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Abstract

Fas-mediated signaling pathway is an important mechanism for apoptosis in a variety of cells, including cholangiocarcinoma and other cancer cells. The formation of death inducing signaling complex (DISC) is a critical step for Fas-mediated signaling. Recent experimental studies showed that calmodulin (CAM) binds to Fas and regulates Fas-mediated (DISC) formation and the binding of (CAM) to Fas is inhibited by the (CAM) antagonist, trifluoperazine (TFP). The present study sought to characterize (CAM)/Fas binding affinity and the effects of Fas mutations and the effects of (CAM) antagonist, trifluoperazine (TFP), on (CAM)/Fas binding affinity. The results will be valuable to further elucidate the role of (CAM) in Fas-mediated death inducing signaling and provide a molecular mechanism for drug design of apoptosis mediators via (CAM) antagonism in the future. Isothermal Titration Calorimetry (ITC) binding experiments were used to quantify (CAM)-Fas binding. Recombinant (CAM) and Fas DD proteins were expressed in Escherichia coli cells and purified. Purified (CAM) and Fas were gel-filtered prior to ITC experiments. We have quantified the wildtype (CAM)/Fas binding with ITC experiments. We will further quantify the binding of (CAM)/Fas V254N mutant and the binding of (CAM)/Fas C-terminal deletion mutant to determine the effects of Fas mutations on (CAM)/Fas binding. The effects of (CAM) antagonist, TFP, on (CAM)/Fas binding will be also determined with ITC experiments. Results from these studies will facilitate the identification of novel strategies and drugs capable of effectively regulating Fas-mediated apoptosis in cancer cells.

Introduction

(CAM)

Calmodulin is a cytoplasmic protein with a high affinity for calcium ions (Ca^{2+}). (CAM) functions as an intracellular mediator of biological pathways and requires a certain concentration of intracellular calcium for signal transduction to occur. Higher expression of Ca^{2+} bound (CAM) has been associated with cancer [1, 2]; furthermore, the Ca^{2+}-(CAM) plays a key role in the propogation cholangiocarcinoma cells via inhibition of the Fas-Mediated apoptotic pathway [3]. Previous studies have shown that (CAM) binds to the Fas on its cytoplasmic death domain (DD) [10], specifically on residues 231-254 of the Fas [11]. Also it has been recently demonstrated that (CAM) is recruited into the (DISC) formation [2]. Computational modeling and simulations inspected the binding thermodynamics and conformation of the (CAM)-Fas complex, and provided evidence for the structural role of (CAM) and Fas binding in Fas induced (DISC) formation and ultimately Fas mediated apoptosis [5].

Fas

The Fas Receptor (Fas), alternatively known as APO-1/CD95, is a type-1 membrane protein categorized into tumor necrosis factor family [1]. The Fas is a key component in the Fas regulated apoptotic signaling pathway that regulates apoptosis in a variety of Fas expressing cells. Activation of the Fas occurs via the binding of the Fas ligand protein (FasL) or Fas agonistic antibodies [1].

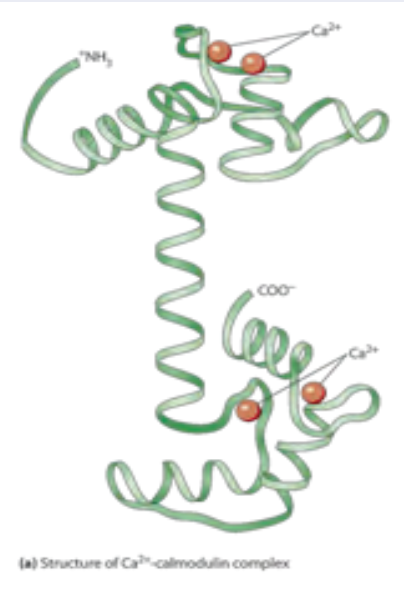


Figure 1: A structure of Calmodulin binding with four Ca2+ ions.

