Published in final edited form as:
Anticancer Agents Med Chem. 2013 May 1; 13(4): 584–594.

FAK and HAS Inhibition Synergistically Decrease Colon Cancer Cell Viability and Affect Expression of Critical Genes

Melissa Heffler[1], **Vita Golubovskaya**[1], **Jeffrey Conroy**[2], **Song Liu**[3], **Dan Wang**[3], **William Cance**[1], and **Kelli B. Dunn**[4,*]

[1]Department of Surgical Oncology Roswell Park Cancer Institute and the University at Buffalo/ State University of New York, Buffalo, NY

[2]Microarray Core Facility, Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY 14263

[3]Bioinformatics Core Facility, Biostatistics, Roswell Park Cancer Institute, Buffalo, NY 14263

[4]Division of Colon and Rectal Surgery, University of Louisville, Louisville, KY

Abstract

Focal adhesion kinase (FAK), hyaluronan (HA), and hyaluronan synthase-3 (HAS3) have been implicated in cancer growth and progression. FAK inhibition with the small molecule inhibitor Y15 decreases colon cancer cell growth *in vitro* and *in vivo*. HAS3 inhibition in colon cancer cells decreases FAK expression and activation, and exogenous HA increases FAK activation. We sought to determine the genes affected by HAS and FAK inhibition and hypothesized that dual inhibition would synergistically inhibit viability. Y15 (FAK inhibitor) and the HAS inhibitor 4-methylumbelliferone (4-MU) decreased viability in a dose dependent manner; viability was further inhibited by treatment with Y15 and 4-MU in colon cancer cells. HAS inhibited cells treated with 2μM of Y15 showed significantly decreased viability compared to HAS scrambled cells treated with the same dose ($p<0.05$) demonstrating synergistic inhibition of viability with dual FAK/HAS inhibition. Microarray analysis showed more than 2-fold up- or down-regulation of 121 genes by HAS inhibition, and 696 genes by FAK inhibition ($p<0.05$) and revealed 29 common genes affected by both signaling. Among the genes affected by FAK or HAS3 inhibition were genes, playing role in apoptosis, cell cycle regulation, adhesion, transcription, heat-shock and WNT pathways. Thus, FAK or HAS inhibition decreases SW620 viability and affects several similar genes, which are involved in the regulation of tumor survival. Dual inhibition of FAK and HAS3 decreases viability to a greater degree than with either agent alone, and suggests that synergistic inhibition of colon cancer cell growth can result from affecting similar genetic pathways.

Keywords

Colon cancer; FAK; gene expression; hyaluronan; silenced RNA; viability

INTRODUCTION

Colorectal cancer is the third leading cause of cancer death in the United States [1]. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is overexpressed in many types

*Address correspondence to this author at the Division of Colon and Rectal Surgery, University of Louisville, Louisville, KY; Tel: (502) 583-8303; Fax: (502) 584-0302; kbdunn01@louisville.edu.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

of cancer, including colon cancer [2–5]. FAK was initially identified at sites of contact between cells and the extracellular matrix, and is known to play a central role in signaling pathways involved in cell proliferation, survival, motility and invasion; several process intrinsic to tumorigenesis [6]. FAK's role in these processes requires its autoactivation, or tyrosine autophosphorylation at the Y397 site. This unique manner in which FAK auto-activates allows for selective inhibition of FAK signaling by blocking the Y397 site and thus blunting its activation. Previous work has demonstrated that Y15 (1,2,4,5-benzenetetraamine tetrahydrochloride) targeted Y397 site of FAK, inhibited FAK autophosphorylation and colon cancer cell viability and growth *in vivo*, and increases apoptosis (not published). Therefore, we sought to determine the genes that are affected by FAK inhibition in this manner, and identify mechanisms underlying its efficacy as an anti-tumor agent.

Hyaluronan (HA) is another cellular element that is involved in cancer growth and progression. A high molecular weight cell surface glycosaminoglycan, HA is synthesized by three enzymes, or hyaluronan synthases (HAS1, 2, and 3) [7]. HAS3 in particular is overexpressed in the metastatic colon cancer cell line, SW620 [8]. We have shown previously that HAS3 inhibition in the primary colon cancer cell line HCT116, by way of transfection with siRNA to HAS3, decreases tumor growth and increases apoptosis [9]. We have also demonstrated that inhibition of HAS3 decreases FAK expression and activation (phosphorylation) in SW620 colon cancer cells, and that the addition of HA to these cells increases FAK phosphorylation, or activation (not published). Taken together, these data suggest that HAS/HA inhibition can blunt tumor growth by affecting apoptosis and HA is involved in the activation of FAK signaling, thus explaining the tumorigenic effects of these molecules. With previous work as a foundation, we determined the effect of HAS inhibition alone and in combination with FAK inhibition with Y15, on the viability of the metastatic cell line, SW620. We utilized two different methods of HAS inhibition: transfection with siRNA to HAS3, and treatment with the HAS inhibitor 4-methylumbelliferone (4-MU). In this manner we analyzed both genetic and pharmaceutical means of HAS inhibition. We also analyzed the gene expression analysis in cells transfected with siRNA to HAS3 and in cells treated with FAK inhibitor Y15 to identify the mechanisms underlying its anti-tumor efficacy and reveal common genetic pathways that link HA and FAK signaling in tumor cells. HAS inhibited cells treated with as little as 2μM of Y15 showed significantly decreased viability compared to HAS scrambled cells treated with the same dose ($p<0.05$) demonstrating synergistic inhibition of viability with dual FAK/HAS inhibition. Microarray analysis showed more than 2-fold up- or down-regulation of 121 genes by HAS inhibition, and 696 genes by FAK inhibition ($p<0.05$) and reveal common genes affected by both inhibition suggesting that FAK and HAS signaling pathways are genetically linked in colon cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

To study the effect of FAK and HAS inhibition in colon cancer, we chose to focus our work on the metastatic cell line, SW620. Derived from lymph node metastasis, this cell line over-expresses HAS3, one of the enzymes responsible for the synthesis of HA [8]. We purchased cells from ATCC and maintained them according to the company's recommendations. Cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (D-MEM) + 10% fetal bovine serum + 1% penicillin/streptomycin. HAS3-inhibited cells were maintained under identical conditions.

Antibodies

FAK monoclonal antibody (FAK 4.47) was obtained from Millipore Inc and anti-pY397 FAK antibody was obtained from Invitrogen, Carlsbad, CA.

HAS Inhibition

We used two methods to inhibit HA. Small interfering (si) RNA to HAS3 was transfected into our cells (HAS-silenced) and a scrambled sequence was used as control (HAS-scrambled). PCR after transfection was done to confirm HAS inhibition. We also inhibited HA by treating our cells with the HAS inhibitor 4-methylumbelliferone (4-MU; Sigma-Aldrich, St. Louis, MO). 4-MU is thought to function by inhibiting HAS2 and HAS3. It has shown efficacy in decreasing cell migration, invasion, proliferation and tumor growth [10–12]. 4-MU was suspended in methanol as per company recommendations, and maintained at room temperature. It was then diluted in D-MEM at the time of cell treatment.

FAK Inhibition

The small molecule Y15 (1,2,4,5-Benzenetetraamine tetra- hydrochloride; Sigma-Aldrich, St. Louis, MO) was used to inhibit FAK autophosphorylation in SW620 cells. It was diluted in PBS to a stock concentration of 25mM, and then further diluted in D-MEM at the time of cell treatment. Y15 was stored at −20°C.

