TARGET IDENTIFICATION OF CYTOTOXIC

1,4-BENZODIAZEPINE-2,5-DIONES

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This thesis has been read and approved by _____ Date: __/__/____

Abstract

This study aims to elucidate the apoptotic target of cytotoxic 1,4-benzodiazepine-2,5-diones. Previous studies have identified certain 1,4-benzodiazepine-2,5-diones as competitive ATP-binding site inhibitors of Rho-associated protein kinase (ROCK). Through sequence analysis of the ROCK ATP-binding cleft a novel potential apoptotic target, cell cycle related kinase (CCRK), was identified. CCRK is thought to activate cyclin dependent kinase 2 (CDK2) by phosphorylating Thr160. CDK2 in turn functions as a key cell cycle regulator of the G_1 to S cell cycle phase transition. We report here that cellular treatment with *S*-UM-BD-29, one such cytotoxic 1,4-benzodiazepine-2,5-dione, results in G_1 to S phase cell cycle arrest and a decrease in CDK2 phosphorylation. Furthermore, we report a robust relationship between differing natural protein levels of CCRK across cell lines and sensitivity to *S*-UM-BD-29. Additional *in vitro* kinase inhibition and siRNA CCRK knockdown experiments proved unsuccessful and definitive evidence that CCRK is the cellular target of cytotoxic 1,4-benzodiazepine-2,5-diones that induces apoptosis remains elusive. The findings reported here do suggest that CCRK is the likely target of cytotoxic 1,4-benzodiazepine-2,5-diones, though, and future research will serve to validate this. CCRK is quickly emerging as an important physiological target of certain cancers and a compound that is capable of treating cancer cells that rely on CCRK for survival has very promising therapeutic potential.

Introduction

Previous studies in the Glick lab have demonstrated that cells treated with (*R*,*S*)-1,4-benzodiazepine-2,5-diones such as UM-BD-29 are cytotoxic (Figure 1, EC_{50} 500 nM in Jurkat T cells) [1]. The goal of my research project is to identify the cellular target of UM-BD-29 that is ultimately responsible for the cellular apoptosis observed with UM-BD-29 treatment.

UM-BD-29 has one chiral center. The *S*-enantiomer has two cellular targets: Rho-Kinase (ROCK) and an unknown target that leads to apoptosis. The *R*-enantiomer is not cytotoxic and targets only ROCK. Treatment of cells with the *S*-enantiomer results in signal transduction due to the inhibition of ROCK, which interferes with identification of the cytotoxic target. Therefore, in order to identify the cytotoxic cellular target of UM-BD-29, libraries of compounds were first screened to identify analogues that were either cytotoxic, inhibitors of ROCK, or both. These experiments revealed that UM-BD-54, a regioisomer of UM-BD-29 (Figure 2), does not inhibit ROCK but has similar cytotoxicity as UM-BD-29 (Figure 1, EC_{50} 600 nM in Jurkat T cells). As such, UM-BD-54 will be used to identify the cellular target of this class of compounds that leads to apoptosis.

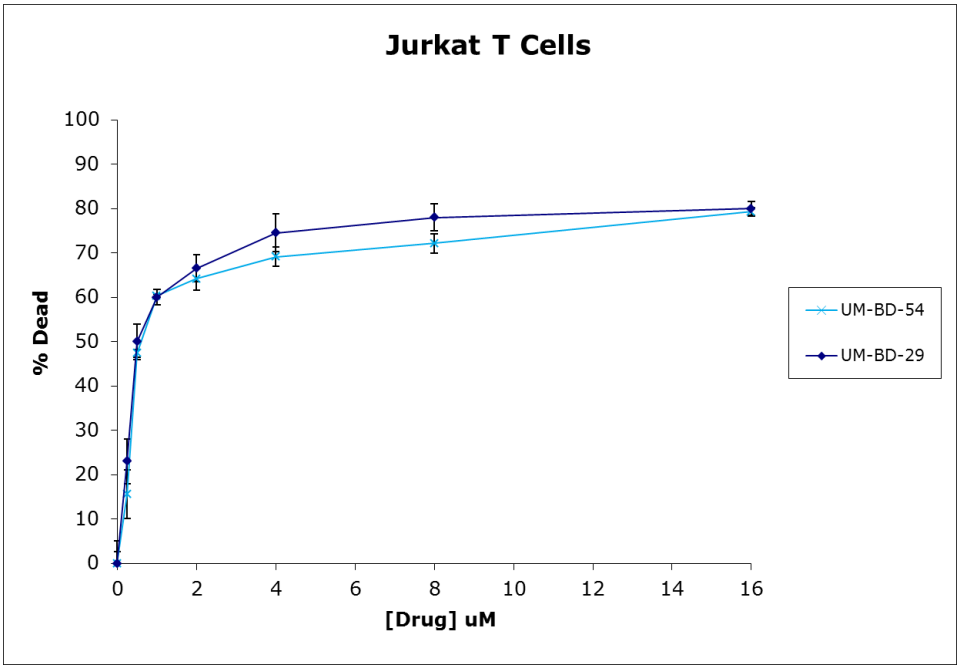

Figure 1 Cytotoxicity data for UM-BD-29 (EC_{50} 500 nM) and UM-BD-54 (EC_{50} 600 nM) in Jurkat T cells as determined by Alamar Blue.

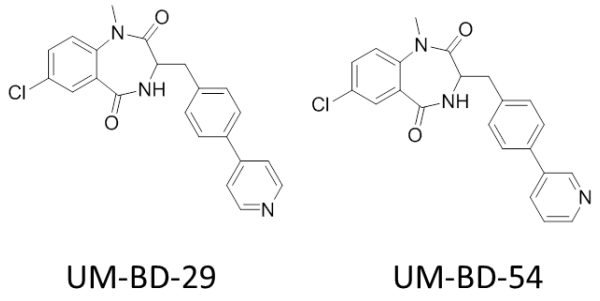

Figure 2 Chemical structures of UM-BD-29 and UM-BD-54.

UM-BD-29 was found to be a competitive ATP-binding site inhibitor of ROCK through Ambit's KINOMEscan, where a compound's ability to compete with an immobilized proprietary ligand for the ATP-binding site of many kinases was tested using 10 µM UM-BD-29. UM-BD-29 was found to inhibit ROCK ≥60% in this scan. Since the *S*-enantiomer of UM-BD-29 inhibits both ROCK and the cytotoxic target, it is likely that the binding clefts of the two targets are

similar. Breitenlechner et al. found that ROCK inhibitor selectivity of ROCK versus Protein Kinase A ATP-binding site is dictated by a unique combination of 4 binding site residues, and their sequence analysis indicates that there are only 6 of the 491 known human kinases that possess this same combination of residues: MUSK, MET, MST1R, CDC2L5, CRK7, and CCRK [2]. Of these 6 kinases, MUSK, MET, MST1R, and CDC2L5 were tested in the Ambit KINOMEscan and showed <20% inhibition at 10 μM UM-BD-29. Although little is known about CRK7, Iorns et al. found that it shares 89% sequence identity with CDC2L5 and it appears to be involved in RNA splicing and/or RNA polymerase II transcription [3]. The remaining kinase, cycle-related kinase (CCRK), is thought to phosphorylate CDK2, an essential CDK for the G_1 to S phase transition, at Thr160 [4]. It is important to note that when CDK2 is phosphorylated at Thr160 this causes structural changes in the T-loop of the enzyme that increases the kinase activity by several fold [5].

Cyclin-dependent kinases (CDKs), like CDK2, are enzymes that control the entry of a cell into the S and mitosis phases of the eukaryotic cell cycle. As the name indicates, a CDK must be cyclin-bound and, in most cases, needs to be phosphorylated by a CDK-activating kinase (CAK) in order to be active. In eukaryotes, the major CAK, which is active throughout the entire cell cycle and capable of phosphorylating all CDKs dependent on T-loop phosphorylation, is, itself, a CDK, and consists of three subunits: CDK7, cyclin H, and the RING finger protein MAT1 [6]. CCRK is thought to be a second eukaryotic CAK, phosphorylating CDK2 at Thr160 along with the more general CAK, the CDK7/cyclin H/MAT1 complex [4]. CCRK, unlike CDK7, is thought to exist as a monomeric protein [6]. CDK2, along with its cyclin partner cyclin E, is thought to affect the transition from G_1 to S phase by activating the retinoblastoma protein Rb by phosphorylating serine and threonine residues (Figure 3) [7]. Activating Rb

stimulates the transcription of genes required for the G_1 to S phase transition in addition to genes required for S phase progression [8]. Research by An et al. maintains that CCRK does likely phosphorylate the CDK2/cyclin E complex, which in turn phosphorylates Rb and promotes the transition from the G_1 to S phase, although they maintain the possibility that CCRK might directly phosphorylates Rb [9].

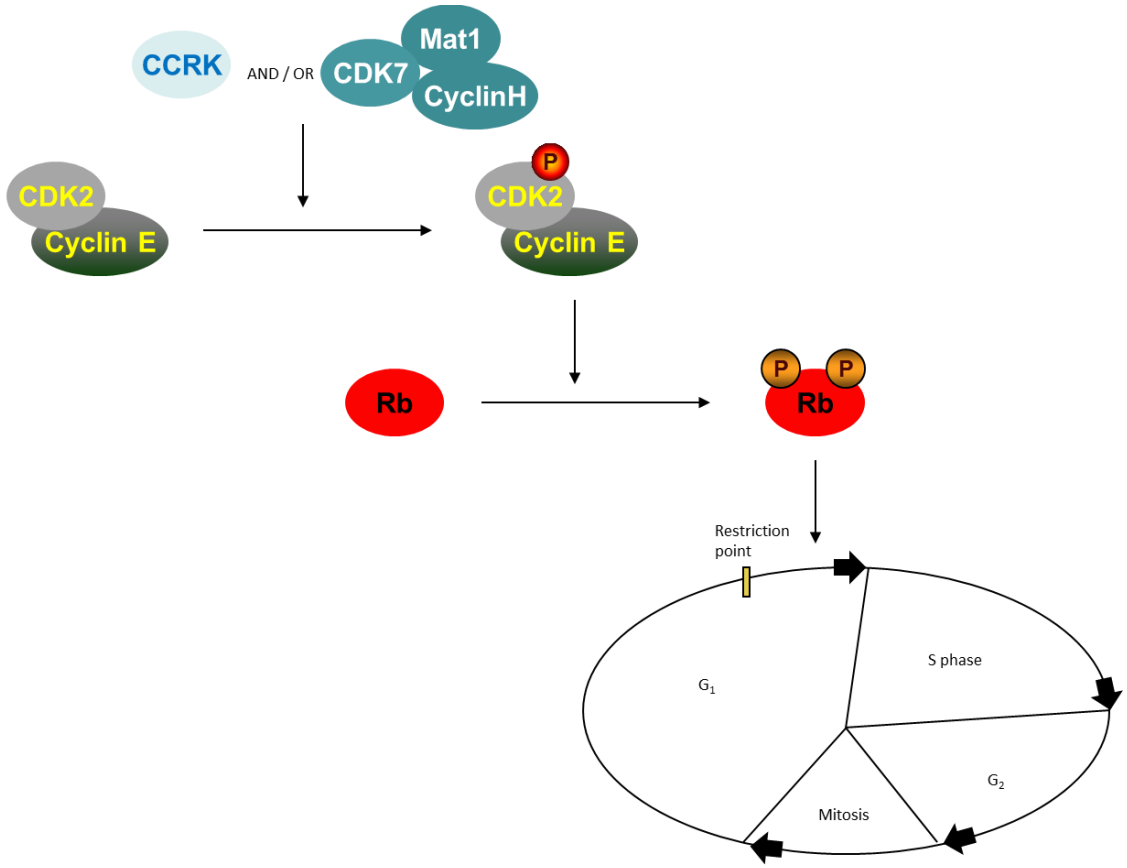

Figure 3 Model illustrating how CCRK and/or the CDK7 complex activates CDK2 by phosphorylation which, in turn, phosphorylates Rb and allows G_1 to S phase transition in the cell cycle. Adapted in part from [9].