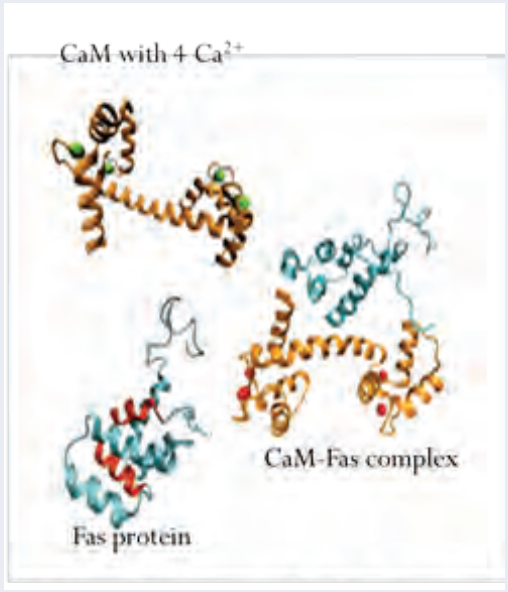


Figure 2: Molecular structures of CaM, Fas, and CaM-Fas complex generated via Visual Molecular Dynamic (VMD)

Upon activation, the Fas conveys the ligand signal by recruiting and activating the Fas-associated death domain protein (FADD), which induces the formation of the death-inducing signaling complex (DISC) composed of certain effector caspases and FLICE-like inhibitory protein (FLIP). These effector caspases activate pro-apoptotic proteins downstream in the pathway leading to apoptosis of the cell [2, 6]. Atypical expression or mutation of the Fas in cell types is associated with the propagation of a variety of diseases including lymph proliferative syndrome [5], and several types of cancers: breast cancer, hepatocellular carcinoma, and glioma [2].

Breast Cancer

A 2009 publication by the American Cancer Society ranked cancer as the second leading cause of mortality in the United States [7]. 15 percent of these cancer deaths were in females who had developed breast cancer [7]. These statistics make breast cancer treatment imperative to the improvement of patient survival rates. In April of 1997, the Federal Drug Administration approved the use of trifluoperazine (TFP), an organic phenothiazine, for cancer treatment [8]. The antipsychotic drug has been shown to inhibit the function of protein regulators in the Fas-associated death domain (FADD), and in vitro studies have revealed that continuous exposure to TFP inhibits the growth of breast cancer cells [9,10,11].

(CAM) and breast cancer

The primary protein that TFP affects is calmodulin (CAM), a small (Mr≈16700, ca.148 amino acids) acidic protein consisting of four calcium (Ca^{2+}) binding loops surrounded by α-helix structures [9]. When calcium levels reach a certain point, the three-dimensional structure of (CAM) opens up, allowing the protein to preferentially bind other molecules and activate cell signaling pathways [12]. One crucial point in Fas-mediated apoptosis is (CAM) binding to Fas ligand (FasL). Data from proton NMR studies and micro calorimetric titrations suggest that TFP induces structural changes to the Ca^{2+} bound (CAM) upon binding which could further affect (CAM) binding to other proteins [4, 7]. Affinity chromatography has been used to find the dissociation constant, Kd, of (CAM)/TFP binding to be between 4.5 and 5.8 [8]. Another group of researchers have found Kd to be 4.73 using back-scattering interferometry (BSI) [9]. Although different methods of experimentation have demonstrated that TFP affects (CAM)-Fas binding, no quantitative data characterizing the full binding kinetics of (CAM)-Fas/TFP binding has been reported.

Fas and breast cancer

In 1996, the National Cancer Institute (NCI) discovered that several breast cancer lines from original MCF-7 line are resistant to Fas-mediated apoptosis. Further research proved that Fas and its apoptotic pathway are down-regulated in breast cancer patients [10]. Current hypothesis suggest that tumor progression may be aided by decreased Fas sensitivity; therefore, modulation of Fas expression may be an approach to induce apoptosis in breast cancer cell [11].

Aims of the current study

This study aims to characterize (CAM)/Fas binding affinity and the effect of Fas mutations and the effect of (CAM) antagonist, trifluoperazine (TFP), on (CAM)/Fas binding affinity. It was hypothesized that (CAM) antagonist, TFP, would bind to (CAM) alone, and inhibit (CAM) binding to Fas in a TFP-concentration dependent manner. To test this hypothesis, this study will characterize (CAM)/Fas binding and the effect of TFP at different concentrations on (CAM)-Fas binding, in vitro, with Isothermal Titration Calorimetry (ITC) experiments.

The results of this study will lay the basis to clarify the cell signaling pathway by which TFP acts to inhibit malignant tumor growth. These findings will have a direct impact on cancer research, and specifically breast cancer mainly because knowledge about the pathway will allow modifications with other enzymes and in such create strong cell signals that will help pinpoint the exactly location of a cancer cell and thereby eradicate it using drug therapy. Understanding the molecular mechanisms of how TFP influences protein interactions will enable scientists to optimize the compound and design the new drug candidates so that the drug can act more effectual and side effects can be minimized. Future studies will characterize the effect of TFP on (CAM)-Fas binding in regulating the formation of the death-inducing signaling complex (DISC) to modulate apoptosis in breast cancer cells.

