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Glyceollin, a novel regulator of mTOR/p70S6 in estrogen receptor positive breast cancer

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Abstract

An estimated 70% of breast cancer tumors utilize estrogen receptor (ER) signaling to maintain tumorigenesis and targeting of the estrogen receptor is a common method of treatment for these tumor types. However, ER-positive (+) breast cancers often acquire drug resistant or altered ER activity in response to anti-estrogens. Here we demonstrate glyceollin, an activated soy compound, has anti-estrogen effects in breast cancers. We demonstrate through estrogen response element luciferase and phosphorylation-ER mutants that the effects of glyceollin arise from mechanisms distinct from conventional endocrine therapies. We show that glyceollin suppresses estrogen response element activity; however, it does not affect ER-alpha phosphorylation levels.

Additionally we show that glyceollin suppresses the phosphorylation of proteins known to crosstalk with ER-alpha signaling, specifically we demonstrate an inhibition of Ribosomal Protein S6 Kinase, 70kDa (p70S6) phosphorylation following glyceollin treatment. Our data suggests a mechanism for glyceollin inhibition of ER through the induced suppression of p70S6 and demonstrates novel mechanisms for ER inhibition.

Keywords

Glyceollin; endocrine therapy; p70S6; estrogen receptor (ER); mTOR; anti-estrogen; kinase signaling

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1. Introduction

A member of the nuclear steroid receptor superfamily, estrogen receptor alpha (ER α) is a key player in normal mammary development; however, ER α 's role in breast cell physiology imparts upon it an equally significant function in deregulated cell growth and breast carcinoma. As a transcription factor, ER α either directly or indirectly regulates the expression of many genes involved in cell growth and proliferation [1–3], requiring its expression and activity to be tightly controlled [4–5]. One major mechanism by which the cell regulates ER α is through post-translational modifications such as phosphorylation. There are many phosphorylation sites within ER α , including serines 118 (ERK1/2) [6–7], 167 (AKT, RSK, Ribosomal Protein S6 Kinase, 70kDa (p70S6), IKK ϵ) [8–10], 104/106 (ERK1/2) [7], 305 (PKA) [11], threonine 311 (p38 α) [12], and tyrosine 537 (src) [13]. Additional layers of ER α regulation exist at the level of the kinases, which target these sites within ER α , and co-activator proteins that bind the receptor and alter its function [4].

Its role in breast cancer pathogenesis makes ER α one of the most popular targets for medical intervention. In fact, most first-line treatments for women with ER α (+) breast cancer involve an ER α antagonist [14]. However, there are inherent drawbacks to these therapies, mainly that ER α (+) breast cancers often become drug resistant, making them extremely hard to treat [15–16]. Additionally some anti-estrogens such as tamoxifen can have both antagonistic and agonistic interactions with the estrogen receptor [17]. Therefore, discovery of novel compounds that can be used to treat ER α (+) breast tumors is critical. Our lab has recently characterized an activated soy compound, glyceollin, which possesses marked anti-cancer properties. Glyceollin is produced in the soybean plant in response to stress stimuli (UV light, exposure to microorganisms, and low temperature), and our previous *in vitro* and *in vivo* breast cancer studies as well as our primate studies have collectively shown that glyceollin is anti-estrogen [18–20]. Glyceollin inhibits the transcriptional activity of ER α , and our *in vitro* binding studies confirm that glyceollin can physically bind ER α , most probably in the ligand binding pocket [18]. Interestingly, we have recently shown that glyceollin can inhibit the proliferation of ER α (–) cells, demonstrating glyceollin's ability to affect multiple targets in breast cancer cells [21]. Therefore, the current study aims to identify alternate sites either within ER α or targets tangentially associated with the receptor that glyceollin exploits to inhibit ER α -dependent breast cancer cell growth.