Liu et al. ruled out the possibility of CDK7 being activated by CCRK and then, in turn, phosphorylating CDK2 by co-expressing CCRK and CDK7 or kinase-dead CCRK and CDK7 in osteosarcoma U2OS cells; this experiment showed that neither the wild type nor the kinase-dead CCRK mutant had any influence on co-expressed CDK7 activity [4]. Further evidence of

CCRK's direct interaction with CDK2 was found when CDK2 was co-immunoprecipitated with an antibody that was specific for CCRK in U-373 and U-87 MG cell lines [10]. This finding suggests that CDK2 has a strong interaction with CCRK, either through direct or indirect binding. In addition, CCRK protein expression levels have been positively associated with CDK2 phosphorylation levels in five glioma cell lines (U-373 MG, U-87 MG, U-118 MG, SW-1088, U-138 MG) and overexpression studies of CCRK in U-138 MG cells showed an increase in CDK2 phosphorylation with total CDK2 protein remaining completely unaffected [10]. Gene silencing experiments further support these initial findings with Liu et al. finding that CCRK is absolutely essential for CDK2 activity and cellular growth and that siRNA silencing of CCRK causes a decrease in CDK2 activity through a lack of phosphorylation at Thr160 [4]. Another gene silencing study further supports CCRK's role on G_1 to S transition through phosphorylating CDK2 in finding that CCRK knock-down affects the phosphorylation of Rb as well as the expression of cyclin E [9].

The clear role that both the CDK7, cyclin H, MAT1 complex and CCRK have on CDK2 phosphorylation, with CCRK seemingly doing the same job as the CDK7 complex, begs the question why a cell would need such redundancy. Liu et al. speculate that, since both CCRK and the CDK7 complex are nuclear proteins, it is possible that they each reside in different sub-nuclear compartments and have different substrates available to them, and, even though CDK7 can phosphorylate all known CDKs, it could be specific to a few designated CDKs [4]. If this were the case then any additional CAKs would serve to complement and share the work of the main CAK, the CDK7 complex, opening up additional avenues for cell cycle regulation. This theory is supported by experiments that have shown that the CDK7 complex has a substrate preference for the CDK/cyclin complex while CAK1 family proteins, such as CCRK, have a

preference for monomeric CDKs [11, 12]. It should be noted, however, that CDK2 can be phosphorylated in both the monomeric and cyclin-bound forms by the CDK7 complex [13].

CCRK has been found to be indispensable for the cell line proliferation of cervical carcinoma HeLa cells, osteosarcoma U2OS cells, and colorectal carcinoma HCT116, LoVo, and DLD1 cells [4, 6, 9]. Furthermore, CCRK siRNA transfection inhibited the growth of U-373 and U-87 MG cells in both a time and dose dependent manner [10]. An and colleagues postulate that there are two possible mechanisms responsible for the growth inhibition observed with CCRK knockdown -- the suppression of CCRK could directly induce apoptosis and thereby inhibit growth or CCRK suppression may inhibit cell cycle progression thereby inhibiting growth [9].

In support of growth inhibition through cell cycle disruption, U-373 and U-87 MG cells with CCRK silenced have been shown to have a higher percentage of cells in the G_0/G_1 phase than control transfected cells and this suggests that the growth-inhibiting effect of CCRK knockdown by siRNA occurs by arrest at the G_1 to S phase transition [10]. Similarly, LoVo and DLD1 cells were found to suffer G_1 to S phase cell cycle arrest upon CCRK knock-down [9]. In addition to these, however, depletion of CCRK has been shown to promote cell death in cancer cells [6, 14]. This evidence indicates that it isn't entirely clear what mechanism CCRK induces growth inhibition. It is possible that a combination of induced apoptosis and G_1 to S phase cell cycle arrest result in the growth inhibition or the mechanism responsible for growth inhibition could depend on the particular cell type.

Five splice variants of CCRK have been discovered. Four of the five variants are not known, at this point, to have distinguishable roles. The fifth variant, however—the cardiac splice variant—is unique. This novel variant has confirmed expression mainly in the heart, but also has detectable levels in the liver and kidney, and is 100% identical to the mouse cardiac

splice variant [15]. This incredible conservation suggests that the cardiac splice variant has an important biological role. Qiu et al. found that overexpression of the cardiac isoform of CCRK in cardiac myocytes did not increase the levels of CDK2 Thr160 phosphorylation while overexpression of generic CCRK in cardiac myocytes did increase the levels of CDK2 Thr160 phosphorylation [15]. This lack of cell cycle regulation suggests that the cardiac variant must have some other vital role. CCRK has been identified as a negative regulator of apoptosis [16], and both the general and cardiac isoforms of CCRK showed a 50% reduction in apoptosis after addition of chelerythrine, a cytotoxic protein kinase C inhibitor, in cardiac myocytes when compared to a control, which suggests that the cardiac isoform, while lacking CDK2 phosphorylation activity and retaining pro-survival activity, is unique and biologically important [15]. This establishes not only CCRK's role in the cell cycle but also an independent role as an anti-apoptotic protein.

It is important to understand that CCRK is a novel protein and its physiological role is not completely understood. Wu et al. suggests that the mechanism by which CCRK regulates cell cycle progression may well be specific to the cell line [17]. For example, CCRK has also been found to phosphorylate male germ cell-associated kinase, a key regulator of the cell cycle in the testis, at Thr157 in mammalian cells [4, 18]. Furthermore, Wu et al. found that CCRK knockdown by siRNA reduced cyclin D1 expression in TOV-21G ovarian carcinoma while neither CDK2 nor phosphorylated CDK2 levels were affected and that overexpression of CCRK caused overexpression of cyclin D1, again with no effect on CDK2 or phosphorylated CDK2 protein levels [17].

CCRK is a potential therapeutic target because of the potent role it plays in cell cycle progression and promoting cell survival. CCRK expression was elevated at least two-fold in 18

of 29 glioblastoma multiform tissue samples in a recent study and was also found to be elevated in 70% of colorectal cancer patient samples [9, 19]. Furthermore, Wu et al. found that the average overall survival time for patients with ovarian carcinomas found to be overexpressing CCRK was 37.4 months compared to an average overall survival time of 69.1 months for those patients with ovarian carcinomas expressing normal levels of CCRK [17]. There is scientific evidence to suggest that there is a link between CCRK expression and carcinogenesis. What is intriguing is that Ng et al. found that the mean tumor volumes of mice injected with CCRK shRNA transfected U-373 and U-87 cells were statistically significantly smaller than those of control transfected cells by 4-6 weeks [10]. This shows, *in vivo*, that inhibition of CCRK, in this case by shRNA, results in a physiologically and therapeutically significant effect – a slower growing tumor.

Taken together, these data indicate that CCRK is a promising possible target of *S*-UM-BD-29 and UM-BD-54. In order to determine whether CCRK is the target, experiments that look for classic signs of CCRK inhibition, such as cell cycle arrest at the G_1 to S phase transition and a decrease in CDK2 phosphorylation will be completed first. A caspase inhibitor experiment to verify that a possible decrease in CDK2 phosphorylation is a contributor to, and not a result of, cellular apoptosis will follow. Then an *in vitro* kinase assay with CCRK, its substrate CDK2, and treatment with or without UM-BD-54 will rule out any potential confounding cellular affects. Finally, knock-down of CCRK by siRNA should sensitize a cell line to *S*-UM-BD-29 and UM-BD-54. These experiments would be able to conclusively demonstrate that CCRK is the cellular target of *S*-UM-BD-29 and UM-BD-54 that is responsible for the apoptotic phenotype.

Materials and Methods

Compounds – Stock solutions of *S*-UM-BD-29, UM-BD-54, and UM-BD-61 were prepared in 16 mM DMSO and diluted to the appropriate concentration using culture media. Drug solutions were applied to cell cultures with the final concentration of DMSO present at 0.5% in all experiments.

Cell Culture – A549, ACHN, CCRF-CEM, Jurkat, U251, LN18, 786-0, NIH-3T3, Ovcar 3, Ovcar 4, PC-3, SW620, DU145, SK-Mel-2, SK-Mel-5, K562, MDA-MB-231, U87, NCI-ADR-RES, NCI-H460, Ovcar 8, SNB-75, HOP-62, IGROV-1, and HCT-116 were purchased from the ATCC. A549 and PC-3 cells were maintained in F12K media (ATCC). ACHN, DU145, SK-Mel-2, SK-Mel-5, and U87 were maintained in EMEM media (ATCC). CCRF-CEM, Jurkat, 786-0, Ovcar 3, Ovcar 4, SW620, NCI-ADR-RES, NCI-H460, Ovcar 8, SNB-75, HOP-62 and IGROV-1 were maintained in RPMI 1640 media (Mediatech). U251, LN18, NIH-3T3 and MDA-MB-231 were maintained in DMEM media (ATCC). K562 was maintained in IMDM media (ATCC). HCT116 was maintained in McCoy's 5A media (ATCC). All cell lines other than NIH-3T3 cells had their media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mediatech), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (290 µg/mL) (Invitrogen). NIH 3T3 cells had their media supplemented with 10% heat-inactivated calf serum (Colorado Serum Co), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (290 µg/mL) (Invitrogen). Cells were propagated in a humidified incubator (37 ºC, 5% CO_2).

Detection of cellular DNA content by flow cytometry – Hypodiploid DNA content measurement was made after incubating cells, already having been treated with test compound for 24 hours, in a labeling solution (50 µg/mL propidium iodide in phosphate buffered saline (PBS) containing

0.2% Triton X-100 and 10 µg/mL RNAse A) at 4 ºC for 12 hours. PI fluorescence was then measured in the FL2 channel on a linear scale by flow cytometry (Beckton Dickinson). Data were analyzed, excluding aggregates, using the software CellQuest.

Preparation of whole cell lysates – Cells were pelleted and supernatants aspirated. Cell pellets were washed twice with PBS, followed by pelleting and supernatant aspiration, before lysis with WCE lysis buffer (25 mM Hepes pH 7.7, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.5 mM DTT) with protease inhibitors (1 mM PMSF, complete protease inhibitor cocktail tablet (Roche)) and phosphatase inhibitors (3.3 mM NaF and 0.1 mM sodium orthovanadate). Cells were incubated on ice for 30 minutes in the lysis buffer. After incubation, cells were centrifuged (12,000g, 30 minutes, 4 ºC) to separate cellular protein from cell debris. The supernatant containing cellular proteins was collected and protein concentration was determined by the Bradford protein assay [20].