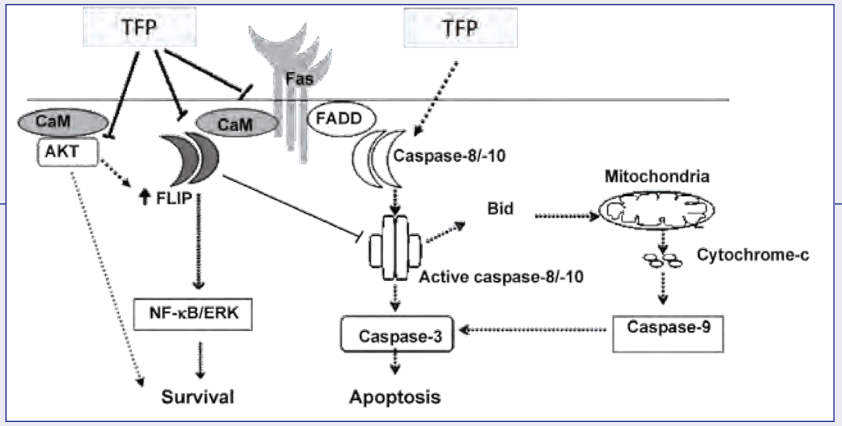


Figure 3: Hypothesized mechanism involved in CaM-Fas-TFP apoptotic pathway. This pathway can be used for survival as well as cell death.

Materials and Methods

Computational molecular simulation and biochemical techniques reported that (CAM) binds to residues 231-254 on Fas [11]. A recombinant protein His-Sumo-Fas 191-335 along with a recombinant His-Sumo-(CAM) protein will be used in ITC experiments. The molecular weight of 6XHis-Sumo-Fas (30621.71g/mol) and 6XHis-Sumo-Fas (30429.07 g/mol) were calculated using ApEA plasmid Editora Tcl/Tk script written by M. Wayne Davis Copyright 2003-2004.

Bacterial cell culture

Kanamycin and chloramphenicol-resistant Rosetta Escherichia coli cells line plasmids were transformed with the full human (CAM) protein sequence (pET28-His-SUMO-(CAM)) and the FADD-binding portion of Fas protein (pET28-His-SUMO-Fas 191-335-1). Separate 1 L bacterial cultures of 6XHis-SUMO-(CAM) and 6XHis-SUMO-Fas were inoculated with 20 mL starter cultures respectively, 1M kanamycin sulfate, and 0.3M chloramphenicol. Cultures were grown in suspension at 37°C. Once an optical density measured at 600nm reached approximately 0.6 units, 1mM isopropyl α-D-1-thioglactopyranoside (IPTG) was injected into cultures to begin overexpression of target protein. Induction lasted 3.5 hrs at 37°C after which cultures were incubated at 4°C overnight and spun down into cell pellets at 4200 rpm for 25min the following day.

Bacterial cell lysis, purification, and storage

Lysis buffer composed of 20mM Tris base, 10mM imidazole, and 300mM NaCl was adjusted to pH 8.0 with HCl and cell pellets were resuspended in 80 mL buffer per liter cultured. Cell suspensions were French pressed once at 1000 psi, 5000 psi, 10000 psi, and three times at 15000 psi. Cell lysate was centrifuged 30min at 13200 rpm. Supernatant containing soluble proteins was then filtered and purified the same day. Proteins carrying the 6XHis-SUMO tag were purified using nickel-nitriloacetic acid (Ni^{2+}-NTA) affinity chromatography and eluted into pH 8.0 buffer composed of 20mM Tris-HCl, 250mM imidazole, and 300mM NaCl. Elution purity was further increased by gel filtration, then quantified using a Pierce Bicinchoninic acid (BCA) assay, and concentrated. Concentrated protein samples stored for more than 1wk were flash frozen at -80°C and quickly thawed with a 20°C water bath when needed; however, samples used for ITC experiments within 1 week were kept at 4°C.

Protein tag cleavage

Elution samples used for without-tag experimental runs were dialyzed into pre-cleavage buffer following purification. Pre-cleavage buffer was composed of 20mM Tris-HCl, 10% glycerol, pH 8.0 and dialysis was performed at 4°C for 24 hrs. Buffer was changed at least twice to effectively remove imidazole. SUMO protease was added at 1unit/10μg target protein and incubated at 37°C for 1 hr in 20mM Tris-HCl, 1mM Tris(2-carboxyethyl)phosphine (TCEP), pH 8.0 (cleavage buffer). Protein was then re-purified on Ni^{2+}-NTA columns to remove tag pieces, subjected to gel filtration, and quantified using BCA.