2. Material and Methods

2.1 Reagents

The following antibodies were purchased from Cell Signaling (Danvers, MA): p-p70S6 (T389) (cat. # 2905), p-S6 (S235/236) (cat # 4858), total-S6 (cat #2217), p-EF2K (cat #3691), t-EF2K (cat #3692), p-eIF4B (cat #5399), t-eIF4B (cat #3592). The antibody against total ER α (HC-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (cat # sc543). Antibodies against ER α p-S167 and ER α p-118 were purchased from Novus Biologicals (Littleton, CO) (cat # NBP1-12613 and NBP2-12613, respectively). Infrared-labeled secondary antibodies were purchased from LiCor (Lincoln, NE) (cat # 926-32210 and 926-32211). Dulbecco's modified Eagle's medium (DMEM, cat # 11965-118), phenol

red free DMEM (cat # 31053-028), MEM non-essential amino acids (cat # 11140-050), MEM essential amino acids (cat # 11130-051), L-glutamine (cat # 25030081), penicillin/streptomycin (cat #15070-063), and sodium pyruvate (cat # 11360070) were obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) (cat # SH30071.03) and charcoal stripped fetal bovine serum (cs-FBS) (cat # SH30068.03) were purchased from Hyclone (Thermo-Scientific, South Logan, UT). Porcine insulin was purchased from Sigma (St. Louis, MO) (cat # I5523-10MG). Effectene was purchased from QiaGen (Valencia, CA) (cat # 301427). MPER (mammalian protein extraction reagent) was purchased from Pierce (Thermo Scientific, Rockford, IL) (cat # 78501). Protease and phosphatase inhibitors were purchased from Sigma (St. Louis, MO) (cat. #'s P1860-1ML, P0044, and P5726). The proteome profiler human phospho-kinase array kit was purchased from R & D Systems (Minneapolis, MN) (cat # ARY003).

2.2 Cell Culture

MCF-7 cells were cultured as previously described [34]. Briefly, the MCF-7 cell line used is a subclone of MCF-7 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) generously provided by Louise Nutter (University of Minnesota, MN) [35]. The MCF-7, T-47D, and HEK293 culture flasks were maintained in a tissue culture incubator in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For estrogen studies, cells were grown in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (5% CS-FBS) for 48 hrs before the addition of hormones.

2.3 Western Blot Analysis

MCF-7 cells were seeded in 10 cm² plates at a density of 60 to 70% confluence (5 to 10 × 10⁶ cells) and were allowed to attach overnight. For estrogen studies, media were replaced with media containing 5% charcoal-stripped FBS and cells were incubated for 48 hrs before the addition of hormones. For glyceollin studies, the media was exchanged with fresh media containing glyceollin for the indicated times. The media was then removed and the cells were scraped into 1 mL of PBS plus 3 mM EDTA. The cell suspensions were spun for five minutes at 2,000 × g and the supernatant was aspirated. The cell pellets were lysed by vortexing in 200 µL of M-PER buffer containing protease and phosphatase inhibitors. The samples were then spun in a microcentrifuge for five minutes at 12,000 × g and the supernatants were collected. Protein concentrations were determined using a nanodrop spectrophotometer (Thermo Life Sciences) and 50 µg of total protein was loaded and run on a 4 to 12% polyacrylamide gel (Invitrogen, cat # NP0335BOX). The gels were blotted onto nitrocellulose using the iblot transfer system (Invitrogen, cat # IB3010-01). The blots were blocked for one hour at room temperature in 1 × TBST (Affymetrix, Santa Clara, CA, USA, cat # 77500 5 LT) containing 5% non-fat milk. The blots were then washed in 1 × TBST and were incubated overnight at 4°C in 10 mL of primary antibody at a 1:500 dilution (Santa Cruz antibodies) or 1:1000 dilution (Cell Signaling, Novus antibodies) in 5% BSA/TBST (Sigma cat # A7906-1 KG). Blots were then washed in 1 × TBST and incubated with IR-labeled secondary antibodies for 30 minutes at room temperature. The blots were then washed in 1 × TBST and scanned using the Odyssey infrared imaging system (LiCor, Lincoln, NE). Bands were quantified using the Odyssey software (LiCor) and normalized to

bands corresponding to the housekeeping Rho-GDI protein. Three independent samples were prepared for each condition assayed and statistical significance was determined using the t-test function of Origin graphing software (Northampton, MA).