Western Blotting – Cell lysates, as prepared above, were denatured by boiling for 5 min with one-fourth volume of 4x SDS sample buffer (250 mM Tris Cl pH 6.8, 40% glycerol, 8% SDS, 8% 2-mercaptoethanol, and 0.2% bromophenol blue). Proteins were then electrophoresed by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad), and reacted with the appropriate antibodies at the recommended concentration: CCRK (Santa Cruz Biotech., #104830), phospho-CDK2 (Cell Signaling, #2561S or Santa Cruz Biotech., #12914), in PBS containing bovine serum albumin (3%) and Tween 20 (0.1%) and GAPDH (Millipore, #MAB374), β-tubulin (Invitrogen, #32-2600), PARP (Cell Signaling, #9542) in PBS containing non-fat milk (1%) and Tween 20 (0.1%). Blots were then incubated with horseradish peroxidase conjugated secondary species-specific antibodies and visualized by chemiluminescence reagents (Amersham Biosciences).

Dot Blot – Protein solutions were denatured by boiling for 5 min with one-fourth volume of 4x dot blot sample buffer (PBS, 8% SDS, 8% 2-mercaptoethanol). Proteins were then directly applied onto a PVDF membrane (Bio-Rad), and reacted with phospho-CDK2 (Cell Signaling, #2561S or Santa Cruz Biotech., #12914) antibody, in PBS containing bovine serum albumin (3%) and Tween 20 (0.1%). Blots were then incubated with horseradish peroxidase conjugated secondary species-specific antibodies and visualized by chemiluminescence reagents (Amersham Biosciences).

Cytotoxicity Assay – Cells were plated at 10,000 cells per well in 96-well plates in growth media supplemented with 5% FBS and treated with the appropriate compound in triplicate for a final volume of 190 µL. Cells were then incubated for 21 hours in a humidified incubator (37 ºC, 5% CO_2). After 21 hours of treatment, 10 µL Alamar Blue (Invitrogen) was added per well and cells were incubated for an additional 3 hours. The wells absorbances were read at 540 and 630 nm using a universal microplate reader (Molecular Devices). Alamar Blue reduction was then calculated according to the manufacturer's protocol. The number of viable cells correlates directly with the magnitude of Alamar Blue dye reduction [21].

Protein Level Assessment – Relative protein levels and comparative protein analyses were calculated using software ImageJ, which enables quantitative image analysis.

In Vitro Kinase Assay – Recombinant CCRK (Abnova, #H00023552-P01) or CDK7/cyclin H/MAT1 (Invitrogen, #PV3868) was incubated with 200 µM ATP and recombinant CDK2 (Abnova, #H00001017-P01) or T160 peptide (Abgent, #SP2014d) in kinase buffer (25 mM Tris, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, pH 7.5) for 30 or 60 min at 25, 30, or 37 ºC. The enzyme reaction was then stopped by addition of SDS

and boiling for 5 min. Phosphorylated CDK2 or phosphorylated T160 peptide was then probed for by either western blotting or dot blot.

Immunoprecipitation – Protein lysates were prepared as described above substituting WCE lysis buffer for IP Lysis/wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4) supplemented with a protease inhibitor cocktail tablet (Roche). 1500 µg cell lysate was incubated with 20 µg CCRK antibody raised in rabbit (Abnova, #H00023552-D01P) at 4 ºC for 12 hours. The antibody-protein complex was captured by binding to protein A/G Plus agarose beads (Pierce). The agarose beads were then washed three times with IP Lysis/wash buffer. The antibody-protein complex was eluted first using a high salt gentle elution buffer (Pierce, #21027), second using a low pH elution buffer (pH 2.8), and finally by boiling for 5 minutes with a reducing buffer (20 mM DTT, 0.12 Tris, 2% SDS, 20% glycerol, lane marker dye, pH 6.8). The protein concentrations of the fractions were determined by optical density as described by Scopes [22]. CCRK presence was then confirmed by western blotting CCRK antibody raised in goat (Santa Cruz Biotech., #104830).

Small interfering RNA (siRNA) – ON-TARGETplus SMARTpool CCRK siRNA (targeting 5'-UCACUGAGCUGCCGGACUA-3', 5'-GAUGGAGGACAAUCAGUAU-3', 5'-GAACAGCUUUGCUAUGUGC-3', 5'-AGGCACAGGUCAAGAGCUA-3'), siGENOME SMARTpool CCRK siRNA (targeting 5'-GGAAGGACUUACGGUAUCA-3', 5'-GAAGGAAGAUUUGGUUUGG-3', 5'-UCAGGUGGCUCUGCCUUAU-3', 5'-UCACUGAGCUGCCGGACUA-3'), and custom CCRK siRNA (targeting 5'-GAAGGUGGCCCUAAGGCGG-3', 5'-GGCGGUUGGAGGACGGCUU-3') were synthesized and purified by Dharmacon. ON-TARGETplus GAPDH control pool (Dharmacon, #D-001830-

10-05) and ON-TARGETplus non-targeting pool (Dharmacon, #D-001810-10-05) were also used.

Transient Transfection with siRNAs – One day before transfection, cells were detached by treatment with 0.25% trypsin-EDTA (Invitrogen) and plated onto 60 x 15 mm tissue culture dishes at 3 x 10^5 cells per plate. Cells were then transfected by incubation with appropriate siRNA using DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's instructions in normal growth media supplemented with 10% FBS. Mock transfection cells were incubated with just DharmaFECT 1 transfection reagent in normal growth media supplemented with 10% FBS. Transfection media was replaced with normal growth media supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (290 µg/mL) 6 hours after transfection. Cells were harvested and lysed at 24, 48, 72, or 96 hours after transfection.

Results

UM-BD-29 Effect on Cell Cycle

One hallmark of CCRK inhibition is cell cycle arrest at the G_1 to S checkpoint. To examine cell cycle disregulation caused by UM-BD-29, one can analyze the DNA fragmentation pattern induced by UM-BD-29 using propidium iodide staining assessed through flow cytometry. Analysis of the $SubG_0$ DNA content of Jurkat T cells after 24 hours of treatment with 10 µM UM-BD-29 shows that there is an accumulation of G_0/G_1 DNA accompanied by a reduction in $S/G_2/M$ DNA (Figure 4).

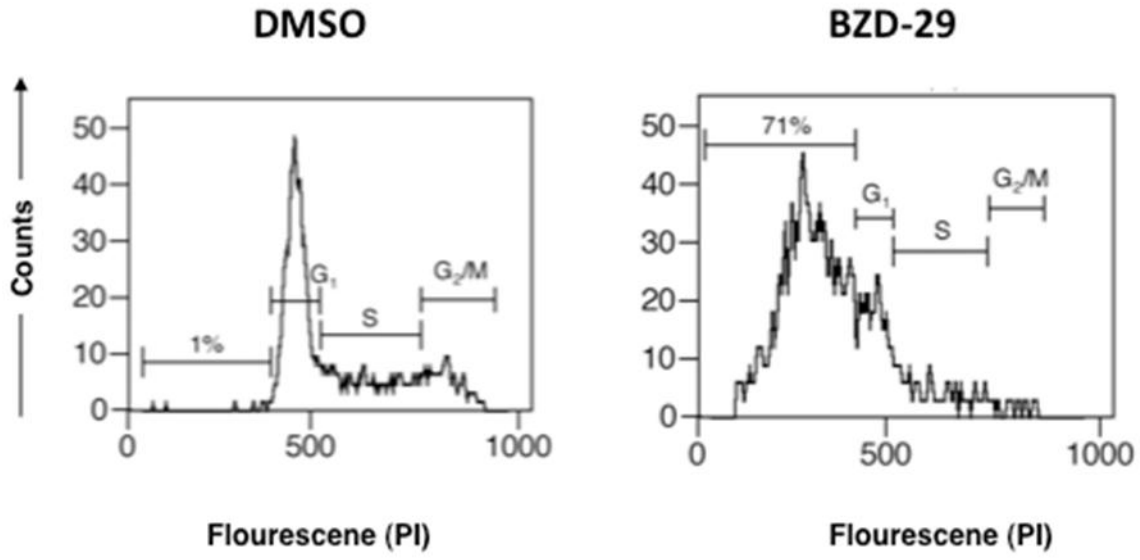

Figure 4 $SubG_0$ analysis of Jurkat T cells treated with 10 µM UM-BD-29 for 24 hours. DNA quantified by flow cytometry using PI staining. Figure adopted from [23].

This DNA fragmentation pattern, wherein the percentage of cells in the G_0/G_1 phases increase and the $S/G_2/M$ phases decrease, is consistent with what Ng et al. observed with CCRK knockdown in glioblastoma cells and is indicative of arrest at the G_1 to S phase transition [10]. In addition, UM-BD-29 has previously been observed to cause growth arrest in all of the NCI-60 cell lines [23]. Growth arrest is normally a prelude to apoptosis and so these data suggest that

UM-BD-29 induces apoptosis through a vital regulator of the cell cycle, likely one that regulates the G_1 to S phase checkpoint, as CCRK does [9, 10].

<u>*S*-UM-BD-29 and UM-BD-54 effect on CDK2 phosphorylation in Human Glioblastoma</u>

Another unique result of CCRK inhibition is a marked decrease in CDK2 phosphorylation [4, 10]. In order to determine whether or not treatment with UM-BD-54 and *S*-UM-BD-29 would induce this phenotype human glioblastoma U251 cells were treated with *S*-UM-BD-29 or UM-BD-54 and phosphorylated CDK2 protein expression was then examined by western blotting. Exposure to both concentrations of *S*-UM-BD-29 and UM-BD-54 had an extremely robust response, with phosphorylated CDK2 levels decreasing by approximately 80% as measured by ImageJ when compared to the DMSO vehicle controls (Figure 5).

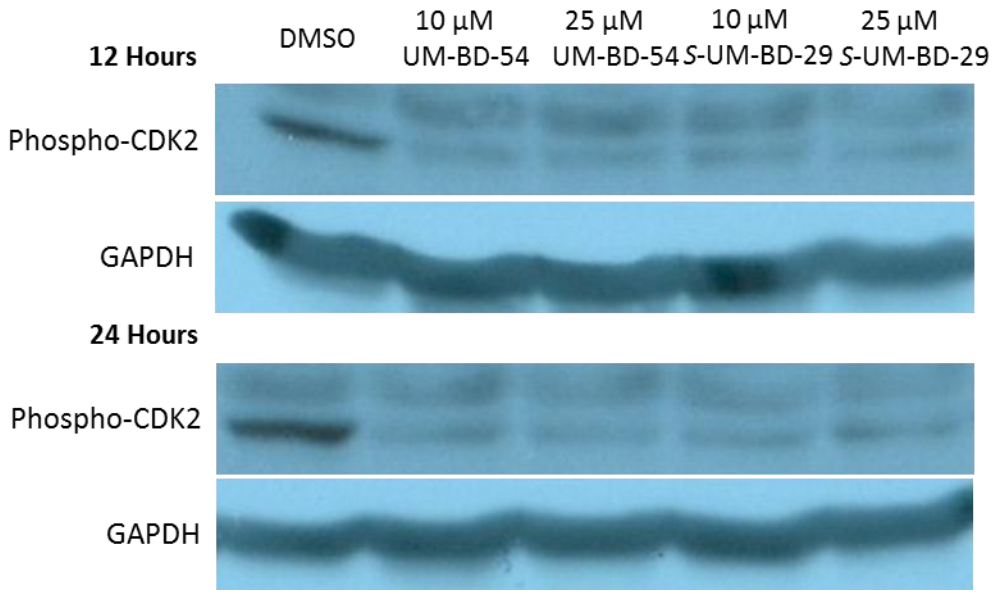

Figure 5 Western blot analysis of Phospho-CDK2 protein in U251 cells. Cells were treated with 10 µM *S*-UM-BD-29, 25 µM *S*-UM-BD-29, 10 µM UM-BD-54, 25 µM UM-BD-54, or DMSO for 12 or 24 hours.