Other methods and materials

Twelve percent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify 1) protein expression after culture, 2) sufficient bacterial cell lysis after French press, and 3) protein purity after purification. Dihydrochloride trifluoperazine was purchased from Fisher Scientific. A MicroCal VP-ITC calorimeter was used in conjunction with MicroCal Origin data analysis software to perform ITC experiments.

ITC determines binding thermodynamics and kinetics

ITC plays an important role in finalizing the characterization behind the (CAM)-Fas binding by providing quantitative data in regards to thermodynamics and kinetics of the interactions. Furthermore, ITC has become a perfect choice in determining the binding energies of protein-protein and protein-ligand interactions. ITC is also capable of measuring the magnitude of the two thermodynamic components that contribute to binding affinity, enthalpy and entropy. The result of ITC is an Isothermal plot of Kcal/mole (injectant) vs molar ratio of injectant to macromolecule in the cell.

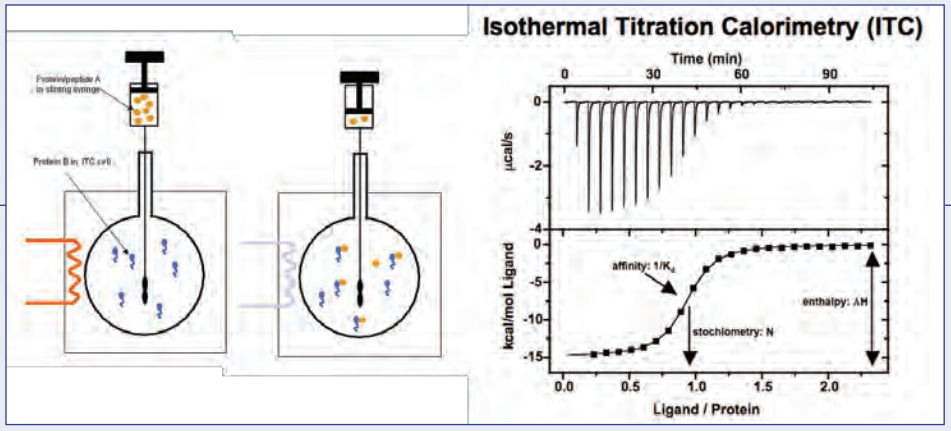


Figure 4: Isometric Calorimetry method and result format. Injection into ITC machine (left), data delivered from a standard ITC run right.

ITC Experimental controls

1. Controlled for the effect of the 6xHis-SUMO tag.

Protease was used to cleave the 6xHis-SUMO tag at the SUMO recognition site from 5 mg of (CAM) and 5 mg of Fas protein. These untagged samples underwent the same experimental conditions as tagged samples for the (CAM)/Fas and (CAM)/TFP binding reactions so that any possible effect of the tag on protein binding may be observed.

2. Establish positive and negative control for experimental cases:

a. Case #1: (CAM)/Fas binding
b. Case #2: (CAM)/TFP binding
c. Case #3: (CAM)-TFP/Fas binding

Positive Control: Because calcium binding has been shown to promote the active form of (CAM) protein, interactions between Ca^{2+}-saturated (CAM) and ligand in the absence of any drugs were used as the positive control in each of the three cases [7]. Protein interaction between (CAM) and ligand (Fas or TFP) in the absence of calcium and any drugs were used as the positive control. A solution of 100mM Ca^{2+} and 5mM (CAM) was used for this scenario.

Negative Control: The common chelating agent, ethylene glycol tetraacetic acid (EGTA), was used to strip calcium molecules from (CAM). A calcium-devoid scenario served as the negative control in all three cases. Calcium-devoid protein interaction served as the negative control in all three cases. A solution of 400mM EGTA and 5mM (CAM) was used for this scenario. These controls are important since calcium has been shown to increase the enthalpy of (CAM) reactions during ITC experiments [11].

3. Control for heat of dilution.

The ligand was inserted into the sample cell full of buffer without the presence of the macromolecule. The heat of dilution was found from this reaction. The heat of dilution is normally small and negligible, but it was checked anyways as a precaution. If heat of dilution is found to be signiticant, it is because the ligand dimerized or aggregated with itself. If it is found to be significant, it is because the ligand has dimerized or aggregated with itself. The heat of dilution should be subtracted from the heat of reaction found in experiments when ligand is injected into sample cell with macromolecule present.