Cell Viability

The wild type SW620 cells were seeded onto a 96 well plate (5×10^3 cells per well in 100μL of D-MEM+10% FBS+1% penicillin/streptomycin) and incubated overnight. Cells were then treated with various doses of Y15 (1, 2, 4, 10μM) alone or in combination with the HAS inhibitor 4-MU (1, 10, 50, 100, 200μM). After 24 hours of incubation, 20μL of Cell Titer 96 Aqueous One Solution Proliferation Assay (Promega Corporation, Madison, WI) was pipetted into each well. Cells were incubated for one hour with the reagent, and then the plates were read using the Gen 5 1.07 microplate reader at 490nm. Similarly, HAS-silenced and HAS scrambled cells were seeded onto 96 well plates at 5×10^3 cells per well in 100μL of medium. After allowing the cells to attach overnight, we treated them with various doses of Y15. Cells were again left to incubate for 24 hours following treatment, and then 20μL of the MTT reagent (Promega Corporation, Madison, WI) was added to each well. Again, after 1 hour of incubation with the reagent, the plates were read using the microplate reader at 490nm.

DNA Microarray Analysis

After determining the effect of FAK/HA inhibition on cell viability we looked at genetic alterations following treatment to explore a potential mechanism. 100mm plates were seeded with 6×10^6 wild type, HAS-silenced and HAS-scrambled SW620 cells and left overnight in the incubator to attach. After 24 hours of incubation, one plate of wild-type cells was treated with 4μM of Y15 and another was left untreated. Both plates were then left to incubate for another 24 hours. After 24 hours, cells were collected and submitted to the gene microarray facility for gene expression profiling using the HumanRef 8 whole genome gene expression array and direct hybridization assay (Illumina, Inc., San Diego, CA). cDNA was derived from 500ng of total RNA. Then using the Ambion Illumina Total Prep RNA Amplification Kit (Ambion, Inc.), the cDNA was used to make biotin-labeled cRNA *via in vitro* transcription. Illumina HumanRef-8 v3 Bead Chips were produced by labeling and hybridizing probes overnight at 58°C, and then to measure intensity of fluorescence of each probe, the Bead Chips were analyzed using the Illumina Bead Array. Data are deposited into the NCBI Database with GEO accession number GSE39168.

Bioinformatics and Statistical Analysis

The data generated from microarray were analyzed by the R-based Bioconductor package. Applying the lumi model, the expression intensity was converted to a log2 scale and then normalized with the Quantile normalization algorithm. Then, the degree of expression for each gene was calculated with the limma program, and a significant difference in gene expression was determined using at least a 1.2- fold change and a p-value <0.05.

RT-PCR

RNA that was isolated from our Y15-treated and untreated SW620 cells, as well as the HAS-silenced and HAS-scrambled cells was used with the TaqMan One-Step RT-PCR Master Mix (Applied Biosystems; Branchburg, NJ) for DNA amplification. RT-PCR primers were purchased from Eurofins MWG Operon (Hunstville AL) and probes were purchased from Biosearch Technologies (Novato, CA). Table 1 shows the sequences of the primers and probes used. GAPDH was used as an endogenous control. 2ng/μL of RNA diluted in DEPC H_2O from each RNA sample was combined with 40x Multiscribe Reverse Transcriptase and RNase Inhibitor Mix, and 2x Master Mix (without UNG), as well as 10μM of forward primer and reverse primer, and 20μM of TaqMan probe and amplified by RT-PCR standard protocol. Samples were analyzed in triplicate and the threshold cycle number (Ct) was calculated for each amplification using the ABI PRISM 7700 cycler's software.

Immunohistochemistry

1×10^5 SW620 cells were seeded onto circular cover slips that were placed into the wells of a 24-well plate. Cells were attached overnight in the incubator, and then were fixed in 0.5ml of 3.7% formaldehyde. Then over ice, permeabilizing solution was added to each well (0.5ml). After 3 minutes on permeabilizing solution, the cells were washed and then blocked in 10μL of 25% goat serum for 20 minutes. Primary anti-FAK or anti-pY397 FAK antibody (1:200) was then applied for 30 minutes. Following primary antibody incubation, cells were washed and incubated in secondary antibody (1:200) for 30 minutes in dark conditions. For cells exposed to anti-FAK primary, Texas Red goat anti-mouse secondary was used, and for cells exposed to anti-pY397 FAK primary, Alexa Fluor 594 goat anti-rabbit secondary was used. FITC conjugated phalloidin (1:25; Molecular Probes, Eugene, OR) was used to identify actin and Hoechst was used for nuclear staining. After transferring the circular coverslips to slides, the samples were examined using a Zeiss Observer.A1 microscope at 100X magnification.

RESULTS

FAK and HAS Inhibition Synergistically Affect Cell Viability in a Dose-dependent Manner

We tested the effect of inhibiting FAK and HA on the viability of the metastatic SW620 colon cancer cell line. We looked at the efficacy of each inhibitor alone and in combination to see if dual FAK/HA inhibition would synergistically inhibit viability. We did a series of MTT viability assays on cells treated with Y15 and cells with decreased HAS *via* transfection with siRNA to HAS3 or treatment with the HAS inhibitor 4-MU.

Y15 and 4-MU inhibited cell viability in a dose-dependent manner (Fig. 1A, left). Y15 significantly inhibited cell viability starting at a dose of 2μM (p<0.05), and as the dose was increased to 10μM, viability decreased further (Fig. 1A). Similarly, 4-MU decreased SW620 viability in a dose-dependent manner, with a significant decrease in viability seen at a dose of 100μM that was further decreased at 200 μM dose (Fig. 1A, right; p<0.05). Thus, inhibition of FAK and inhibition of HAS decreased viability of cells in a dose-dependent manner.

Because our data showed an effect of HAS inhibition on viability, and we also showed that Y397 FAK inhibition with Y15 also decreases viability, immunocytochemistry was done on cells to visualize the effect of 4-MU on FAK and Y397 FAK (Fig. 1B). Untreated cells demonstrated robust FAK as evidenced by red staining in the top panel. FAK is clearly cytoplasmic, and at the periphery of the cell at focal adhesions. However after treatment with 4-MU, focal adhesions, and FAK staining in general are dramatically reduced. When probed for Y397 FAK, the untreated cells also demonstrated visible activated protein and the speckling represents focal adhesions. However, after treatment with 4-MU, there is significantly less Y397 FAK in the cells and focal adhesions are diminished. Thus, 4Mu inhibitor decreased Y397-FAK and FAK at focal adhesions and caused cell rounding.

We have shown that FAK inhibition decreases viability, and HAS inhibition decreases viability and FAK and Y397 FAK in colon cancer cells. Therefore, we next looked at the effect of dual FAK and HAS inhibition on cell viability. When 2μM of Y15 was added to 10μM of 4-MU, there was a significant decrease (greater than 50%) in cell viability ($p<0.05$; Fig. 1C). These data were further supported by the MTT viability assay on cells treated with Y15 following transfection with siRNA to HAS3. Fig. (1D) shows that 2μM of Y15 significantly decreased the viability of HAS-silenced cells compared to scrambled or untreated controls ($p<0.05$). These data show that dual FAK/HAS inhibition synergistically and significantly inhibits the viability of colon cancer cells.