While this experiment shows that *S*-UM-BD-29 and UM-BD-54 can strongly decrease CDK2 phosphorylation by at least 12 hours, this does not show that the decrease in CDK2 phosphorylation precedes the onset of apoptosis. Presumably, treatment with these drugs causes apoptosis by inhibiting CCRK and resulting in decreased CDK2 phosphorylation, which is known to then cause apoptosis [6]. It is possible, however, that treatment with these drugs causes apoptosis by some other means and CDK2 phosphorylation is decreased as a result of apoptotic signaling and not directly by *S*-UM-BD-29 and UM-BD-54 drug treatment.

In order to dispel this possibility, the general caspase inhibitor Z-VAD-FMK, which can block cellular apoptosis by inhibiting caspases, was utilized. U251 cells were treated with either *S*-UM-BD-29, UM-BD-54, or UM-BD-61 (Figure 6).

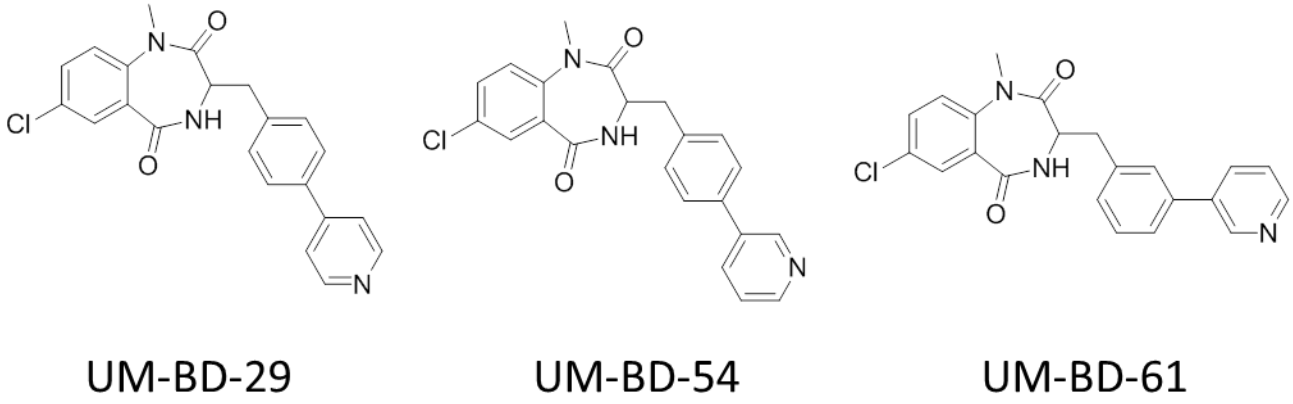

Figure 6 The chemical structures of UM-BD-29, UM-BD-54, and UM-BD-61.

UM-BD-61, a non-cytotoxic, non-ROCK inhibiting derivative of the (*R*,*S*)-1,4-benzodiazepine-2,5-dione family, was used to see whether the decrease in CDK2 phosphorylation is dependent on the cytotoxic element of the compounds and not just the scaffold. Following treatment, phosphorylated CDK2 protein levels were examined by western blotting (Figure 7). Exposure to *S*-UM-BD-29 and both concentrations of UM-BD-54 results in an approximately 60% decrease

in phospho-CDK2 protein levels when compared to the DMSO vehicle controls, as measured by ImageJ. In addition, Z-VAD-FMK did not inhibit the reduction in phosphorylated CDK2 protein levels, indicating that this reduction occurs upstream of apoptosis. Lastly, UM-BD-61 treatment did not cause a reduction in phosphorylated CDK2 protein levels, suggesting that this reduction is linked to the cytotoxic element of the family of compounds and not the shared scaffold.

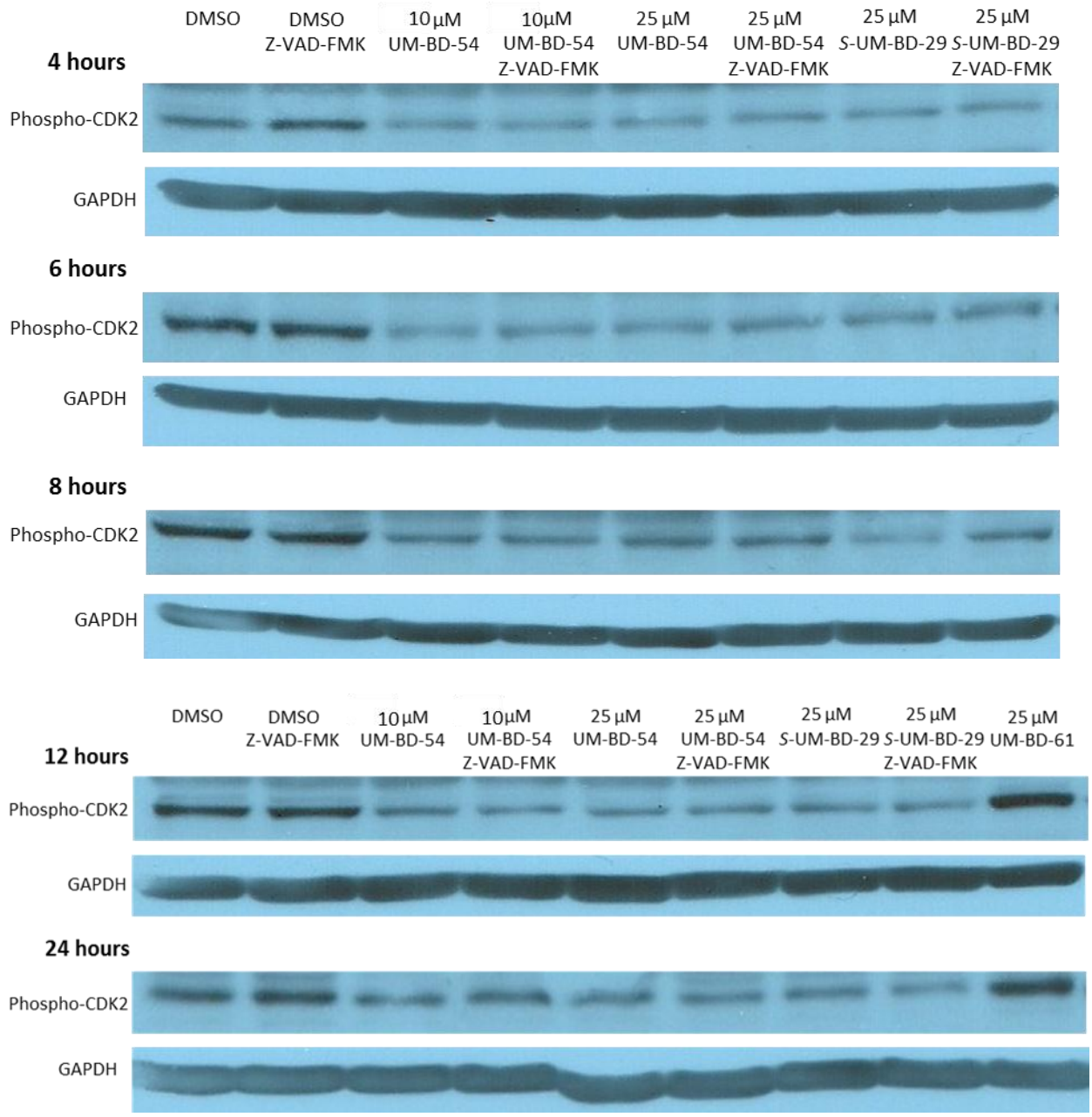

Figure 7 Western blot analysis of Phospho-CDK2 protein in U251 cells. U251 cells were treated with DMSO, 10 µM UM-BD-54, 25 µM UM-BD-54, or 25 µM *S*-UM-BD-29 for 4, 6, 8, 12, or 24 hours. Of these conditions, one copy was subjected to 50 µM Z-VAD-FMK treatment for 1 hour prior to experiment. The 12 and 24 hour time points were also treated with 25 µM UM-BD-61, a non-cytotoxic non-ROCK inhibiting derivative.

In order to verify that Z-VAD-FMK worked, PARP protein levels were examined at the 24 and 48 hour time points by western blotting. PARP cleavage is one of the cellular indicators of apoptosis. Exposure to both concentrations of *S*-UM-BD-29 and UM-BD-54 induced apoptosis, with more cell death occurring at the 48 hour time point (Figure 8). As expected, the non-cytotoxic UM-BD-61 did not induce apoptosis. Most importantly, samples that were pretreated with Z-VAD-FMK had no PARP cleavage at conditions that would otherwise result in apoptosis. This indicates that the treatment with Z-VAD-FMK did inhibit apoptotic signaling, which validates the earlier that decreases in phosphorylation of CDK2 by *S*-UM-BD-29 and UM-BD-54 occurs upstream of apoptosis.

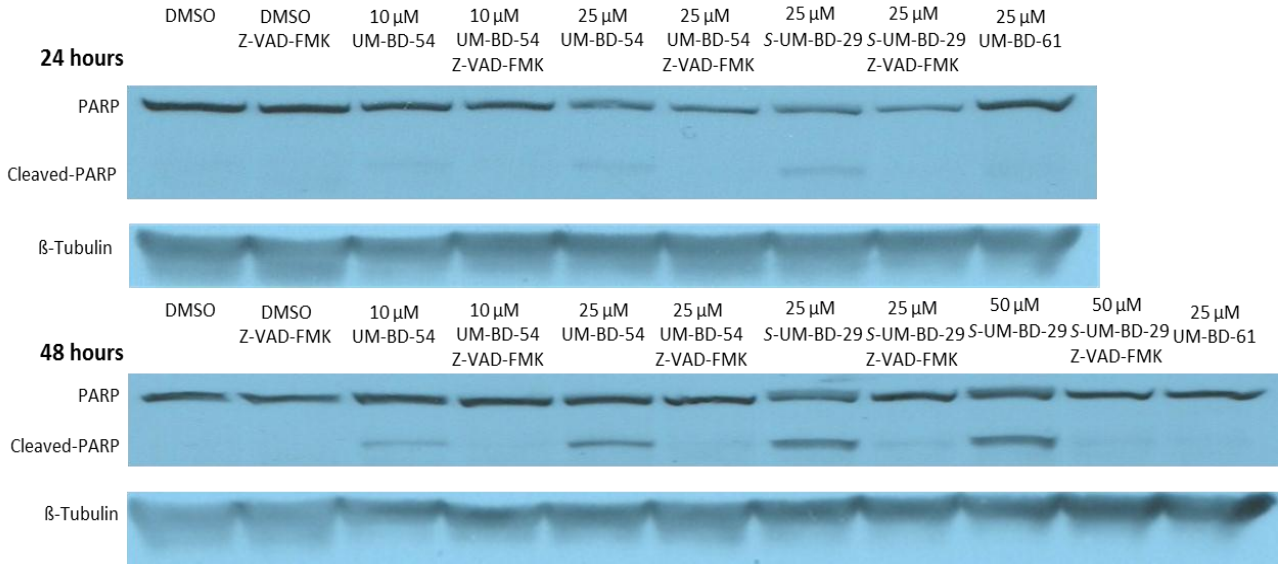

Figure 8 Western blot analysis of PARP protein in U251 cells. U251 cells were treated with DMSO, 10 µM UM-BD-54, 25 µM UM-BD-54, or 25 µM *S*-UM-BD-29 for 24 or 48 hours. The 48 hour time point was also treated

with 50 µM *S*-UM-BD-29 to assure cell death. Of these conditions, one copy was subjected to 50 µM Z-VAD-FMK treatment for 1 hour prior to experiment.