4. Controlled for complex binding.

The backward and forward reaction kinetics were determined by simply reversing the ligand/macromolecule roles during ITC runs. For instance, in Case #1, (CAM) was put in the reference cell and Fas was injected into the cell as the ligand. Once the binding kinetics for that experiment took place, Fas was put in the reference cell and (CAM) was injected into the cell as the ligand. Unless complex binding is taking place, the backward and forward reaction kinetics should be the same.

ITC sample preparation

1. purified samples of (CAM) and Fas protein were separately eluted and then dialyzed against buffer consisting of(CAM) and Fas protein and then dialyze them against buffer consisting of 20mM Tris base, 1M NaCl, and 250mM imidazole. Samples of TFP were dissolved into the same buffer. Once all solutes were dissolved, each buffer solution was checked for a pH of 7 and subsequently equilibrated to pH 7 if needed.

2. The sample cell was adjusted to the desired temperature of 30°C to allow it to equilibrate while samples of macromolecule and ligand were individually stirred at medium speed and placed in the ThermoVac for 5-10 mins at 30°C.

3. Once bubbles were no longer visible in the samples, the knob on the ThermoVac (bleeder) was twisted to allow gas pressure to equilibrate and release the cap.

4. After cleaning reference cell and flushing the syringe with DI H_2O, the ligand buffer was positioned in the holder beneath the syringe on the outer ITC machine, and "Open Fill Port" was selected on the ITC software.

5. The syringe was filled with ligand, avoiding formation of air bubbles. "Close Fill Port" was selected.

6. The reference cell was slowly loaded with the macromolecule, ensuring that the cell was full and no bubbles had formed. The syringe was slowsly twisted to dislodge any bubbles that may have formed.

7. The run times parameters were set, "Run" was pressed, and the ITC machine was allowed to establish a baseline ΔH° and run.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Pre-induction as well as Post-induction samples were taking via SDS-PAGE.

ITC runtime parameters

Table 1 shows a list of runtime experimental and injection parameters to be held constant at all times.

Table 1: ITC constants

Experimental Parameters		Injection Parameters	
Total # injections	10	Volume (µL)	8
Cell temperature (°C)	30	Duration (sec)	13.7
Reference power (µCal/sec)	15	Spacing (sec)	150
Initial delay (sec)	200	Filter period (sec)	2
Stirring speed	260		

Purification

The (CAM) and Fas proteins were purified using Ni^{2+}-NTA (nickel nitriloacetic acid) affinity chromatography. In the first step, all proteins that were not 6xHis-tagged flowed through column while proteins with the tag bound to resin. In the second step, the bound proteins were washed to eliminate inclusion body remnants and other unwanted materials. Finally, elution exposed that bound proteins are unbound from resin with high concentrations of imidazole and collected for dialysis into appropriate ITC buffer.

Preliminary Results

Recent ITC trials were ran on (CAM)-Fas and (CAM)-TFP binding. Although validation of the binding mechanism is still being studied, the results of the ITC trials that were run on (CAM)-Fas and (CAM)-TFP binding validate that validates that 1) there is determinant binding between (CAM) and Fas, and 2) there are multiple binding points in the (CAM)-TFP complex, as seen in figures 8 and 9 respectively.

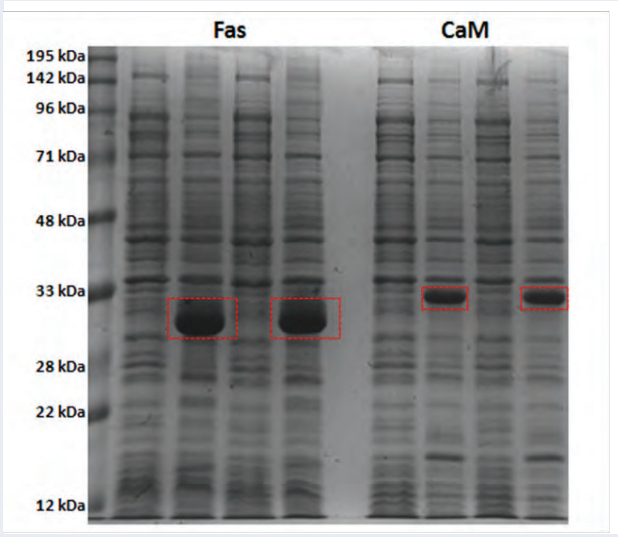


Figure 5: SDS-PAGE results of samples from 1L bacterial cultures of Fas and CaM protein, pre and post induction (2.1.11).