2.4 Luciferase Assay

As previously described [36], HEK293T cells were seeded in 24-well plates at a density of 5×10^5 cells/well and allowed to attach overnight. The cells were then transfected for 5 hours with 300 μ g pERE-luc plasmid (Panomics, Santa Clara, CA cat # LR0020) using the Effectene kit according to the manufacturer's protocol. After 5 hours, either vehicle or indicated compounds were added to the cells and cells were incubated at 37 °C overnight. The following morning, the media was removed, and 100 μ l of MPER lysis buffer was added per well and then incubated for 5 min at room temperature. Cell debris was pelleted by centrifugation at $15,000 \times g$ for 5 min. Luciferase activity of 100 μ L of cell extracts was determined using an equal volume of Bright Glo luciferase assay reagent (Promega Corp., Madison, WI, cat # E2620) in an Autoluminat Plus luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.5 Proteome profiler assay

MCF-7 cells were seeded in 10 cm² plates at a density of 60 to 70% confluence (5 to 10×10^6 cells) and were allowed to attach overnight. The next day, either vehicle (DMSO) or glyceollin was added to the cells to a final concentration of 10 μ M. The cells were incubated for 30 minutes at 37 °C and subsequently lysed according to the manufacturer's instructions. A single blot was used for each vehicle and glyceollin sample and the blots were processed according to the kit's instructions but instead of using the streptavidin-HRP antibody, the blots were incubated with infrared-tagged streptavidin (Licor, cat # 926-32230) followed by imaging on the Odyssey infrared imaging system. Individual dots were quantified using the Odyssey software and normalized using the positive controls.

2.6 Statistical Analysis

Statistical Analysis was performed using Graph Pad Prism 5. Student's t test was used to determine p values and statistically significant values had a p-values of <0.05.

3. Results

3.1 Glyceollin inhibits the estrogen receptor through multiple mechanisms

We have previously reported that glyceollin can deter the growth of ER α (+) MCF-7 breast cancer cells, at least in part by binding to and inhibiting ER α [18]. Our steady-state binding studies have shown that glyceollin I binds ER α and our docking analyses predict the binding occurs within the ligand binding pocket of the receptor, similar to tamoxifen [18,22]. We decided to explore the possibility that glyceollin affects other regions of ER α , independently of the ligand binding domain. Therefore, we tested the ability of glyceollin to inhibit the transcriptional activity of a mutant ER α (ER (AF-1)) that has no functional ligand binding (AF-2) domain [23]. To test this hypothesis, we transfected human embryonic kidney cells, which do not express ER α , with either the wild-type or the mutant ER (AF-1), along with an ERE-luciferase reporter. We incubated the cells with vehicle, 10 pM estradiol (E₂), 10 μ M

glyceollin, or E₂ + glyceollin. Our results show that glyceollin inhibits the transcriptional activity of E₂-stimulated wild-type ER α by ~ 70%, similar to what we have seen in the past (Fig. 1A) [18]. We further found that glyceollin inhibited the ER (AF-1) mutant to similar levels (Fig. 1B), suggesting glyceollin, in addition to targeting the ER ligand binding pocket, may inhibit the ER through an alternative mechanism, one involving the AF-1 domain. One common method of regulating ER function is through post-translational modifications, particularly phosphorylation of serine, threonine, or tyrosine residues. ER α is phosphorylated within the AF-1 region at serine 118 by ERK1/2 kinase [6, 24] and at serine 167 by AKT kinase [9]. Therefore, we tested the ability of glyceollin to inhibit the activity of ER mutants in which serine 118 or serine 167 was changed to a glutamic acid residue, mimicking constitutive phosphorylation. Our results show that glyceollin is capable of inhibiting the activity both ER S118E and ER S167E (Figs. 2A and 2B). These data suggest that if glyceollin affects the phosphorylation status of the AF-1 region within ER α , it does so at sites other than serine 118 or 167. To confirm these results, we looked at direct phosphorylation of each site using Western blot analysis of MCF-7 cells that had been treated with vehicle, tamoxifen, or glyceollin. As expected, glyceollin had little effect on the phosphorylation status of serine 118 or 167 (Fig. 3A and 3B).