Native CCRK Protein Levels and *S*-UM-BD-29 Resistance

If *S*-UM-BD-29 causes apoptosis by inhibition of CCRK then there may well be a relationship between natural CCRK protein levels and a cell line's resistance to *S*-UM-BD-29. In order to determine this, 25 cell lines were selected for cytotoxic analysis by Alamar Blue with treatment by *S*-UM-BD-29 and CCRK protein analysis by western blotting. Of these 25 cell lines, we were unable to obtain a representative EC_{50} for 8 of them (U87, NCI-ADR-RES, NCI-H460, Ovcar 8, SNB-75, HOP-62, IGROV-1, and HCT-116) due to cell death maxing out at 60% or below even at high (32 µM) treatment. For the 17 cell lines (A549, ACHN, CCRF-CEM, Jurkat, U251, LN18, 786-0, NIH-3T3, Ovcar 3, Ovcar 4, PC-3, SW620, DU145, SK-Mel-2, SK-Mel-5, K562, and MDA-MB-231) that were able to obtain an accurate EC_{50} value for there is a clear correlation between *S*-UM-BD-29 resistance and relative level of CCRK protein (Figure 9).

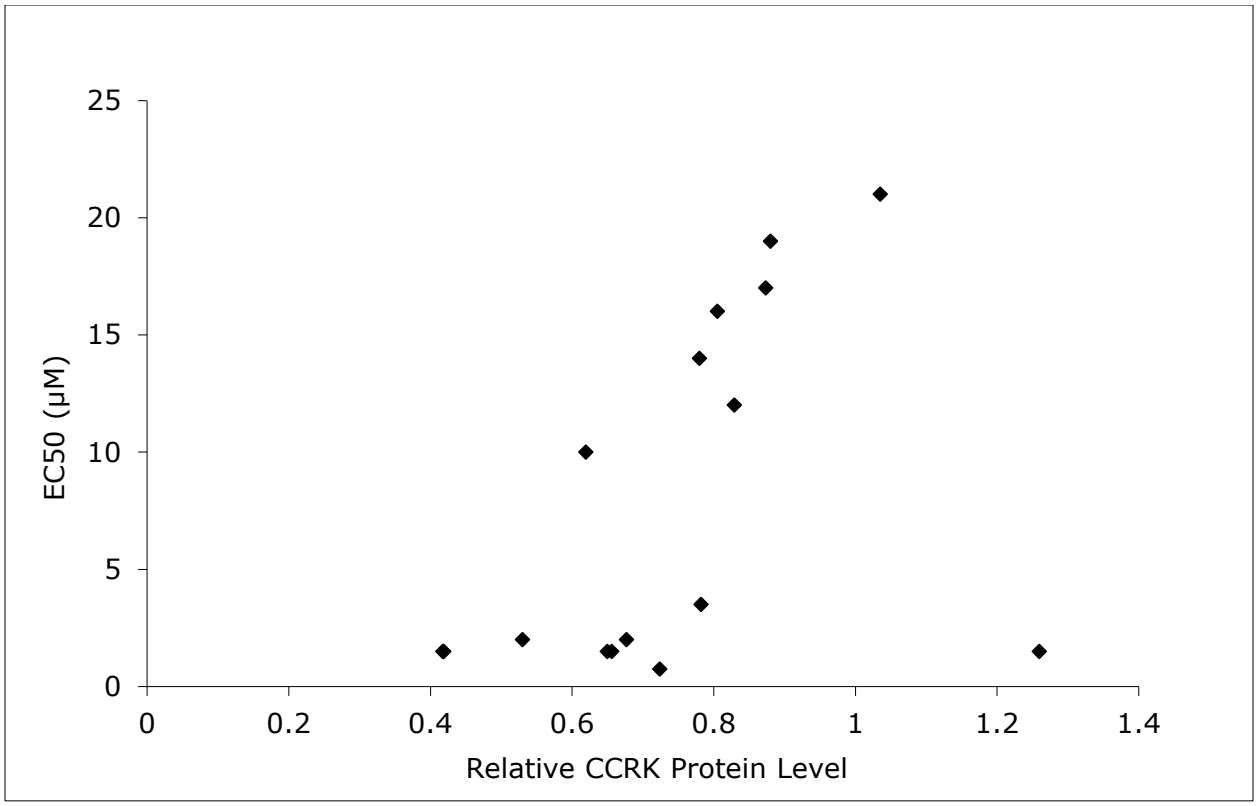

Figure 9 Graphical representation of Alamar Blue cytotoxicity and western blotting CCRK protein levels, as measured by ImageJ, for 17 cell lines. **Data produced together with Joanne Cleary.

There was one outlier in the relationship between *S*-UM-BD-29 sensitivity and relative CCRK protein, MDA-MB-231. It is unclear why, despite a relatively high CCRK protein level, this strain is so sensitive. It is possible that these cells have a greater dependence on CCRK and any disruption is cytotoxic to the cell. With the exclusion of the outlier MDA-MB-231, there is an R squared value of 0.6264 (Figure 10).

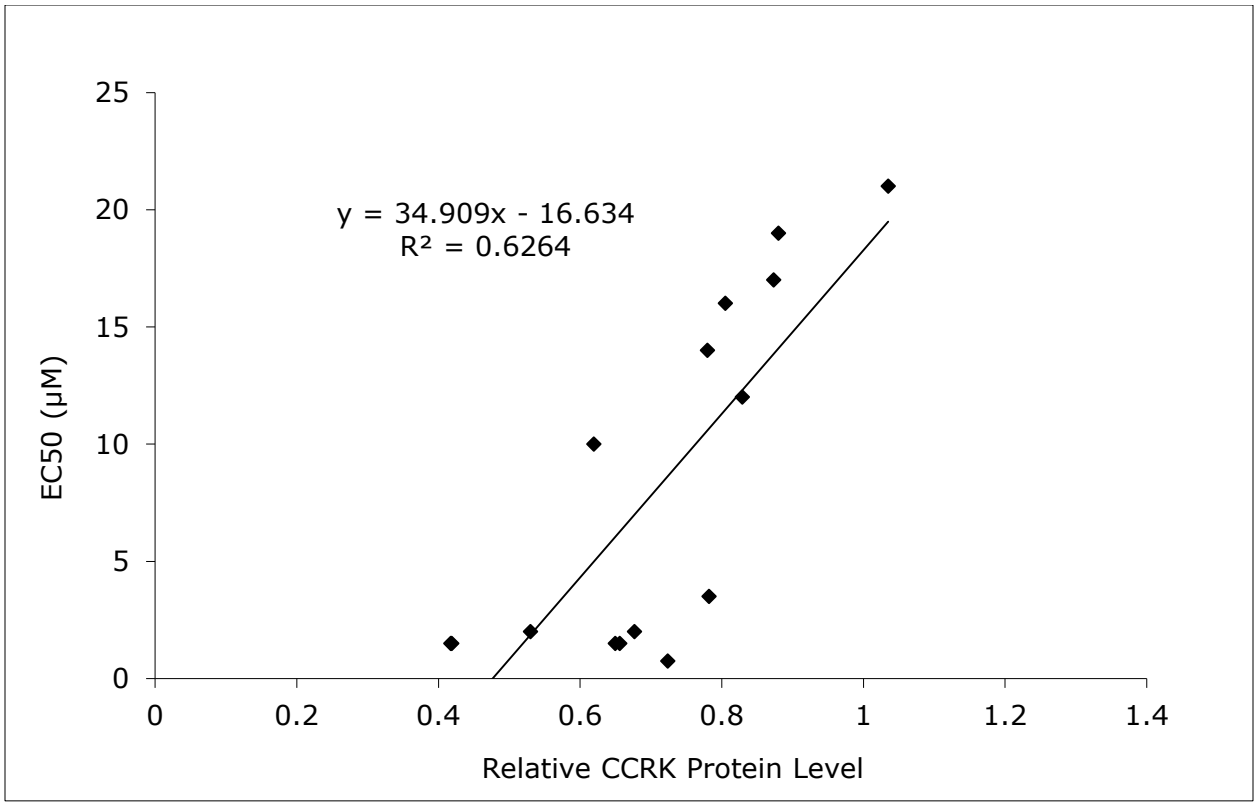

Figure 10 Graphical representation of Alamar Blue cytotoxicity and western blotting CCRK protein levels, as measured by ImageJ, for 16 cell lines – excluding one outlier. The linear trend line shows an R squared value of 0.6264. **Data produced together with Joanne Cleary.

This correlation value suggests that there is a relatively strong direct relationship between levels of CCRK protein and resistance to *S*-UM-BD-29 as measured by EC_{50}. These data suggest that CCRK plays an important role in the cytotoxicity induced by *S*-UM-BD-29.

<u>*In Vitro* Kinase Inhibition</u>

Although all of the data thus far suggests that *S*-UM-BD-29 and UM-BD-54 elicit apoptosis through inhibition of CCRK, it is possible that there are some confounding events that occur within the cell. It is possible that the apoptotic phenotype is being caused by some other mechanism and CCRK might not be involved at all. The most direct way to definitively show

that CCRK is inhibited by our compounds is to perform an *in vitro* kinase inhibition assay. These assays are often done at the industrial scale scanning many kinases for inhibition by a particular drug, such as Millipore's KinaseProfiler and Ambit's Kinomescan. Unfortunately, CCRK is such a novel kinase an industrial option is not available and the assay must be created from scratch.

There were a lot of initial experiments focused on creating this assay. Commercial sources of full length recombinant CDK2 proved to be partially phosphorylated at some point before or during purification, rendering it unusable for our assay (data not shown). A T160 peptide of CDK2, where CCRK phosphorylates CDK2, was chosen instead. It should be noted that industrial kinase scans normally utilize peptides in lieu of the full length substrate. Phosphorylated T160 peptide was also acquired in order to verify that the phospho-CDK2 antibodies that had been used would be able to detect the T160 peptide (data not shown). It still wasn't clear if the T160 peptide would be able to be phosphorylated *in vitro*, however. An *in vitro* kinase assay using full length recombinant CCRK and CDK7/Cyclin H/MAT1 was attempted. Phospho-T160 was probed for by dot blot (Figure 11).