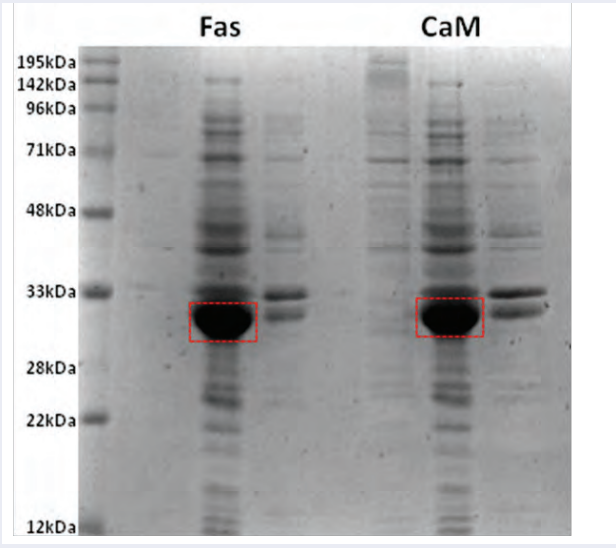


Figure 6: SDS-PAGE results of samples from 1L bacterial cultures of Fas and CaM protein, pre and post cell lysis.

Discussion

While still preliminary, the results from this ITC study will contribute to the current literature on (CAM)/Fas binding and the effect of TFP on (CAM)/Fas binding by shedding light onto the following parameters: 1) the number of binding sites for ligand to macromolecule, n, 2)the change in heat capacity, ΔCp, 3) the standard change of enthalpy, ΔH°, 4) the standard change of free energy, ΔG°, 4) the standard change in entropy, ΔS°, and 5) the number of binding sites for ligand to macromolecule, n. Because most biological reactions are exothermic and follow the rules of thermodynamics, a negative change in enthalpy is expected along with a positive change in entropy in each of the binding cases.

Possible sources of error

If the stoichiometric relationship between the (CAM)-Fas macromolecule and TFP ligand is not indicative of at least a 1:1 binding ratio (i.e., no binding occurs), then four possible sources of error should be considered: 1) All the ligand binding sites on (CAM) are not identical and independent, 2) the expressed protein is not thoroughly pure, 3) the calculated protein concentrations are not correct, and 4) all of the protein used was not properly folded and active.

Other potential sources of error stem from the use of bacteria to express human genetic material encoding for the proteins, Fas and (CAM), discussed in this study. It must be noted that human expression of these proteins may differ from bacterial expression resulting in alterations in protein conformation, configuration, and function. No true or quantitative testing of protein purity, activity, or functionality was performed. It should also be noted that only the FADD-binding portion of Fas protein (res191-335) was used to complete this study; therefore, the binding effects of (CAM) and full, trimerized Fas protein have yet to be quantified and could differ from the effects found in the present study.

Conclusion

The goal of this research was to characterize (CAM)/Fas binding affinity and the effect of Fas mutations and the effect of (CAM) antagonist, trifluoperazine (TFP), on (CAM)/Fas binding affinity. This project shows an in vitro representation of how well the proteins bind and dissociate under a range of given conditions from a thermodynamic perspective. The results from this study could help to understand Fas and (CAM) binding in Fas-mediated death inducing signaling complex formation and potentially useful for developing the novel strategy or novel drug candidates for the chemotherapy of breast cancer. The next step of the research will be to perform biological studies that focused on the cellular level to determine how the protein binding dynamics shown in this study affect breast cancer cells.