3.2 Glyceollin treatment of breast cancer cells results in a downregulation of p70S6 kinase activity

Our data suggest glyceollin either directly or indirectly targets ER α at a site(s) within the ER's N-terminal or AF-1 region. Because ER α contains numerous potential phosphorylation sites, we used a proteome profiler phospho-kinase array (R & D Systems) to screen potential upstream effectors of ER α that may be inhibited by glyceollin. We incubated MCF-7 cells with either vehicle or 10 μ M glyceollin for 30 minutes and found that, compared to vehicle, glyceollin inhibited several intracellular kinases, notably the p70S6 kinase at multiple sites (Table 1). To confirm these results, we performed Western blot analyses of three separate samples of MCF-7 cells that were treated for 0, 10, 30, 60 and 120 minutes with 10 μ M glyceollin and again found that glyceollin inhibited phosphorylation of p70S6 kinase (T389) by approximately 40% (Fig. 4A). We next tested what effect glyceollin had on several p70S6 target proteins, including S6, eIF4B and eEF2K. In each case, glyceollin inhibited phosphorylation of the p70S6 kinase targets by approximately 40–50% (Fig. 4A). To further confirm these data, we incubated the ER (+) ductal carcinoma cell line T-47D with 10 μ M glyceollin. Again, glyceollin decreased the phosphorylation of mTOR pathway proteins, especially S6 kinase (Figure 4B and 4C). This data further supports a role for glyceollin in inhibition of the p70S6 kinase pathway.

3.3 Glyceollin blocks p70S6 kinase- mediated increase in ER α transcriptional activity

Recently, Yee's group found that IGF promotes a direct, physical interaction between p70S6 kinase and ER α in MCF-7 cells [25]. Based on this study and our findings that glyceollin inhibits both transcriptional activity of the estrogen receptor and phosphorylation of p70S6 kinase, we tested the ability of p70S6 kinase to affect ER α transcriptional activation. We co-expressed constitutive active p70S6 kinase and an ERE-luciferase reporter in MCF-7 cells followed by addition of vehicle, 100 pM estradiol, 10 μ M glyceollin, or estradiol + glyceollin. In the presence of estradiol, overexpression of p70S6 kinase increased activity of

the ER by approximately 1.7 fold compared to estradiol alone and glyceollin was able to block the p70S6 kinase effect (Fig. 4D). These data, in light of the recent report by Yee's group, strongly suggest that p70S6 kinase directly targets ER α , and that this interaction can be abrogated by glyceollin.

4. Discussion

Our group previously isolated the soy product glyceollin and characterized it as an anti-estrogenic compound that can directly bind to and inhibit ER α [18, 22]. Using a proteome profiler array of phosphorylated protein kinases, we found glyceollin to be an inhibitor of p70S6 kinase in MCF-7 breast cancer cells. In fact, glyceollin inhibited p70S6 kinase on three separate sites, T229 and T389 within p70S6 kinase's catalytic domain and T421/S424 within the C terminal auto inhibitory domain (Table 1). We not only confirmed the array results using Western blot analyses, but also found glyceollin could inhibit the phosphorylation and activation of several p70S6 kinase target proteins, including eIF4B, S6, and eEF2K. These data reveal a novel role of glyceollin in breast cancer cells, and our luciferase data point to a direct effect of glyceollin on ER α transcriptional activity (Fig. 4D). A recent study by Yee's group showed an IGF-mediated direct interaction between p70S6 kinase and ER α that leads to phosphorylation of ER α at serine 167 [25]. However, our Western blot data show glyceollin has no inhibitory effect on serine 167 phosphorylation of ER α and it even results in a small increase at this site. In addition to p70S6, phosphorylation of ER α serine 167 can occur through multiple mechanisms including phosphorylation by IKK ϵ , RSK, and AKT. As p70S6 is known to inhibit AKT through a feedback mechanism, the loss of p70S6 and therefore loss of inhibition on AKT may be one possible mechanism for the increase in ER α phosphorylation observed in our cell system [26].

As one of the best characterized downstream targets of mTOR, p70S6 kinase is an important regulator of cell size, protein translation, and cell proliferation [27]. p70S6 kinase has recently been linked to hormone receptor positive breast cancer. One report ties phosphorylated p70S6 kinase to poor disease outcome in patients with hormone receptor positive breast cancer and potentially to progression of tumors to hormone independence [28]. The regulation of mTOR and downstream effectors such as p70S6 can come from multiple avenues. As suggested in figure 6, regulation of p70S6 by glyceollin could occur through any number of mTOR/p70S6 upstream activators, including TSC1/TSC2, p53, AMPK, PRAS40, and PP2A [26, 29]. Yoon *et al* recently showed in skeletal muscle that glyceollin activates AMPK through a Ca²⁺/CaMKK dependent mechanism [30]. Additionally AMPK is known to repress CREB activity; along with p70S6, CREB was one of the top dephosphorylated proteins from our phospho-kinase array. While the regulation of AMPK through glyceollin is an intriguing possibility, further studies will be required.