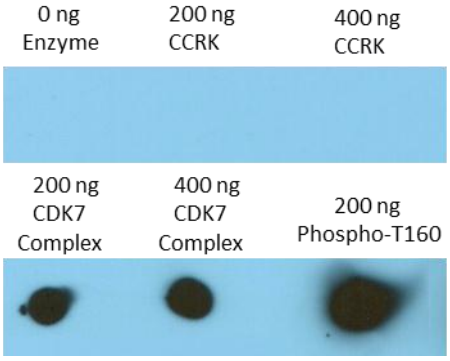

Figure 11 Dot blot analysis of phosphorylated T160 peptide. 200 or 400 ng of CCRK or CDK7/Cyclin H/MAT1 was allowed to react with 200 ng unphosphorylated T160 peptide for 30 minutes at 25ºC.

While CDK7/Cyclin H/MAT1 phosphorylated the T160 peptide in the assay, CCRK did not. In order to have CCRK produce detectable phosphorylation many different variables were altered. The reaction temperature was tested at 25ºC, 30ºC and 37ºC, different ratios of CCRK to T160 peptide (1:2, 1:1, 2:1), an increase in the overall amount of CCRK (400 ng, 800 ng), and different reaction times (30 min, 60 min) were all tried to increase CCRK activity. Despite this, CCRK did not produce any detectable phosphorylation of the T160 peptide (data not shown).

There are currently only two CAKs known to phosphorylate CDK2 on T160, CCRK and the CDK7 complex [4]. Although previous industrial kinome scans, like those of Ambit and Millipore, have suggested UM-BD-29 does not inhibit the CDK7 complex, these experiments were done using racemic UM-BD-29, of which only the *S* enantiomer is cytotoxic. This means that the kinome scans only effectively tested inhibition at half the 10 µM concentration of UM-BD-29 provided. It is thus within reason that the CDK7 complex could be inhibited by higher doses of the cytotoxic UM-BD-54 and this could be the cause of the CDK2 phosphorylation decrease observed. Recombinant CDK7/Cyclin H/MAT1's ability to phosphorylate the T160 peptide with pretreatment of UM-BD-54 was tested by our *in vitro* kinase assay (Figure 12).

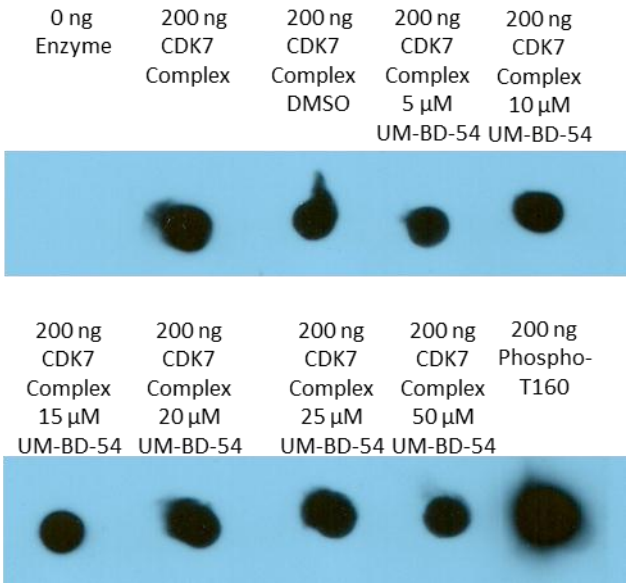

Figure 12 Dot blot analysis of phosphorylated T160 peptide. 200 ng of CDK7/Cyclin H/MAT1 was allowed to react with 200 ng unphosphorylated T160 peptide for 30 minutes at 25ºC. Some samples of enzyme were pretreated with DMSO, 5, 10, 15, 20, 25, or 50 µM UM-BD-54 for 30 minutes prior to the kinase assay.

As the CDK7 complex was unaffected by UM-BD-54, this reaffirms that the decrease in phospho-CDK2 observed upon treatment with UM-BD-54 is caused by some other mechanism.

As the recombinant CCRK that had been used previously was produced in a cell-free system [24], it is possible that there are some posttranslational modifications that are necessary for CCRK's activity. In order to account for this, we ordered mammalian CCRK from human embryonic kidney (HEK 293) cells. This CCRK, too, proved to be kinase-dead, however (data not shown).

It is possible that CCRK needs certain cofactors to be active. Other CCRK researchers have suggested that, although CCRK is thought to be a monomer, it may have unknown cofactors that activate it [4]. Obtaining a kinase-active form of CCRK is vital to showing that UM-BD-29 and UM-BD-54 inhibit CCRK and so immunoprecipitation of CCRK was elected as

the next step. Immunoprecipitation should provide active CCRK along with any associated cofactors and post-translational modifications to make it active.

Immunoprecipitation proved to be very difficult. Three different elution methods were attempted with U251 cell lysates: elution by high salt, elution by lowering pH, and elution by denaturing. The high salt elutions were buffer exchanged by dialysis into 100 mM NaCl, 250 mM Tris-HCl pH 7 solutions and the low pH elution was immediately neutralized with a high pH buffer. All of these methods provided fractions that were known to have protein by optical density readings [22]. These fractions were probed for CCRK by western blotting (Figure 13).

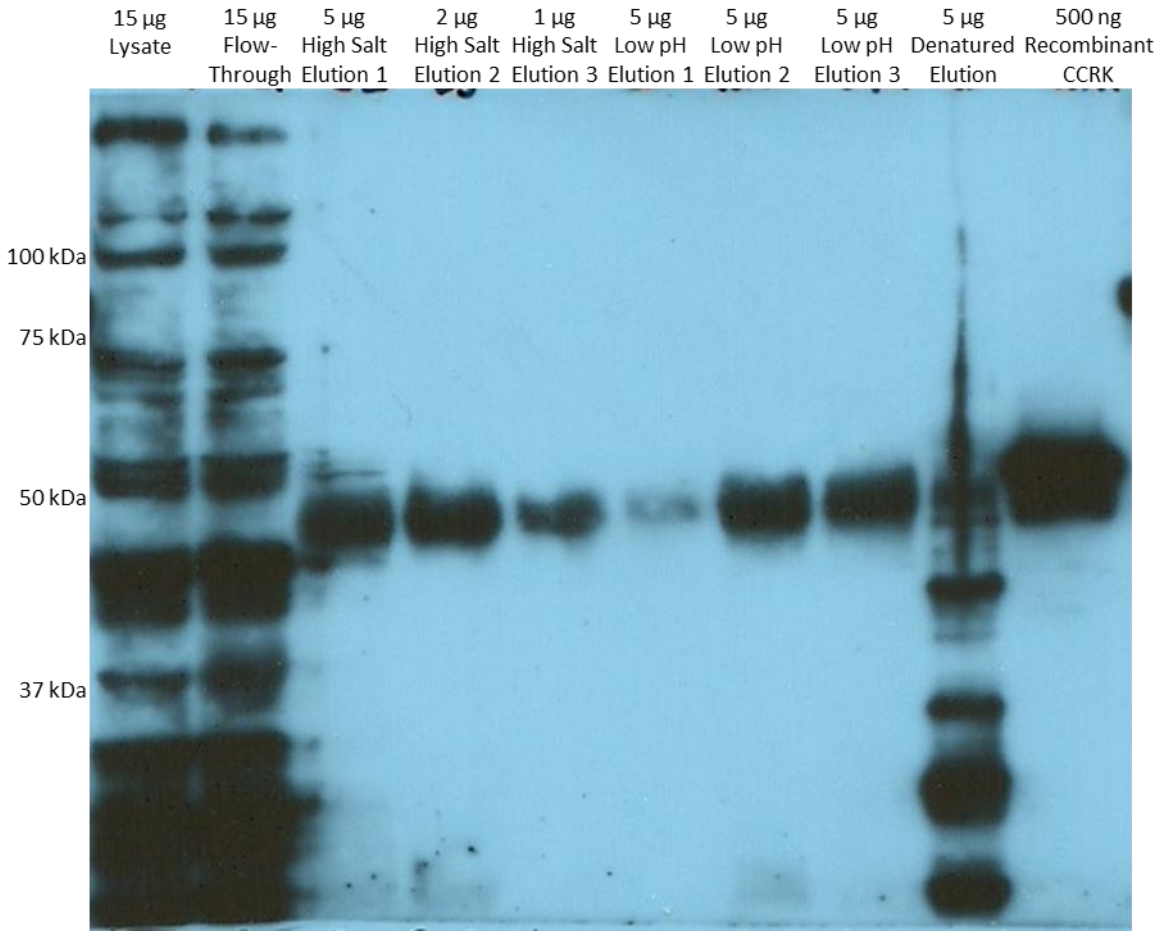

Figure 13 Western blot analysis of CCRK protein in U251 cells following immunoprecipitation.

Unfortunately, CCRK runs right at 50 kDa along with the heavy chain of IgG. CCRK was immunoprecipitated with a rabbit IgG and probed for western blotting with a goat CCRK IgG. The donkey anti-goat secondary that we used was reported to cross react <0.1% with other species. As this could be a problem the validity of this claim was tested by western blotting (Figure 14).

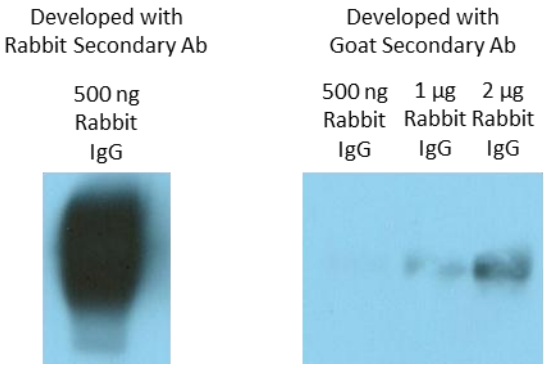

Figure 14 Western blot analysis of CCRK rabbit IgG. 500, 1000, or 2000 ng of CCRK rabbit IgG was probed with anti-goat secondary while 500 ng of CCRK rabbit IgG was probed with anti-rabbit secondary to visualize cross reactivity.

While cross-species reactivity is apparent, it is relatively limited. Following western blot verification that fractions contain CCRK, all salt elutions and all pH elutions are combined and the protein concentration is determined by optical density reading [22].

An *in vitro* kinase assay using immunoprecipitated CCRK either from the salt or pH elutions was probed for phospho-T160 by dot blot (Figure 15).

