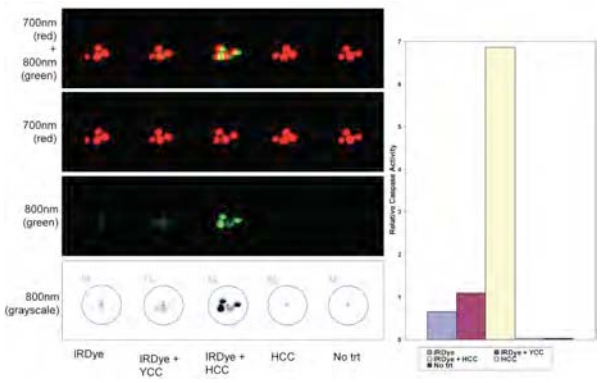

Figure 1. Fluorescence in oocytes co-injected with the IRDye® and cytochrome *c*. Oocytes were injected with either IRDye, IRDye and yeast cytochrome *c* (YCC), IRDye and horse cytochrome *c* (HCC), HCC alone, or nothing (No trt). Oocytes were imaged using the LI-COR Odyssey® thirty minutes after injection. Resulting images are shown on the left, demonstrating oocyte autofluorescence in the red channel (700nm) and IRDye signal in the green channel (800nm). 800nm signal from each group of oocytes was quantitated in the grayscale image (blue circles), and a graph from the obtained values is displayed on the right. As shown, significant signal is obtained only oocytes co-injected with IRDye and HCC.

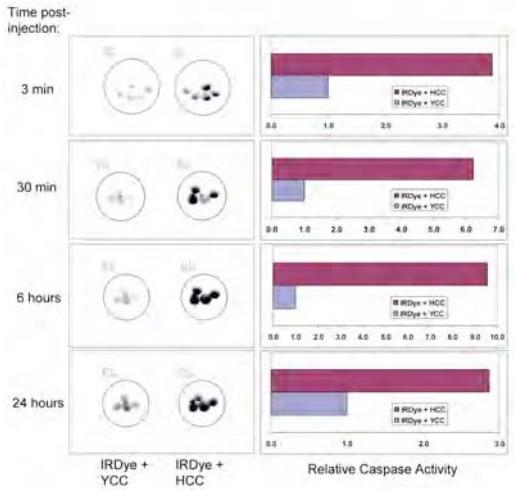

Figure 2. Oocyte fluorescence can be detected from three minutes to twenty-four hours after injection. Oocytes were microinjected with either IRDye + YCC or IRDye + HCC. Images of the injected oocytes were obtained on the LI-COR Odyssey® over time. Quantitated grayscale images of the 800nm signal are shown on the left for several time points, with corresponding graphs displayed on the right. Significant 800nm signal is obtained from as early as three minutes post-injection and is maintained for at least twenty-four hours selectively in the HCC-injected oocytes.

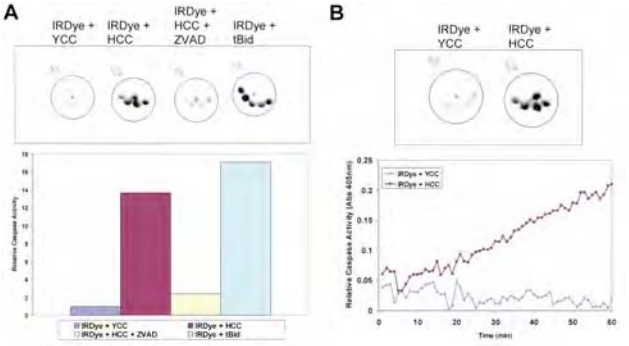

Figure 3. Fluorescence in IRDye-injected oocytes is due to activation of effector caspases. A, Oocytes were injected with either IRDye + YCC, IRDye + HCC, IRDye + HCC + the general caspase inhibitor ZVAD-FMK (ZVAD), or IRDye + a truncated form of Bid (tBid). A quantitated grayscale image of the 800nm signal and the corresponding graph are displayed. **B**, Oocytes injected as indicated (top) were flash-frozen thirty minutes following injection, lysates were prepared and caspase activation was measured using a colorimetric substrate (bottom).

with the caspase inhibitor zVAD-fmk (Fig 3). We have acquired a library of bioactive compounds and intend to test groups of these (in pools) by manual injection to determine if we can identify pools that either rapidly induce apoptosis or that inhibit cytochrome c-induced apoptosis. Pools would then be deconvoluted by breaking down into smaller pools, etc. This is far more labor intensive than the automated screens we have ongoing, but this may be a more sensitive assay, allowing us to detect novel small molecule apoptotic modulators more readily.