Recent studies have reported that the dual inhibition of both mTOR and ER α signaling has advantages over singular inhibition of either pathway. The mTOR signaling pathway is known to be integral to E₂ induced cell proliferation and sole inhibition of mTOR through the use of the mTORC1 specific inhibitor RAD001 abolishes E₂ stimulated tumorigenesis and cellular proliferation [31–32]. Here we have shown that glyceollin, in addition to binding to and inhibiting ER α may function as a p70S6 kinase inhibitor. If so, the

combination of ER α and p70S6 kinase inhibition could result in a beneficial outcome in patients with ER α (+) breast cancer, as these patients are prone to recurrence and this recurrence may involve upregulation of p70S6 kinase activity. Glyceollin then provides a unique therapeutic opportunity by inhibiting two key signaling components without the use of multiple drugs. Furthermore, patients who experience a recurrence of breast cancer that is refractory to endocrine therapy may benefit from a specific p70S6 kinase inhibitor as opposed to a more general mTOR pathway antagonist, both in efficacy and general side effect tolerance. Additionally as an inhibitor of ER α , glyceollin offers a mechanism of action that is different from other ER α antagonists. To date there is not a record of glyceollin-induced ER α activity and based on our phospho-kinase array data we show loss of phospho-src levels. Src is a known co-activator of ER α and its increased activity has been attributed to the agonistic effect of tamoxifen and tamoxifen resistance [33]. Additionally we have shown that glyceollin treatment of triple negative cell lines produced an antitumor effect *in vivo* and altered miRNA expression profile, increasing conventional tumor suppressive miRNAs.²¹

In conclusion, our study suggests a novel target of the activated soy compound glyceollin. Using a proteome profiler array, we found glyceollin inhibits the mTOR pathway protein p70S6 kinase. We not only confirmed this result in MCF-7 cells but also in the ER α (+) cells line T-47D. Interestingly, our data show that glyceollin blocks activity in an ER mutant lacking an active AF-2 domain. This result is intriguing and although no link has been shown between p70S6 kinase and the AF-1 domain of the ER (other than S167), there may be another p70S6 targeted posttranscriptional modification of the ER that is blocked by glyceollin. However, further studies would be necessary to confirm this. Taken together our data suggest that glyceollin could be a treatment option for patients with either ER α (+) or perhaps more importantly ER α (–), more aggressive, breast cancer.

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Highlights

- Glyceollin suppresses phosphorylation of proteins which crosstalk with ER α
- p70S6 is a novel target of glyceollin in ER+ breast cancer cell lines
- The effects of glyceollin are distinct from conventional endocrine therapies

Figure 1. Glyceollin inhibits ER- α Independent of the Ligand Binding Domain

Human embryonic kidney cells were transfected with an estrogen response element (ERE) and either a (A) wild type (WT) ER- α or (B) mutant ER- α (AF-1) which contains a non-functional AF-2 ligand binding domain. Cells were then pretreated with 10 μ M glyceollin (GLY), 100 nM fulvestrant (ICI), or 100 nM tamoxifen (OHT) for 1 hour prior to treatment with 10pM estrogen (E₂). After 18 hours cells were lysed and luminescence was read. Results represent relative luminescence. Normalization was to HEK cells treated with E₂ alone. Error bars represent SEM. * Significantly different from E₂ treatment $p < 0.05$.

Figure 2. Glyceollin inhibits ER- α Independent of ER- α Phosphorylation

Human embryonic kidney cells were transfected with an estrogen response element (ERE) and either a **(A)** constitutively active ER- α S118E mutant or **(B)** a constitutively active ER- α S167E mutant. Cells were then pretreated with 10 μ M glyceollin (GLY), 100 nM fulvestrant (ICI), or 100 nM tamoxifen (OHT) for 1 hour prior to treatment with 10 pM estrogen (E₂). After 18 hours cells were lysed and luminescence was read. Results represent relative luminescence. Normalization was to HEK cells treated with E₂ alone. Error bars represent SEM. * Significantly different from E₂ treatment $p < 0.05$.