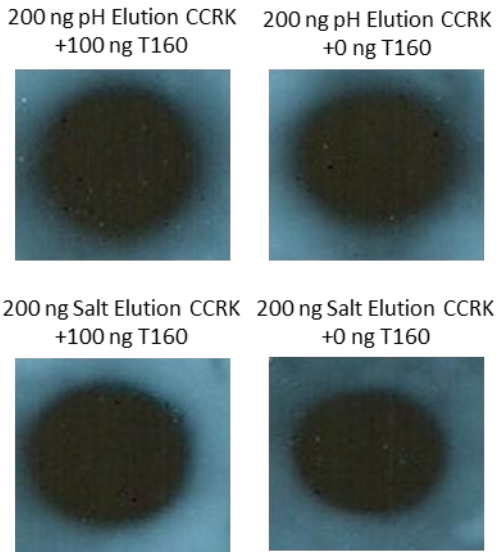

Figure 15 Dot blot analysis of phosphorylated T160 peptide. 100 or 200 ng of CCRK derived from either salt or pH elutions was allowed to react with 200 ng unphosphorylated T160 peptide at 30ºC or 37ºC for 30 or 60 minutes. All enzyme containing fractions (including those without peptide) had a strong chemiluminescent reading.

There was such a high amount of IgG present in the enzyme samples that the background cross-species IgG interaction was greater than any possible T160 phosphorylation. At this point, it is clear that the IgG must be separated from the CCRK to avoid this cross-species reactivity.

Although CCRK runs on an SDS-PAGE gel at around 50 kDa it is actually only 40 kDa in size. This size difference between the undenatured IgG (150 kDa) and CCRK (40 kDa) appeared to be one of the few ways to separate the two. On the next round of immunoprecipitation, the combined salt elution fraction and combined pH elution fraction were subjected to filtering by Amicon 50 kDa centrifugal filters according to the manufacturer's instructions. The resultant split fractions were then probed for CCRK via western blotting (Figure 16).

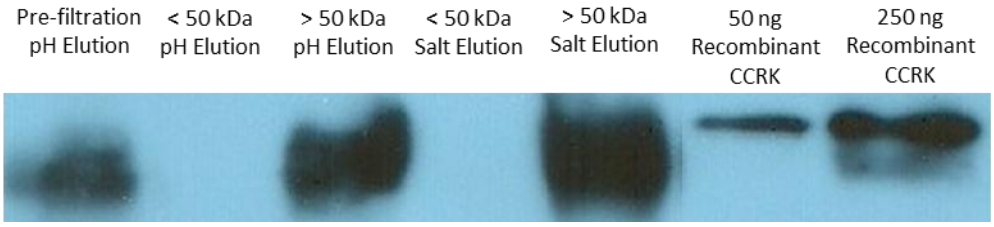

Figure 16 Western blot analysis of CCRK protein in U251 cells following immunoprecipitation and separation by a 50 kDa filter.

The under-50 kDa fractions, which should have had CCRK, did not have any apparent CCRK while the over-50 kDa fraction had IgG and what may be CCRK. This brings about three possibilities: the immunoprecipitation reaction never worked, the antibody/CCRK interaction is too strong to separate without denaturing, or CCRK's native structure prevented it from going through the 50 kDa membrane. Subsequent experiments tried 100 kDa Amicon filters, the addition of 1 M NaCl to destabilize the IgG/CCRK interaction, and multiple different immunoprecipitation antibodies but these experiments were also unable to isolate CCRK (data not shown).

The T160 peptide has a molecular weight of approximately 2 kDa. This makes it very difficult to catch on an SDS-PAGE gel. When ran on a high polyacrylamide % gel the T160 peptide is detectable as a 'smearing' that can be correlated to the amount of phosphorylated T160 (Figure 17).

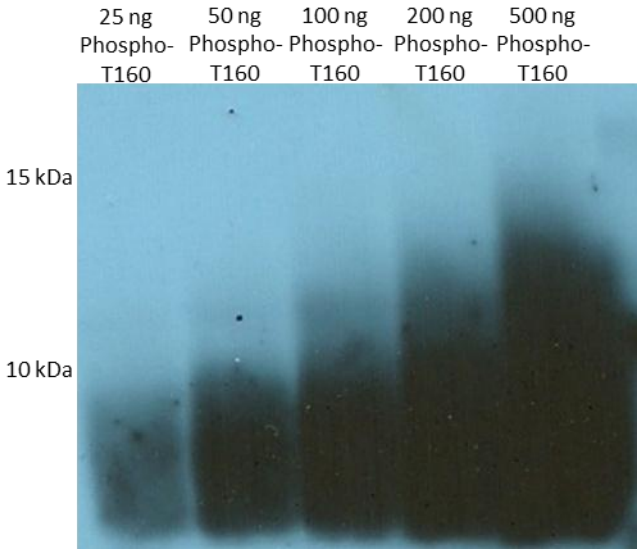

Figure 17 Western blot analysis of phosphorylated T160 protein ran out on an 18% polyacrylamide SDS-PAGE gel. Although 2 kDa proteins might have run off the gel, phospho-T160 causes a 'smearing' effect can be observed.

With this knowledge, an *in vitro* kinase assay was attempted using immunoprecipitation fractions. Phospho-T160 was then probed for by western blotting. The characteristic smear of the phospho-T160 was completely absent in immunoprecipitated CCRK + unphosphorylated T160 lanes (data not shown). This suggests three things: the immunoprecipitation reaction never worked, CCRK bound to IgG is inactive, or CCRK is inactive due to some other reason.

With an active CCRK for *in vitro* studies proving extremely difficult to obtain, we moved on to show that *S*-UM-BD-29 and UM-BD-54 inhibit CCRK through other methods.

<u>siRNA Knockdown of CCRK</u>

We hypothesize that siRNA knockdown of CCRK will cause cells to be more sensitive to apoptosis by *S*-UM-BD-29 and UM-BD-54 as shown by Alamar Blue. This synergistic result would show that CCRK is the apoptotic target of this class of compounds.

The DU145 human prostate cancer cell line was chosen for siRNA experiments because it is a sensitive cell line that is one of Dharmacon's suggested cell lines for transfection. That is, Dharmacon provides optimized transfection conditions and appropriate transfection reagent suggestions. To reduce off-target effects of siRNA knockdown that could confound results, ON-TARGETplus siRNA that contains special modifications were used. Dharmacon has a library of proteins for which there are siRNA target sequences, of which CCRK is one. In addition, Dharmacon offers SMARTpool siRNAs which are a mixture of four targeting sequences to further enhance target knockdown. We elected to proceed with our siRNA experiment using ON-TARGETplus SMARTpool CCRK, GAPDH (positive control), and a non-targeting pool (negative control).

DU145 cells were transfected according to the manufacturer's instructions and then CCRK and GAPDH protein levels were probed by western blotting (Figure 18).

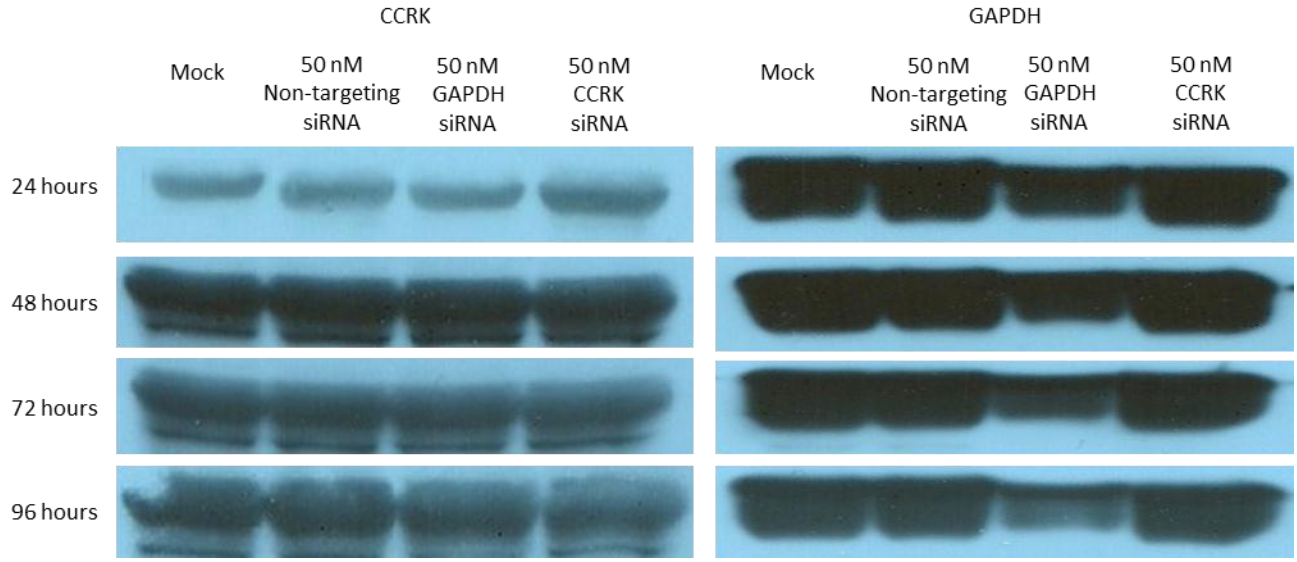

Figure 18 Western blot analysis of CCRK and GAPDH protein of DU145 cells post transfection. DU145 cells were transfected with 50 nM CCRK SMARTpool siRNA, 50 nM GAPDH siRNA, 50 nM non-targeting siRNA, or mock transfected with DharmaFECT 1. Cells were harvested at 24, 48, 72, or 96 hours post transfection.

Although the GAPDH positive control transfection worked, the CCRK transfection failed to knock down CCRK. We elected to try four additional cell lines, U87, HeLa, Ovcar 3, and 786-0 along with the maximal siRNA concentration suggested by Dharmacon, 100 nM. These experiments also did not result in CCRK knockdown (data not shown).

Dharmacon's siRNA library has not been verified experimentally. With this in mind, it is possible that the CCRK siRNA provided was simply ineffective. Dharmacon provided four different sequences from their non-ON-TARGETplus line and the experiment was repeated with DU145 cells and the same result – no CCRK knockdown (data not shown).

A few research labs have successfully knocked down CCRK [4, 6, 9, 10, 17]. Of these successful published sequences we selected two target sequences: 5'-GAAGGUGGCCCUAAGGCGG-3' [9, 10] and 5'-GGCGGUUGGAGGACGGCUU-3' [4, 6, 10, 17] to have synthesized for knockdown of CCRK. DU145 cells were transfected according to the manufacturer's instructions and then CCRK and GAPDH protein levels were probed by western blotting (Figure 19).