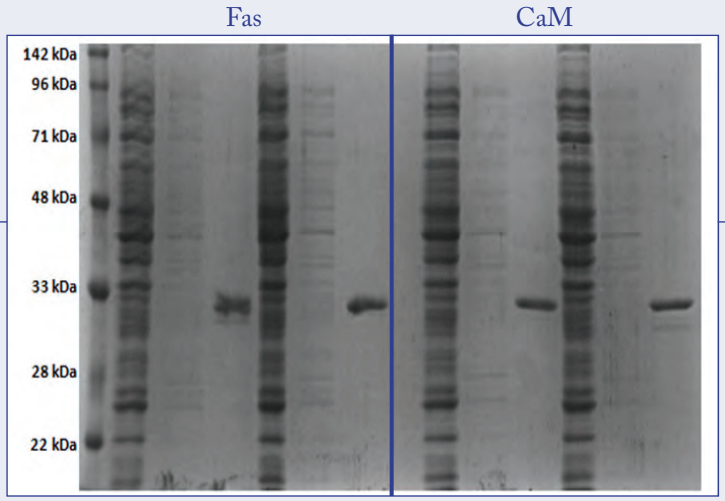
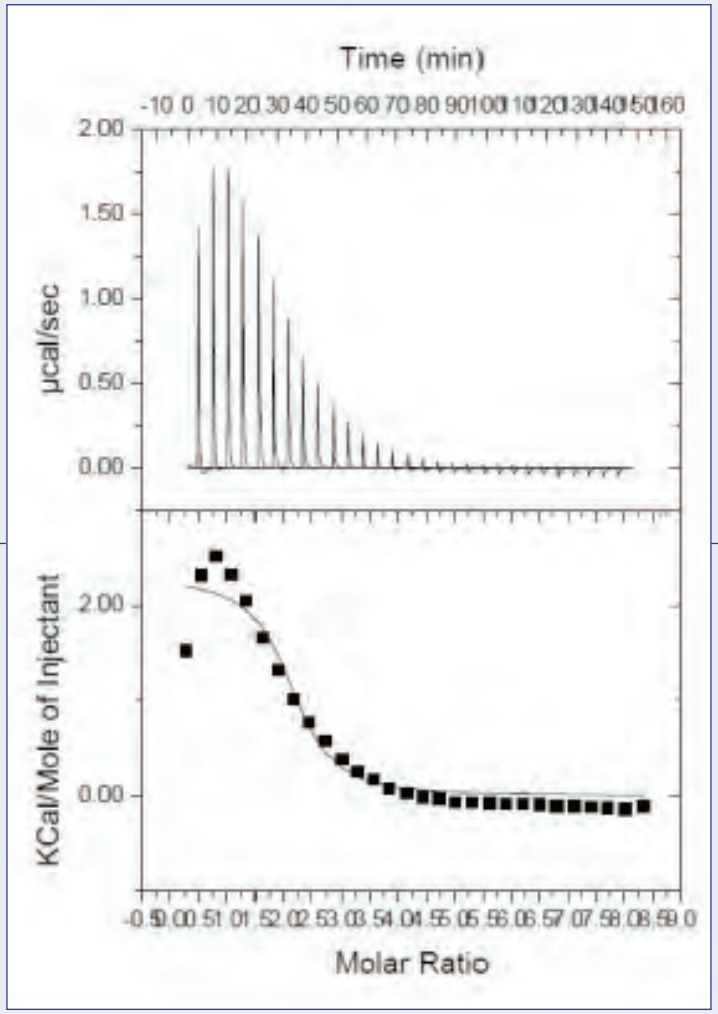


Figure 7: SDS-PAGE results of steps of affinity chromatogrphy protein purification for Fas and CaM (2.10.11).

n=2 for each protein purification's 3 basic purification steps shown above.



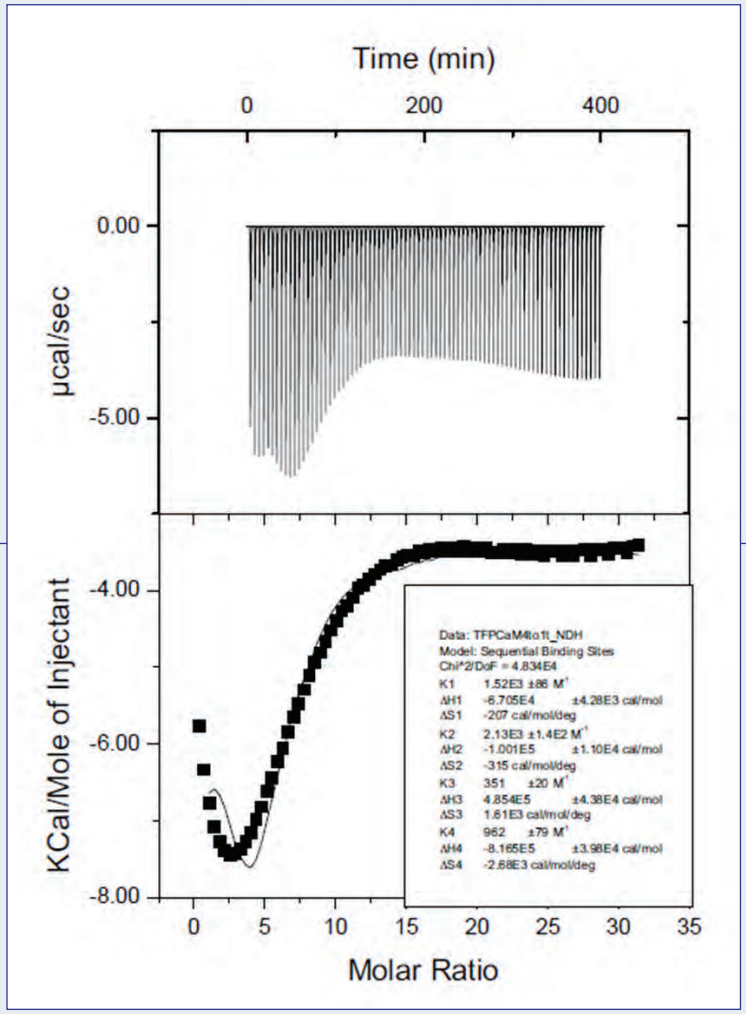
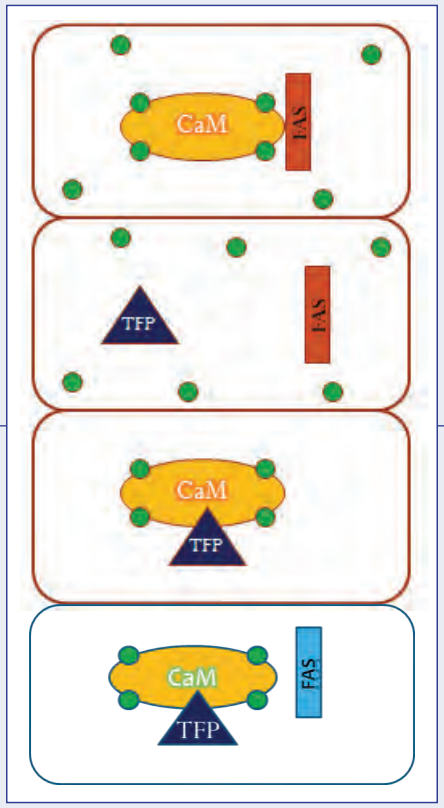