FAK Inhibition with Y15 Significantly Affects Expression of 696 Genes More than two Fold in SW620 Cells

After demonstrating that dual FAK/HA inhibition synergistically inhibits colon cancer cell viability, we looked at the genetic effects of inhibiting FAK to investigate a potential mechanism by which Y15 works. To determine the effect of FAK on gene expression in a malignant phenotype, we performed microarray gene expression analysis on SW620 untreated and treated with the small molecule Y15, inhibiting FAK autophosphorylation. We detected 696 genes that were more than 2-fold up- or down-regulated by FAK inhibition compared with untreated cells ($p<0.05$). Fig. (2A) shows the heatmap generated by Y15-treated versus untreated SW620 cells. Table 2 lists the selected genes that were at least 2-fold up- or down-regulated by FAK inhibition with Y15. These genes included heat shock protein-encoding genes, which are involved in regulation of apoptosis and cell survival under stress, FOX protein-encoding genes, which also regulate apoptosis, and other genes involved in processes like heat-shock response, proliferation, cell cycle regulation, adhesion and growth (Table 2).

RT-PCR confirmed the results of the microarray analysis comparing untreated cells to FAK-inhibited cells. Fig. (2B) shows the up-regulation of 6 genes (SFN, SNAIL, ETS-1, HSPA1, DKK and RGS2), and down-regulation of two genes (CBS and GRTP1), which confirms and validates the findings of microarray assay (Table 2).

Additional support for our microarray data was demonstrated in the Western blot of SW620 cells treated with Y15, and then probed for heat shock protein. Fig. (2C) shows Hsp 70/72 levels in SW620 cells treated for 24 hours with Y15. Expression of Hsp 70/72 significantly increases in a dose-dependent manner in Y15-treated cells compared with untreated cells (Fig. 2C). Thus, Western blotting validated micro array and RT-PCR data and showed that Y15 increases expression of heat shock 70 protein in SW620 cells.

HAS3 Inhibition Significantly Affects Expression of 121 Genes in SW620 Cells

We also analyzed the genes that were affected by transfection with siRNA to HAS3 compared with scrambled control cells by microarray analysis. SW620 cells transfected with

siRNA to HAS3 showed a more than 2-fold up- or down-regulation of 121 genes compared with scrambled control cells ($p<0.05$). The list of selected down- and up-regulated genes is shown in Table 3. Fig. (3A) shows the heat map generated by the HAS3-silenced and scrambled cells. Similar to the Y15-treated cell, we found many genes affected by HAS3 inhibition to be involved in potentially malignant process like control of apoptosis, proliferation, growth, adhesion and cell cycle regulation (SFRP5, ANXA1, FOX genes, TNFRSF4, CEBPB, HSP genes, KLF4, IL8, NBL1, RGS2, WT1, TRIB3, PPP1R14C, CEACAM6, PDLIM1, ALCAM, GRTP1, FGFBP1, HBEGF, INHBE). RT-PCR confirmed the microarray results. Fig. (3B) demonstrates the up-regulation of RGS2 and SNAI2 genes, and the down-regulation of CBS and GRTP1 genes, confirming the microarray data.

FAK and HAS3 Inhibition Affect the Expression of Several Common Genes

To examine if similar genetic pathways were affected by inhibition of either protein, we looked at the genes that were similarly affected by Y15 or by transfection with siRNA to HAS3. We found that 29 genes were common to both inhibitors. Interestingly, among these 29 genes, 9 were down-regulated by FAK and HAS3 inhibition and four were up-regulated more than 2-fold ($p<0.05$; Fig. 4). Several of the genes that were common to both FAK and HAS3 inhibition are of interest with regard to tumorigenesis (ANXA1, RGS2, and GRTP1). Thus, detection of common genes, affected by FAK and HAS3 inhibition demonstrates the cross-signaling and intersection of genetic pathways involved in these cellular processes.

DISCUSSION

FAK and HAS/HA are involved in colon cancer growth and progression. HAS/HA inhibition has been shown to blunt tumor growth and increase apoptosis in a primary colon cancer cell line [9]. Our previous work has shown that decreasing HA in SW620 cells inhibits FAK activation and the addition of HA increases FAK activation. The present data demonstrate a strong correlation between HA and FAK activation and the data presented herein show that blocking both signaling molecules synergistically inhibits colon cancer cell viability. We detected gene profiles in FAK and HAS3-inhibited cells. Some of these genes are involved in malignant process such as protein tyrosine kinase signaling (RGS2), cell cycle regulation, proliferation (ANXA1), and transcription (SNAI2). The synergistic anti-tumor effect seen with dual inhibition of FAK and HA can be either due to the up- or down-regulation of similar genetic pathways or due to up- or down-regulation of divergent genetic pathways that when simultaneously blocked result in a greater anti-tumor effect.

We detected the heat shock proteins (Hsp's) were increased by both FAK and HAS3 inhibition. Hsp's are present in normal tissue, but become overexpressed when environmental or internal stimuli threaten cell viability and are known to be over expressed in many types of cancers, including colorectal cancer [13]. Hsp's perform a number of functions that promote cell viability in the presence of stressors like increased temperature, hypoxia and chemotherapy, or internal abnormalities like genetic mutations. They function in protein folding and chaperoning, transport and stabilization, and sequester abnormal proteins for degradation to allow cellular viability to persist, and the cell to escape apoptosis, under naturally lethal conditions. Hsp's are classified based on their molecular weight, and the proteins that were affected by FAK and HAS3 inhibition in the present study belong to three subfamilies, 40kDa (DNAJA4, DNAJB9, DNAJB1, DNAJC15), 70kDa (HspA1B, HspA1A and Hsp A12A), and 105kDa (Hsph1). While HAS3 inhibition affected HspA12A and DNAJC15, Y15 affected the expression of seven different Hsp proteins (HspA1A, HspA1B, DNAJA4, DNAJB9, DNAJB1, Hsph1 and HspA12A). and HspA12A was up-regulated by HAS3 inhibition but down-regulated by Y15. The 70kDa HspA1A and HspA1B proteins are comprised of an ATP-binding domain and a substrate binding domain, but the HspA12A protein differs from them due to a different substrate binding domain [13].

This permits slightly different function of this specific protein compared to the other 70kDa Hsp's. In general, the substrate binding domain of the 70kDa proteins binds denatured or unfolded proteins and prevents ubiquitination and degradation or facilitates refolding in an effort to maintain their function within the cell. If this approach fails, Hsp70 and Hsp40 promote protein ubiquitination and degradation [13]. When substrate binding occurs, the Hsp induces ATP hydrolysis to fuel protein degradation. Interestingly, the Hsp70 proteins are weak hydrolysers and thus Hsp40 operates as a co-chaperone in this function.

Following HAS3 or FAK inhibition, it is not surprising that heat shock protein expression were enhanced. Such genetic or chemical stress stimulated the rescue efforts of Hsp's to counteract the potentially lethal modifications in cellular homeostasis. As such, an interesting approach to cancer treatment is targeting this cell rescue response and inhibiting the heat shock response in the face of cytotoxic or targeted therapeutics. Several Hsp inhibitors are under investigation and the majority of them target the Hsp90 family of proteins, but inhibition of Hsp90 can induce the up-regulation of Hsp70 proteins [13]. Combination therapy which blocks the heat shock response provoked by cytotoxic or targeted therapeutics, and prevents the intrinsic cellular rescue response in the presence of what should naturally be lethal conditions should enhance our approach to selective cancer treatment.