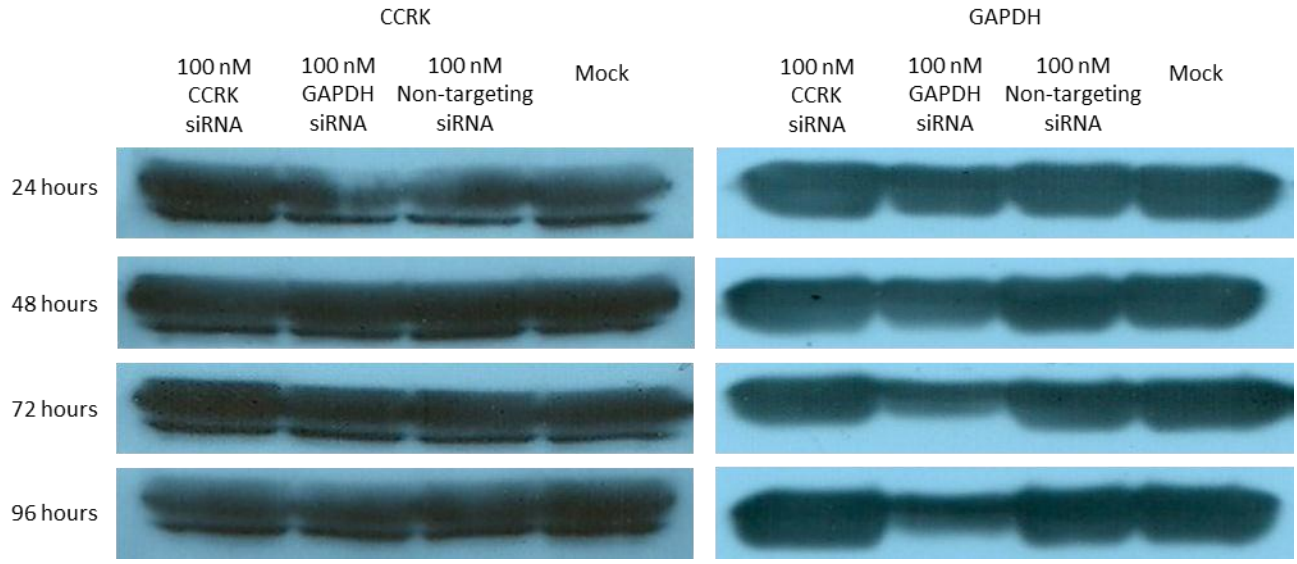

Figure 19 Western blot analysis of CCRK and GAPDH protein of DU145 cells post transfection. DU145 cells were transfected with 100 nM CCRK siRNA (both sequences), 100 nM GAPDH siRNA, 100 nM non-targeting siRNA, or mock transfected with DharmaFECT 1. Cells were harvested at 24, 48, 72, or 96 hours post transfection.

Once again, CCRK was not silenced. The successful knockdown of GAPDH shows that the transfection experiment itself is reliable – the procedure and transfection reagent should appropriately deliver the siRNA. As the transfection works and the sequences have been shown to knockdown CCRK previously, it is difficult to speculate as to why the CCRK knockdown failed. It is possible that this method of transfection is innately inefficient and a less than requisite nM siRNA is actually being transferred to the cell. It is also possible that there was an error in the synthesis of the two CCRK siRNA oligonucleotides. Despite altering variables, using multiple sequences, and having promising positive control results, CCRK knockdown remains elusive.

Discussion

CCRK is a novel, potent pro-survival protein that plays a crucial role in the eukaryotic cell cycle [4, 15]. The purpose of this study was to show that the apoptotic phenotype induced by UM-BD-54 and *S*-UM-BD-29 was caused by inhibition of CCRK. Although there is a significant amount of data that suggests this could be the case, some crucial experiments did not produce the data necessary to definitively show this.

The first set of data that suggests *S*-UM-BD-29 and UM-BD-54 target CCRK lies with *S*-UM-BD-29 already having been found to be a competitive ATP-binding site inhibitor of ROCK [23]. It is likely that the binding cleft of ROCK and the cytotoxic target are similar as *S*-UM-BD-29 targets both of them. Breitenlechner and colleagues found that the sensitivity of a ROCK inhibitor to the ROCK ATP-binding site versus Protein Kinase A's ATP-binding site was dictated by a unique combination of 4 binding site residues and sequence alignment showed that only 6 of the 491 known human kinases possess all 4 binding site residues: MUSK, MET, MST1R, CDC2L5, CRK7, and CCRK [2]. Of these 6 kinases, MUSK, MET, MST1R, and CDC2L5 were tested in the Ambit KINOMEscan and showed <20% inhibition at 10 μM UM-BD-29, while CRK7 shares 89% sequence identity with CDC2L5 and appears to be involved in RNA splicing and/or RNA polymerase II transcription [3]. This left CCRK as the only viable kinase target based on binding cleft similarity to ROCK. Fortunately CCRK proved to be a vital protein whose knockdown lead to cell arrest and cell death [4]. This makes CCRK a prime candidate for being the cytotoxic target.

In order to validate CCRK as the target we had to show that treatment with *S*-UM-BD-29 and UM-BD-54 elicited cellular signaling akin to CCRK inhibition. We began this validation by showing that *S*-UM-BD-29 and UM-BD-54 cause a significant decrease in CDK2

phosphorylation that is independent of caspase activation. Wohlbold et al., while finding that knockdown of CCRK impaired cell proliferation and induced apoptosis, did not find evidence that CCRK activates CDK2 and claimed that CDK7 was the only CAK capable of activating CDK2 [6]. Most other researchers, however, have directly shown that CCRK activates CDK2 by phosphorylation at Thr160 [4, 9, 10, 15]. Still others suggest that the mechanism by which CCRK regulates the cell cycle might be cell-type specific, observing that CCRK knockdown in ovarian carcinoma causes a nearly complete loss of cyclin D1 with no effect on phosphorylated CDK2 or PARP levels while the same group observed CCRK knockdown's hypophosphorylation effect on CDK2 in glioblastoma [10, 17]. Even if there is some debate as to whether CCRK achieves cell cycle regulation solely through phosphorylating CDK2, the only CAKs currently believed capable of phosphorylating CDK2 are CCRK and the CDK7/Cyclin H/MAT1 complex [4, 6]. As treatment with *S*-UM-BD-29 and UM-BD-54 cause a decrease in phosphorylated CDK2 levels it is very likely that either the CDK7/Cyclin H/MAT1 complex, CCRK, or both are inhibited. Our results have definitively shown in an *in vitro* kinase assay that UM-BD-54 has no inhibitory effect on CDK7/Cyclin H/MAT1. This leaves CCRK as the sole known CAK whose possible inhibition could result in the hypophosphorylation of CDK2 observed.

Furthermore, we've shown that UM-BD-29 induces G_1 to S phase cell cycle arrest in treated cells. CDK2 is known to be an important regulator of the G_1 to S phase transition and hypophosphorylation of CDK2 by inhibition of CCRK results in G_1 to S arrest, as Ng et al. observed in cells where CCRK was knocked done by siRNA [10]. Although G_1 to S cell cycle arrest can be brought on by other means, such as cellular stresses mediated by p53 activation,

there are a finite number of signals that can induce such an arrest [25]. This arrest is therefore another step toward verification of CCRK inhibition.

In this study we attempted to derive our own *in vitro* kinase assay with which to test CCRK inhibition by our compounds. All of the CCRK utilized, either from commercial sources or acquired via immunoprecipitation, proved to be kinase dead. It is also possible that, rather than kinase dead, the CAK activity of CCRK was so limited that it was simply undetectable. In fact, Liu et al. found the kinase activity of CCRK to be weak, especially when compared to the CDK7/Cyclin H/MAT1 complex [4]. There are a few reasons that the CCRK used in the *in vitro* kinase assay might have been either kinase dead or had undetectable CAK activity. CCRK might have been exposed to inhibitory factors located in the cytosol that inhibited it upon lysis, CCRK may not have retained the activate confirmation necessary for CAK activity, and it is also possible that CCRK simply doesn't have robust activity *in vivo* and *in vitro* [4]. In addition, it is possible that there is an unknown factor necessary for activity that wasn't able to be captured with CCRK during immunoprecipitation. Although an *in vitro* kinase inhibition assay was not feasible at this present time, it is possible that in the future, as knowledge of CCRK grows, an *in vitro* assay will be more viable. Commercial kinome scans continually increase their selection of human kinases and CCRK may one day become one of those tested, especially as the realization of its physiological importance grows.

Our results also show that there is a direct connection between cellular CCRK levels and sensitivity to *S*-UM-BD-29. If cellular CCRK protein is relatively high, cells tend to be more resistant to apoptosis induced by *S*-UM-BD-29, while if cellular CCRK protein is relatively low, cells tend to be more sensitive to apoptosis induced by *S*-UM-BD-29. It is unlikely that such a

correlation would exist if CCRK is not involved in the apoptotic signal transduction that takes place upon *S*-UM-BD-29 treatment.

In this study we attempted to knockdown CCRK via siRNA in order to assess sensitivity differences to *S*-UM-BD-29 and UM-BD-54. siRNA knockdown proved to be unsuccessful even while the transfection method proved successful for GAPDH knockdown and the CCRK sequences utilized had successfully knocked down CCRK for other groups. Those groups that transiently knocked down CCRK all used a lipid-mediated transfection reagent to achieve knockdown, but none used DharmaFECT transfection reagents as we did [4, 6, 9, 10, 17]. DharmaFECT lipid transfection reagents shouldn't have a vastly different effect than the lipid transfection reagents that other groups utilized, but it is possible that there is something about this transfection reagent in particular that contributed to the unsuccessful knockdown. It is also possible that there was an error in the synthesis of the siRNA CCRK oligonucleotides used.

Future research in this area could try to bring about CCRK knockdown by experimenting with alternative siRNA delivery methods. It should also be possible to show that CCRK is directly involved with *S*-UM-BD-29 and UM-BD-54 induced apoptosis by electing to overexpress CCRK rather than transiently knock it down. The overexpression of CCRK should convert otherwise sensitive cell lines to resistant cell lines and make those already resistant even less sensitive. If either of these experiments were successful one would be able to definitively show that *S*-UM-BD-29 and UM-BD-54 induce apoptosis through inhibition of CCRK.

The relevance of this work lies in the therapeutic possibilities of a potent and specific CCRK inhibitor. Discovering the target that ultimately leads to apoptosis would enable the design of a compound that is capable of treating cancer cells that rely on over-expression of CCRK for survival without targeting the general CAK CDK7 complex. CCRK has been linked

to many cancers and overexpression has been shown to turn otherwise non-carcinogenic cells to carcinogenic cells whilst inhibition has the opposite effect [4, 10]. In addition, once the crystal structure of CCRK is known, it will be possible to improve upon the structures of *S*-UM-BD-29 and UM-BD-54 by making even more potent and specific derivatives guided by molecular modeling.

Acknowledgements

This thesis could not have been completed without the help and support of everyone that I have worked with during these last three years in the Glick lab. I would like to thank Gary Glick first and foremost for his guidance, intellect, and zeal. Gary alone provided this amazing opportunity. None of this would have been possible without him and it is only through his help and understanding that I was able to complete this work.

A very special thank you goes to Joanne Cleary and Tasha Francis, whom I worked with nearly every day at the Glick lab. Joanne and Tasha had nothing but the highest standards for me and inspired excellence. Whenever I had questions Joanne and Tasha were always there to lend a hand. Joanne also taught me virtually every biological assay I needed and was always there to provide experimental advice.

In addition I would like to thank all of the other current and former Glick lab members – Tony Opipari, Lara Swenson, Rodney Morgan, Li Wang, Dan Wahl, Tom Sundberg, and Lauren Dixon – whom I had the pleasure of working with. All of these people provided help and guidance at some point during my journey.

Lastly I would like to thank my family and friends. My parents Bill and Sue and my brother Tim have been a never ending source of encouragement and love, always believing that I could do whatever I set out to do. My friends Ken Childers, David Almassy, Jay Quick, Huiyan Jin, Alina Hritz, and Meghan Wiggins constantly put up with seemingly never-ending hours at the lab and are always there to provide emotional support. I could never have done this without you all.

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