Figure 8: CaM-Fas ITC binding result.

Figure 10: CaM-Fas-TFP binding mechanism. Ca2+ binds to CaM to stimulate CaM-Fas complex (first from top), TFP does not bind directly with Fas (second from top), Ca2+ bind to CaM to stimulate CaM-TFP complex (third from top), but TFP inhibits CaM from binding to Fas.



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References

1. Nagata S: Apoptosis by death factor. Cell 1997(Chen, Pawar et al. 2008)

2. Chen, Y., P. Pawar, et al. (2008). "Calmodulin binding to the Fas-mediated death-inducing signaling complex in cholangiocarcinoma cells." J Cell Biochem 103(3): 788-799

3. Pawar P, ma L, Byon C, Liu H, Ahn E, Jhala N, Arnoletti J, McDonald J, Chen Y: Molecular mechanisms of tamoxifen therapy for cholangiocarcinoma: role of Calmodulin. Clin Cancer Res 2009, 15(4): 1288-1296.-

4. Ahn E, Lim ST, Cook WJ, McDonald JM: Calmodulin binding to the Fas death domain. Regulation by Fas activation. J Biol Chem 2004, 279(7): 5662-5666.

5. Suever J, Chen Y, McDonald J, Song Y: Conformation and free energy analyses of the complex of calcium-bound Calmodulin and Fas death domain. Biophys J 2008, 95(12): 5913-5921.

6. Ashkenazi A: Targeting death and decoy receptors of tumor-necrosis factor superfamily. Nat Rev Cancer 2002, 2(6): 420-430.

7. Chen Y, Pawar P, Pan G, Ma L, Liu H, McDonald J. (2007.) Calmodulin binding to the Fas-mediated death-inducing signaling complex in cholangiocarcinoma cells. J Cell Biochem. 103(3):788-9.

8. Ahn E, Lim S, Cook W, McDonald J. (2004.) Calmodulin binding to the Fas death domain. J Biol Chem. 279: 5661-6.

9. Wu X, Ahn E, McKenna M, Yeo H, McDonald J. (2005.) Fas binding to Calmodulin regulates apoptosis in osteoclasts. J Biol Chem. 280: 29964-70.

10. Keane M, Ettenberg E, Lowrey, G, Russell E, Lipkowitz S. (1996.) Fas expression and function in normal and malignant breast cell lines. Cancer Res. 56:4791-8.

11. Frankfurt O, Sugarbaker E, Robb J, Villa L. (1995.) Synergistic induction of apoptosis in breast cancer cells by tamoxifen and calmodulin inhibitors. Cancer Let. 97(2): 149-54.

12. Breast Cancer Facts & Figures 2009-2010. American Cancer Society. Available at: <http://www.cancer.org/acs/groups/content/@nho/documents/document/f861009final90809pdf.pdf>. Accessed 30 October 2010.

13. Liu G, Sheng H, Wu S: A study on the levels of Calmodulin and DNA in human lung cancer cells. Br J Cancer 1996, 73 (7): 899-901.Sd—Figures: 1, 2,3