The FOX proteins are another example of genes similarly affected by FAK inhibition and HAS3 inhibition. The Forkhead Box (FOX) proteins are derived from 41 known genes and possess tissue-specific roles, functioning as transcription factors, tumor suppressors, oncogenes, or immune regulators. While FoxP proteins are primarily involved in immune function, the FoxO and FoxM family of proteins have been implicated in cancer-related processes like cell proliferation, migration and invasion. Specifically, FOXM1 proteins act as transcription factors that specifically regulate passage from G1-S and G2-M phases of the cell cycle. FOXM1 phosphorylation activates its transcriptional activity, which leads to the production of several proteins that regulate progression through the cell cycle (eg. cyclin A, cyclin B, NEK2 and KIF20A). In addition, FOXM1 has been implicated in breast cancer development because it plays a role in regulating the transcription of the gene that encodes for estrogen receptor (ER). On the other hand, FoxO proteins are involved in cell cycle arrest at the G1 phase and induction of apoptosis. As such, its deletion, or inactivity is involved in cancer cell survival and resistance to apoptosis and this has been observed in many prostate cancers [14]. In the present study, FOXO3 was more than 2-fold up-regulated by FAK inhibition with Y15. Interestingly, this protein in particular has been identified in DLD1 colon cancer cell lines and using microarray analysis, found to induce the up-regulation of the Mad/Mxd tumor repressor family of proteins [15].

The FOXD1 gene was also up-regulated by FAK inhibition with Y15. Much understanding of the role of FOXD proteins centers on normal embryonic development. Interestingly, FAK is required for normal FOXD1 function as a guide for the appropriate migration and development of neural crest cells [16]. The further study the role of FOXD1 in carcinogenesis and determine if inhibition of FAK activation induces its expression or if it is constitutively up-regulated in colon cancer can provide a focus for future targeted therapy.

The gene, RGS2, was also up-regulated by both FAK and HAS inhibition. Encoding for proteins that regulate G protein signaling (RGS), the presence, or up-regulation of these gene is actually of benefit to the cell with regard to escaping malignant transformation. The RGS family of proteins is comprised of 30 members that are grouped into 5 subgroups. The RGS2 protein is of the B/R4 family, so classified by its structure and sequence homology with other similar proteins. Because they control protein kinase signaling, their down-regulation has been implicated in recurrent or metastatic colorectal cancer, and hence, up-

regulation postulated to be a good prognosticator for disease-free survival. Jiang *et al.* [17] studied RGS2 expression in both colorectal cancer cell lines and colorectal cancer tissue. By RT-PCR and immunohistochemical staining, RGS2 mRNA was down-regulated in the metastatic tumor cell lines and the tissue samples associated with recurrent disease. Moreover, tumor samples with lower RGS2 expression were linked to longer disease-free survival [17]. Induction of RGS2 expression by both HAS3 and FAK inhibition shows the potential mechanism by which our two inhibitors function as anti-tumor agents.

Conversely, one gene involved in the regulation of transcription, which was up-regulated by Y15 but not significantly affected by HAS3 inhibition, is the ETS1 gene. This gene encodes for the v-ets erythroblastosis virus E26 oncogene homolog 1. The family of Ets transcription factors is a target of the Wnt signal pathway, which is abnormally activated in many types of cancers, including colon cancer [18]. Munera *et al.* showed that deficiency of Ets2 in colon cancer stem cells actually promoted tumor establishment in mice [18]. In the present study, we showed that Y15 increased ETS2 gene expression that suggests a mechanism by which Y15 inhibits cancer stem cell proliferation and tumoigenesis.

In addition, Y15 increased DKK1 (dickkopf homolog 1), inhibitor of WNT pathway, playing important ole in stem cell regulation that also can explain antitumor activity of FAK inhibitor. Y15 also down-regulated WNT pathway genes, such as LRP5 and frizzled homolog 2, FZD2. Interestingly, down-regulation of FZD2 was also observed in MCF-7 breast cancer cells stably tansfected with FAKsiRNA confirming important role of FAK in WNT pathway. MCF-7 cells with transfected FAK siRNA expressed less beta-catenin mRNA by RT-PCR analysis. The secreted frizzled-related protein 5 (SFRP5) is one of several proteins in the SFRP family that control Wnt pathway signaling, specifically by preventing it from binding to its receptor, frizzled, and thus maintain apoptosis. Wnt signaling induces increased levels of β-catenin to reach the nucleus and induce the transcription of several genes that can promote cancer progression. Many cancer types, including colon cancer demonstrate abnormal activation of Wnt signaling, thus up-regulation of SFRP can be thought to control this tumorigenic process [19]. In the present study, the SFRP5 gene was up-regulated by both FAK inhibition and HAS3 inhibition, thereby shedding light on one of the potential mechanisms by which our intervention effectively inhibited tumor cell viability. These data are consistent with recent report on cross-linked signaling of FAK and WNT3 pathways [16].

The gene encoding for the laminin protein, which regulates cell adhesion, was also up-regulated by Y15. HAS3 inhibition did not affect laminin expression. This singular effect of Y15 exemplifies the role FAK plays in cell adhesion. First identified at contact sites between cells and the extracellular matrix, FAK integrin activity plays a key role in cell unity and locality. Which when lost can promote tumor cell metastasis. Up-regulation of laminin proteins maintains cellular adhesion and given that Y15 increases this activity, our data indicate that at the genetic and protein level, Y15 is actively promoting activities to prevent tumor cell metastasis.

Y15 also affects the expression of several genes that regulate phosphorylation of proteins. One of these genes is the PPP1R15A gene, which encodes for protein phosphatase 1 protein, which inhibits protein phosphorylation. This is integral to the efficacy of Y15 as a FAK inhibitor, because it functions primarily to prevent Y397 autophosphorylation and downstream signal protein phosphorylations. It is in this manner, that Y15 effectively blunts the malignant FAK cascade, and inhibits viability in such an effective manner as we have demonstrated in the present study. PPP1R14C was down-regulated by HAS3 inhibition, and thus one would conclude that the anti-tumor activity of this protein utilizes different mechanisms. In addition, HAS3 inhibition alone decreased the expression of the anti-

apoptotic gene CEBPB. The gene encodes for the CCAAT/enhancer binding protein, which, in addition to regulating transcription, also works as an anti-apoptotic protein. HAS3 increases the expression of a gene that induces apoptosis and decreases the expression of a gene that inhibits apoptosis.

HAS3 inhibition also increases the expression of the TNFRSF4 gene. This gene encodes for tumor necrosis factor receptor 4 protein, which forms a transmembrane receptor on active and senescent T-cells. Signaling *via* this receptor has anti-tumor activity, and agonists can enhance anti-tumor immunity [20]. HAS3 inhibition, as we have shown, similarly promotes this receptor for T-cell activity and as such indicates a role for HAS3 in promoting anti-tumor immunity. While Y15 does not affect this gene in a similar manner, this is an example of how dual FAK/HAS3 inhibition works synergistically to inhibit viability. While blocking one protein affects a certain set of genes, and not others, the addition of inhibiting a second protein in the same signal cascade knocks out supplementary genes that can augment their anti-tumor effects.