During this past year, we have been focused on **Task 1** to produce cytochrome c and heme lyase variants that are cytoplasmic. Initially, we sought to do this systematically through the identification of residues important for mitochondrial import of these proteins. However, careful reading of the literature suggested that mutations disrupting mitochondrial import might also disrupt protein function. Therefore, we took a distinct approach, making fusion proteins that would alter either the N- or C- termini of the proteins, hoping that these might impede import, while allowing retention of function. Towards this end, we utilized an EGFP-cytochrome c, a cytochrome c-EGFP, an mCherry-heme lyase. These fusions offered the added advantage that we could visualize these proteins through fluorescence microscopy and/or sort cells expressing these proteins by fluorescence activated cell sorting. Interestingly, it had been reported previously that GFP-cytochrome c and cytochrome c-GFP could localize to mitochondria. However, we found that this was true in only a minor subset of cells expressing this construct. Rather, the majority was cytoplasmic (Fig. 4). Simlar results were obtained with mCherry-heme lyase (see red cells in Fig. 5). We have begun experiments described in **Task 2**, expressing the cytoplasmic variants of cytochrome c and heme lyase in a variety of cell types to determine if this induces apoptosis. Preliminary results are promising; we see green

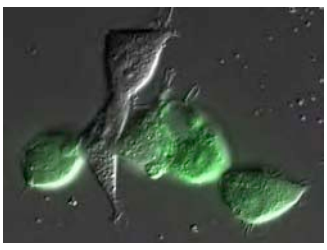

Figure 4. Cytosolic expression of cytochrome c fused to EGFP. HeLa cells were transfected with a construct encoding cytochrome c-EGFP and then visualized twenty-four hours later using fluorescence microscopy. Green fluorescence is detected throughout the cytosol in transfected cells. A similar staining pattern is observed in cells expressing EGFP-cytochrome c.

cells and red cells singly infected with these constructs that appear healthy. However, yellow cells (shown in Fig 5) appear to exhibit apoptotic morphology. These experiments will be repeated and we will also confirm, via caspase assays, that the morphology is truly indicative of apoptotic cell death. If these results appear promising, we will proceed to the originally described animal experiments.

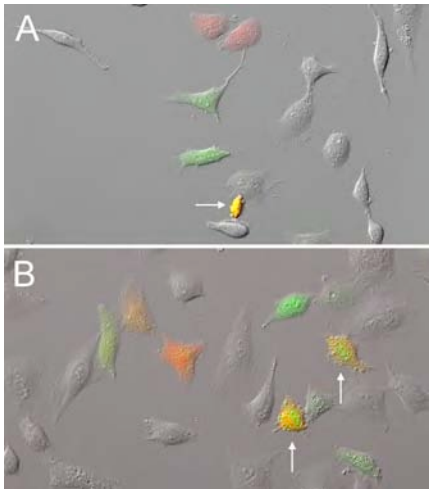

Figure 5. Apoptosis of cells co-expressing cytochrome c-EGFP and mCherry-heme lyase. HeLa cells were transfected with constructs encoding cytochrome c-EGFP and mCherry-heme lyase. Cells expressing either the cytochrome c (green) or heme lyase (red) appear healthy, while cells expressing both constructs (yellow; white arrows) demonstrate apoptotic morphology with membrane blebbing.

Currently **no reportable outcomes**, but we have developed the necessary tools to proceed.

Key research accomplishments:

- Generation of cytoplasmic cytochrome c
- Generation of cytoplasmic heme lyase
- Initial assays on co-expression of cytoplasmic cytochrome c and heme lyase
- Development of a new assay for small molecule screening

Conclusions: We have developed the tools detailed in Task 1 and will proceed to the phenotypic testing of these constructs as detailed in Task 2, while continuing our original Task 3 experiments.