Figure 3. Glyceollin Does not inhibit Phosphorylation of ER- α at S118 or S167

MCF-7 cells were pre-treated with 10 μ M glyceollin (GLY) or 100nM tamoxifen (OHT) for 1 hour prior to treatment with estrogen (E₂) for 30 minutes. Cells were then lysed and total protein was extracted for western blot analysis. Results represent (A) total band density for total ER- α and phospho-ER- α at S118 and S167 and (B) quantified band density. Error bars represent SEM, and for each antibody normalization was to MCF-7 cells treated with vehicle and designated as 1. * Significantly different $p < 0.05$ compared to vehicle control.

Figure 4. Glyceollin inhibits p70S6 phosphorylation as well as the p70S6-mediated activation of ER- α

(A) MCF-7 cells were treated with 10 μ M glyceollin (GLY) at designated time points. Cells were then lysed and total protein was extracted for western blot analysis. Results represent band density for phosphorylation levels of p70S6, S6, EF2K, and eF4B. Error bars represent SEM, and for each antibody normalization was to MCF-7 cells treated with vehicle and designated as 100. (B) T47-D cells were treated with 10 μ M GLY for 30 minutes prior to total protein extraction for western blot analysis for p70S6, S6, EF2K, and eF4B phosphorylation (C) graphical demonstration of protein density for T47-D cells treated with GLY, error bars represent SEM and normalization was to T47-D cells designated as 100, * Significantly different $p < 0.05$ compared to vehicle control. (D) p70S6 kinase and an ERE-luciferase reporter were transiently transfected into MCF-7 cell line. Pre-treatment of increasing concentrations of glyceollin (1, 5, and 10 μ M, indicated by triangle) for 1 hour followed by treatment with 100 pM E2. Cells were lysed after 18 hours and luminescence was read. Error bars represent SEM. * Significantly different $p < 0.05$.

Figure 5. Glyceollin Induces ER/p70S6 Crosstalk

Schematic for proposed model of glyceollin induced alterations of p70S6

Table 1

Glyceollin Induced Alterations in Kinase Phosphorylation Levels

Target Kinase	Phosphorylated residue(s)	% Change (veh=100%)
p38 alpha	T180/Y182	74.7
ERK 1/2	T202/Y204, T185/Y187	74.7
JNK	T183/Y185, T221/Y223	96.5
GSK-3 alpha/beta	S21/S9	97.3
p53 (S392)	S392	102
MEK1/2	S218/S222, S222/S226	89.6
MSK 1/2	S376/S360	83.5
AMPK alpha 1	T174	95.5
Akt (S473)	S473	91.1
Akt (T308)	T308	116
p53 (S46)	S46	95.2
TOR	S2448	78.1
CREB	S133	45.9
HSP27	S78/S82	81.0
AMPK alpha 2	T172	89.8
beta-Catenin	-	60.2
p70 S6 kinase (T389)	T389	66.8
p53 (S15)	S15	87.6
p27	T198	113
paxillin	Y118	101
Src	Y419	64.4
Lyn	Y397	74.6
Lck	Y394	94.6
STAT2	Y689	89.5
STAT5a	Y694	79.6
p70 S6 kinase (T421/S424)	T421/S424	45.4
RSK1/2/3	S380/S386/S377	65.7
p27	T157	97.9
PLC gamma-1	Y783	107
Fyn	Y420	78.7
Yes	Y426	74.2
Fgr	Y412	97.8
STAT3	Y705	103
STAT5b	Y699	114
p70 S6 kinase (T229)	T229	52.7
RSK1/2	S221/S227	70.5
c-Jun	S63	83.4

Target Kinase	Phosphorylated residue(s)	% Change (veh=100%)
Pyk2	Y402	118
Hck	Y411	87.8
Chk-2	T68	78.3
FAK	Y397	104
STAT6	Y641	89.4
STAT5a/b	Y694/Y699	92.1
STAT1	Y701	79.7
STAT4	Y693	78.2
eNOS	S1177	86.6