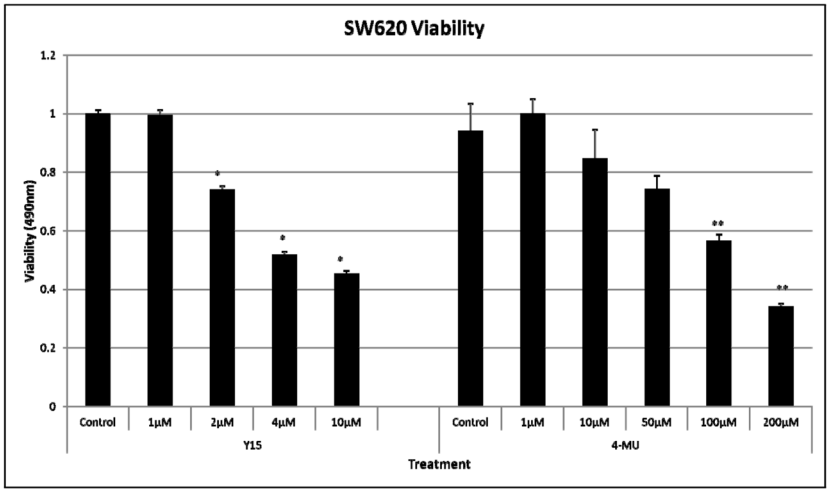
Indeed, our past work has shown the relationship between HA and FAK in colon cancer cells consists of HA-induced FAK activation. And previous work on glioma invasion suggests that tumor cell motility is due to MMP-9 induction *via* HA stimulation of the Ras/FAK/ERK 1,2 pathway [21]. In addition, a study of the relationship between low molecular weight HA and FAK has demonstrated a potential signal cascade by way of the receptor for hyaluronan mediated motility (RHAMM) in fibrosarcoma cells [22]. Certainly, there is data suggesting that HA and FAK signaling in tumorigenesis are linked in different tumor types and the genetic similarities seen following inhibition of FAK and HA in colon cancer cells indicate that there is a common pathway at work. At some juncture in tumor cell signaling, both FAK and HA potentiate common, or parallel malignant signals. There is also up- or down-regulation of several genes that are mutually exclusive to FAK or HA inhibition. Therefore, while there is commonality between the genetic effects of FAK and HA inhibition, blunting of either signaling molecule has a unique genetic impact and thus, when we inhibit both molecules concomitantly, we see synergistic anti-tumor effects. As such, these data indicate that dual FAK/HA inhibition is as an effective therapeutic mode of treating colon cancer.

References

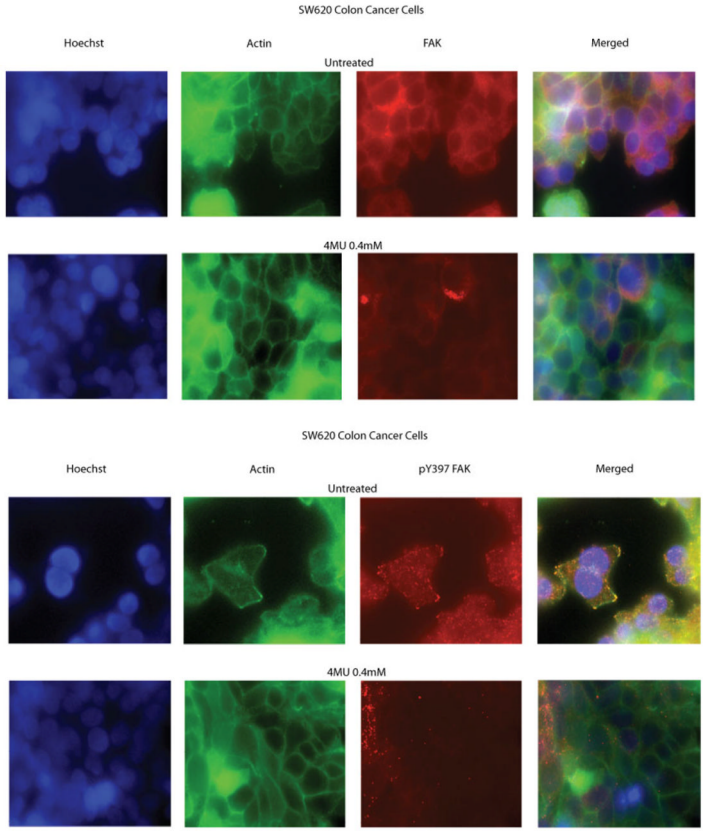
1. American Cancer Society. Colorectal Cancer Facts & Figures 2011–2012. American Cancer Society; 2011.
2. Golubovskaya V, Kweh FA, Cance WG. Focal adhesion kinase and cancer. Histology and Histopathology. 2009; 24(4):503–510. [PubMed: 19224453]
3. Figel S, Gelman I. Focal adhesion kinase controls prostate cancer progression *via* intrinsic kinase and scaffolding functions. Anticancer Agents in Medicinal Chemistry. 2011; 11(7):607–616.
4. Cance W, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, Simkins S, Xu L. Immunohistochemical analysis of focal adhesion kinase expression in benign and malignant human breast and colon tissues: Correlation with preinvasive and invasive phenotypes. Clinical Cancer Research. 2000; 6(6):2417–2423. [PubMed: 10873094]
5. Golubovskaya, V.; Cance, WG. International Review of Cytology. 2007. Focal adhesion kinase and p53 signaling in cancer cells; p. 103-153.
6. Golubovskaya V, Nyberg C, Zheng M, Kweh F, Magis A, Ostrov D, Cance WG. A small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride targeting the Y397 site site of focal adhesion kinase decreases tumor growth. Journal of Medicinal Chemistry. 2008; 51:7405–7416. [PubMed: 18989950]
7. Watanabe K, Yamaguchi Y. Molecular identification of a putative human hyaluronan synthase. Journal of Biological Chemistry. 1996; 271:22945–22948. [PubMed: 8798477]

8. Lai E, Singh R, Teng B, Zhao Y, Sharratt E, Howell G, Rajput A, Bullard Dunn K. Inhibition of hyaluronan synthase-3 decreases subcutaneous colon cancer growth in mice. Diseases of the Colon and Rectum. 2010; 53(4):475–482. [PubMed: 20305449]
9. Teng B, Heffler MD, Lai EC, Zhao YL, LeVea CM, Golubovskaya VM, Bullard Dunn KN. Inhibition of hyaluronan synthase-3 decreases subcutaneous colon cancer growth by increasing apoptosis. Anticancer Agents in Medicinal Chemistry. 2011; 11(7):620–628.
10. Kultti A, Pasonen-Seppanen S, Jauhiainen M, Rilla KJ, Karna R, Pyorla E, Tammi RH, Tammi MI. 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3. Experimental Cell Research. 2009; 315(11):1914–1923. [PubMed: 19285976]
11. Twarcock S, Tammi MI, Savani RC, Fischer JW. Hayluronan stabilizes focal adhesions, filopodia, and the proliferative pheno-type in esophageal squamous carcinoma cells. The journal of biological chemistry. 2010; 285(30):23276–23284. [PubMed: 20463012]
12. Twarock S, Freudenberer T, Poscher E, Dai G, Jannasch K, Dullin C, Alves F, Prenzel K, Knoefel WT, Stoecklein NH, Savani RC, Homey B, Fischer JW. Inhibition of oesophageal squamous cell carcinoma progression by *in vivo* targeting of hyaluronan synthesis. Molecular Cancer. 2011; 10(30)
13. Wang RE. Targeting heat shock proteins 70/90 and proteasome for cancer therapy. Curr Med Chem. 18:4250–4264. [PubMed: 21838681]
14. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. Nat Rev Cancer. 2007; 7:847–859. [PubMed: 17943136]
15. Delpuech O, Griffiths B, East P, Essafi A, Lam EW, Burgering B, Downward J, Schulze A. Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol Cell Biol. 2007; 27:4917–4930. [PubMed: 17452451]
16. Fonar Y, Gutkovich YE, Root H, Malyarova A, Aamar E, Golubovskaya VM, Elias S, Elkouby YM, Frank D. Focal adhesion kinase protein regulates Wnt3a gene expression to control cell fate specification in the developing neural plate. Mol Biol Cell. 2011; 22:2409–2421. [PubMed: 21551070]
17. Jiang Z, Wang Z, Xu Y, Wang B, Huang W, Cai S. Analysis of RGS2 expression prognostic significance in stage II and III, colorectal cancer. Biosci Rep. 2010; 30:383–90. [PubMed: 20001967]
18. Munera J, Cecena G, Jedlicka P, Wankell M, Oshima RG. Ets2 regulates colonic stem cells and sensitivity to tumorigenesis. Stem Cells. 2011; 29:430–439. [PubMed: 21425406]
19. Gotze S, Wolter M, Reifenberger G, Muller O, Sievers S. Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int J Cancer. 2010; 126:2584–2593. [PubMed: 19847810]
20. Jensen SM, Maston LD, Gough MJ, Ruby CE, Redmond WL, Crittenden M, Li Y, Puri S, Poehlein CH, Morris N, Kovacsovics-Bankowski M, Moudgil T, Twitty C, Walker EB, Hu HM, Urba WJ, Weinberg AD, Curti B, Fox BA. Signaling through OX40 enhances antitumor immunity. Semin Oncol. 2010; 37:524–532. [PubMed: 21074068]
21. Park J, Kwak HJ, Lee SH. Role of hyaluronan in glioma invasion. Cell Adhesion & Migration. 2008; 2(3):202–207. [PubMed: 19262113]
22. Kouvidi K, Berdiaki A, Nikitovic D, Katonis P, Afratis N, Hascall VC, Karamanos NK, Tzanakakis GN. Role of receptor for hyaluronic acid-mediated motility (RHAMM) in low molecular weight hyaluronan (LMWHA)-mediated fibrosarcoma cell adhesion. Journal of Biological Chemistry. 2011; 286(44):38509–38520. [PubMed: 21914806]

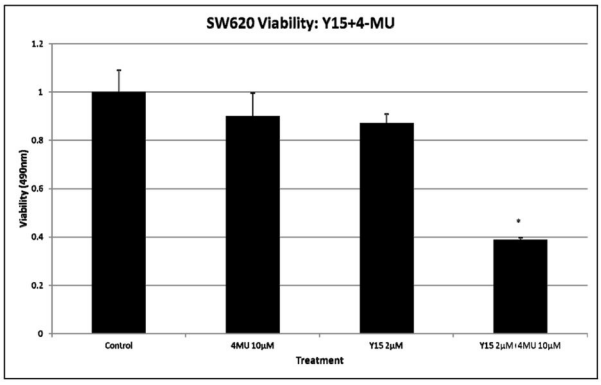
A.



B.



C



D

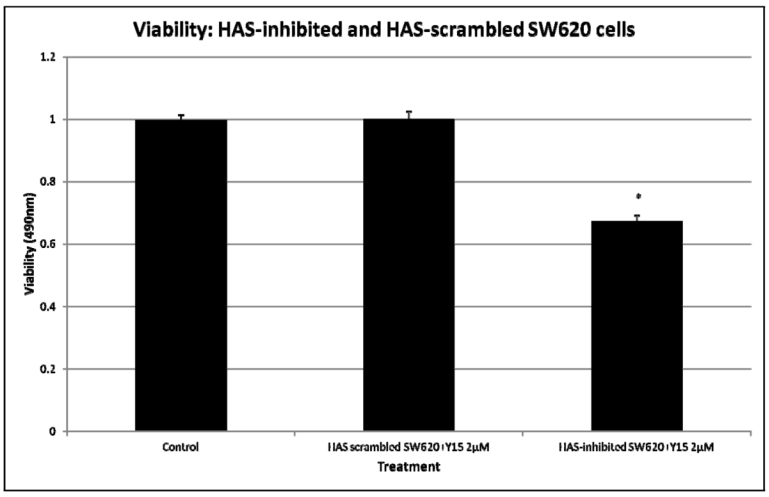


Fig. 1.

Inhibition of FAK and HAS decreases SW620 cell viability in a dose-dependent manner. **A** 5×10^3 of SW620 cells were seeded in 100μL of medium (D-MEM+10% FBS+1% penicillin/streptomycin) and incubated overnight in 96 well plate. Then cells were treated with 1, 2, 4, or 10μM of Y15 or with the HAS inhibitor 4-MU (1, 10, 50, 100, 200μM). SW620 cell viability was decreased in a dose-dependent manner by Y15. A significant decrease in viability was seen at 2μM ($p<0.05$), and persisted as the dose was increased. Similarly, 4-MU inhibited viability dose-dependently, however, a significant decrease in viability was seen at a dose of 100μM and augmented as the dose was increased to 200μM ($p<0.05$). **B.** HAS inhibitor, 4-MU decreases Y397-FAK and total FAK in focal adhesions in SW620 colon cancer cells. Treatment with 4mM of 4-MU decreased the presence of Y397-FAK and FAK at focal adhesions as seen by decreased staining for both total FAK (upper panels) and pY397 FAK (lower panels). **C.** Combination of FAK inhibitor, Y15 and HAS inhibitor, 4-MU decreases SW620 colon cancer cell viability more effectively than each agent alone. When 2μM of Y15 was combined with 10μM of 4-MU, there was a significant decrease in viability ($p<0.05$). **D.** Y15 decreased SW620 colon cancer cell viability more effectively in HAS-inhibited cells than in scrambled control cells.. When HAS-scrambled cells were treated with 2μM of Y15 there was no decrease in viability. However, a significant decrease in viability was seen when HAS-silenced cells were treated with the same dose of Y15 ($p<0.05$).

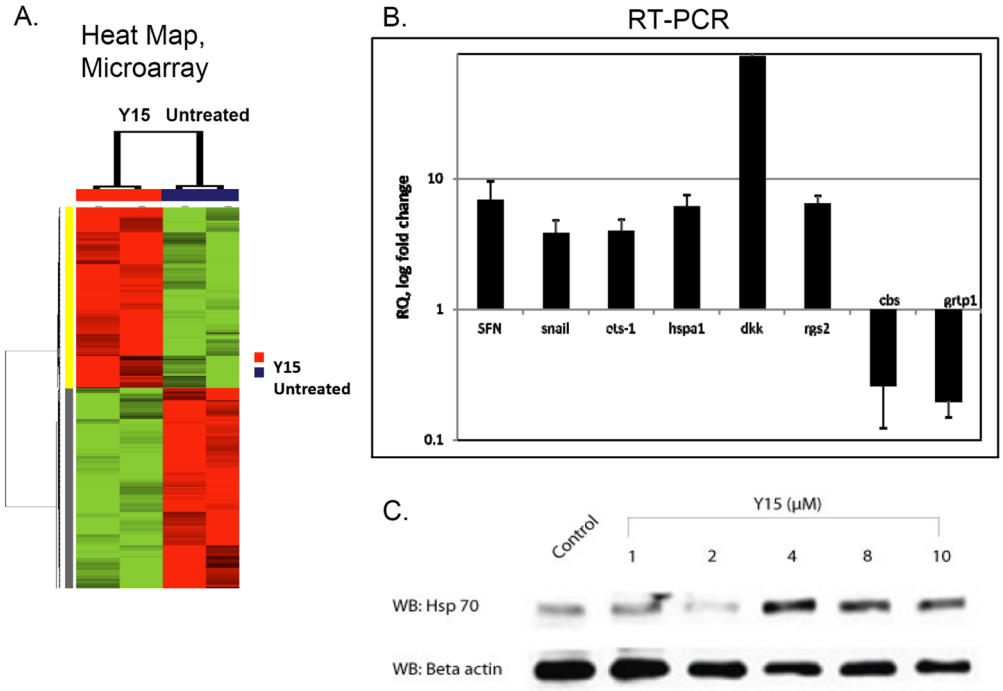


Fig. 2.

DNA microarray analysis revealed many up and down-regulated genes affected more then two fold ($p<0.05$) in Y15-treated SW620 colon cancer cells. **A**. The heat map shows gene expression in untreated SW620 cells and cells treated with 4μM of Y15. Following treatment with Y15, nearly 700 genes were more than 2-fold up- or down-regulated compared to untreated cells and 60 genes were more than 4-fold up- or down-regulated ($p<0.05$). (**B**) RT-PCR from the RNA of cells treated with Y15 confirmed the up-regulation of 6 genes (SFN, SNAIL, ETS1, HSPA1, DKK, RGS2) and down-regulation of 2 genes (CBS, RTP1) that were similarly affected by microarray analysis. **C**. Western blotting demonstrated up-regulated of heat-shock HSP70 protein in Y15-treated cells that was detected by microarray analysis.

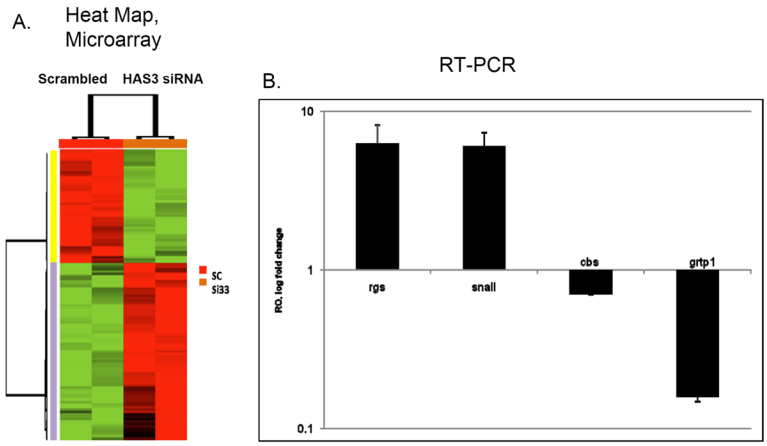


Fig. 3.

DNA microarray analysis revealed many up and down-regulated genes affected more then two fold ($p<0.05$) in HAS-inhibited SW620 colon cancer cells. (**A**) The heat map demonstrates the gene expression profiling following HAS inhibition by transfection with siRNA to HAS3. More than 121 genes were more than 2-fold up- or down-regulated and 9 genes were more than 4-fold up- or down-regulated by HAS inhibition ($p<0.05$). (**B**) RT-PCR confirmed the up-regulation of 2 genes (RGS, SNAIL), and down-regulation of 2 genes (CBS, GRTP1) by HAS inhibition *via* transfection with siRNA to HAS3.

Several common genes affected by FAK and HAS inhibition (p<0.05)

	Gene	Log 2 Fold Change		*p*-value
		HAS inhibition	FAK inhibition	
Down-regulated genes	CBS	-2.26	-3.56	
	DHRS3	-2.09	-2.09	
	EEPD1	-2.04	-3.55	
	ESPN	-2.25	-2.86	
	FAM46C	-2.03	-2.02	***p<0.05***
	GRTP1	-2.59	-2.30	
	IL20RA	-2.54	-2.04	
	INHBE	-4.07	-3.35	
	SCNN1A	-2.17	-2.59	
Up-regulated genes	ANXA1	2.43	3.83	
	MALL	2.06	5.97	*p* ***<0.05***
	RGS2	2.29	5.33	
	SNAI2	2.09	8.23	

Fig. 4. Several common genes affected by FAK and HAS inhibition (p<0.05)

When we analyzed the microarray data from both groups (i.e., identified genes that were affected by both FAK and HAS inhibition), we found that 29 genes were similarly affected by FAK and HAS inhibition ($p<0.05$). Among these genes we found that 9 were down-regulated by FAK inhibition and HAS inhibition and 4 were significantly up-regulated by FAK inhibition and HAS inhibition.

Table 1

Primers and Probes Used for RT-PCR Analysis

Gene	Forward Primer	Reverse Primer
DUPS5	5′GCGGGTCTACTTCCTCAAAG 3′	5′TGAGGGCTCTCTCACTCTCA 3″
probe	5′famCTCGGAATATCCTGAGTGTTGCGTG 3′tamra	
SFN	5′TGGACATCAGCAAGAAGGAG 3′	5′CTGTTGGCGATCTCGTAGTG 3′
probe	5′famTGGGCCTGGCCCTGAACTTT 3′tamra	
SNAIL	5′ACAGAGCATTTGCAGACAGG 3′	5′GTGCTACACAGCAGCCAGAT 3′
probe	5′famTGAGGGCTCATCTGCAGACCCA 3tamra′	
ETS1	5′AGCTTCGACTCCGAGGACTA 3′	5′GACAGGCTTGTCCTTGTTGA 3′
probe	5′famCCCAACCACAAGCCCAAGGG 3′tamra	
RGS2	5′CTTGGCTGTTCAACACGACT 3′	5′CAAACGGGTCTTCCAATCTT 3′
probe	5′famTCGCTCTTGTGGCCACTGCC 3′tamra	
CBS	5′ACTGTCAGCACCATCTGTCC 3′	5′TTGGCTTCCTTATCCTCTGG 3′
probe	5′famCACCGCTCAGGGCCACACTC 3′tamra	
GRTP1	5′GCTACTGCCAGGGAATGAAT 3′	5′CCGGGCTGTAGTAATCTGGT 3′
probe	5′famTCTTCCAACAAGAGCATCTAACAGCCA 3′tamra	
DKK	5′AGCACCTTGGATGGGTATTC 3′	5′CACAATCCTGAGGCACAGTC 3′
probe	5′famAAGGTTCTGTTTGTCTCCGGTCATCA 3′tamra	
HSPA1b	5′CAAGAAGGACATCAGCCAGA 3′	5′AGAAGTCGATGCCCTCAAAC 3′
probe	5′famCTCTTGGCCCTCTCGCAGGC 3′tamra	

Table 2

Selected Genes More than 2-fold up- or Down-regulated by Y15 in SW620 Colon Cancer Cells ($p<0.05$)

Up-Regulated Genes ($p<0.05$)				
Gene Symbol	**Entrez Gene ID**	**Definition**	**Gene Ontology (GO) Biological Process**	**Ratio**
ANXA1	301	annexin A1 (ANXA1)	cell cycle, signal transduction, cell proliferation	3.83
AXUD 1	64651	axin 1 up-regulated	transcription, DNA-dependent apoptosis	6.66
CEACAM6	4680	carcinoembryonic antigen-related cell adhesion	signal transduction, cell-cell signaling	2.42
CDKN1A	1026	molecule 6 (non-specific cross reacting antigen)	G1/2, G2/M transition of mitotic cycle	3.15
DKK1	22943	cyclin-dependent kinase inhibitor 1A	negative regulation of WNT pathway	3.72
DUSP 5	1847	dickkopf homolog 1, DKK1	protein amino acid dephosphorylation	21.6
DUSP 4	1846	dual specificity phosphotase 5	protein amino acid dephosphorylation	3.07
DUSP 1	1843	dual specificity phosphatase 4	protein amino acid dephosphorylation	2.08
		dual specificity phosphatase 1		
ETS1	2113	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1)	regulation of transcription, induction of apoptosis	7.01
FOXD1	2297	forkhead box D1 (FOXD1)	regulation of transcription	1.23
FOXF2	2295	forkhead box F2 (FOXF2)	negative regulation of transcription	2.25
FOXO3	2309	forkhead box O3 (FOXO3)	regulation of transcription	2.16
GADD45A	1647	growth arrest and DNA-damage-inducible, alpha (GADD45A)	regulation of cell cycle; negative regulation protein kinase activity	2.15
HSPA1A	3303	heat shock 70kDa protein 1A	heat-shock response; anti-apoptosis	5.29
HSPA1 B	3304	heat shock 70kDa protein 1B	heat-shock response; anti-apoptosis	6.28
IL8	3576	interleukin 8 (IL8)	inflammatory response; immune response; cell cycle arrest	4.74
LAMA3	3909	laminin, alpha 3 (LAMA3), transcript variant 1, mRNA.	regulation of cell adhesion, regulation of cell migration	6.07
LAMB3	3914	laminin, beta 3 (LAMB3), transcript variant 1	cell adhesion	3.19
LAMC2	3918	laminin, gamma 2 (LAMC2), transcript variant 2	cell adhesion	3.51
PPP1R15A	23645	phosphatase 1, regulatory subunit 15	cystein biosynthetic process from serine; metabolism phosphatase	4.4
RGS2	5997	regulator of G-protein signalling 2, 24kDa (RGS2)	transmembrane receptor protein tyrosine kinase signaling pathway, regulation of G-protein signaling	5.33
SFRP5	6425	secreted frizzled-related protein 5 (SFRP5)	apoptosis, signal transduction	1.31
SNAI2	6591	snail homolog 2 (Drosophila) (SNAI2)	regulation of transcription	8.23
SFN	2810	stratifin (SFN)	release of cytochrome c from mitochondria	8.88

Up-Regulated Genes ($p<0.05$)				
Gene Symbol	**Entrez Gene ID**	**Definition**	**Gene Ontology (GO) Biological Process**	**Ratio**
TNFRSF12A	51330	tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A)	apoptosis, cell adhesion	3.58
Down-Regulated Genes ($p<0.05$)				
CBS	875	cystathionine-beta synthase (CBS)	cystein biosynthetic process from serine; metabolism	
CDH13	1012	cadherin 13	positive regulation of cell-matrix adhesion	0.28
CD99L2	83692	CD99 molecule-like 2 (CD99L2)	cell adhesion	0.31
GRTP1	79774	growth hormone regulated TBC protein	regulation of Rab GTPase pathway	0.31
	11145	HRAS-like suppressor 3 (HRASLS3)	cell cycle	0.43
HRASLS3	3691	integrin, beta 4	cell adhesion	0.43
ITGB4	3714	jagged 2 (JAG2)	cell cycle; Notch signaling pathway	0.49
JAG2	5607	mitogen-activated protein kinase kinase 5	protein amino acid phosphorylation;	0.47
MAP2K5	4602	v-myb myeloblastosis viral oncogene homolog	signal transduction	0.49
	57526	protocadherin 19	G/S transition	0.44
MYB	5300	peptidylprolyl cis/trans isomerase	cell adhesion; homophilic cell adhesion	0.43
PCDH19	389058	SP5 transcription factor	protein folding; cell cycle; regulation of mitosis	0.49
PIN1	64759	tensin 3	transcription	0.32
SP5			positive regulation of cell proliferation, migration	0.56
TNS3				
TNFRSF14	8764	tumor necrosis factor receptor superfamily, member 14, (TNFRSF14)	regulation of apoptosis	0.42
TNFRSF4	7293	tumor necrosis factor receptor superfamily, member 4 (TNFRSF4)	inflammatory response, immune response, regulation of apoptosis	0.55

Table 3

Selected Genes More Than 2-fold up- or Down-regulated by HAS3 Inhibition ($p<0.05$)

Up-Regulated Genes ($p<0.05$)				
Gene Symbol	**Entrez Gene ID**	**DEFINITION**	**Gene Ontology (GO) Biological Process**	**Ratio**
ANXA1	301	annexin A1 (ANXA1), mRNA.	cell cycle, signal transduction, regulation of cell proliferation, anti apoptosis	2.43
FOXL2	668	forkhead box L2 (FOXL2), mRNA.	regulation of transcription, anti apoptosis	2.21
HRASLS3	11145	HRAS-like suppressor 3 (HRASLS3), mRNA.	negative regulation of cell cycle	3.32
HSPA12A	259217	heat shock 70kDa protein 12A (HSPA12A), mRNA.	Apoptosis	2.34
RGS2	5997	regulator of G-protein signalling 2, 24kDa (RGS2), mRNA.	cell cycle, regulation of G-protein coupled receptor protein signaling pathway	2.29
SFRP5	6425	secreted frizzled-related protein 5 (SFRP5), mRNA.	Apoptosis	2.51
SNAI2	6591	snail homolog 2 (Drosophila) (SNAI2), mRNA.	negative regulation of transcription	2.09
TNFRSF4	7293	tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), mRNA.	inflammatory response, immune response, regulation of apoptosis	2.25
WT1	7490	Wilms tumor 1 (WT1), transcript variant A, mRNA.	regulation of transcription	2.13
Down-regulated Genes				
CEACAM6	4680	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen) (CEACAM6), mRNA.	signal transduction cell-cell signaling	0.44
CEBPB	1051	CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), mRNA.	regulation of transcription, anti-apoptosis	0.33
IL8	3576	interleukin 8 (IL8), mRNA.	angiogenesis, cellular component movement, inflammatory response, immune response, induction of positive chemotaxis	0.20
PPP1R14C	81706	protein phosphatase 1, regulatory (inhibitor) subunit 14C (PPP1R14C), mRNA.	regulation of phosphorylation	0.45