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GLP-1 Promotes Angiogenesis in Human Endothelial Cells in a Dose-Dependent Manner, through the Akt, Src and PKC Pathways

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Abstract

Introduction—Novel anti-diabetic medications that mimic or augment the physiological actions of GLP-1 improve cardiovascular risk factors in diabetics and GLP-1 has been proposed to have a beneficial role in the cardiovascular system. GLP-1 may have a direct cardioprotective role by decreasing infarct size and protecting from ischemia-reperfusion injury while prolonging survival in rodent models. The mechanisms underlying these observations remain largely unknown. *In vitro* studies suggest that GLP-1 may promote endothelial cell proliferation, but no study to date has evaluated a potential direct effect of GLP-1 on angiogenesis.

Specific Aim—To evaluate whether GLP-1 affects angiogenesis in humans and to elucidate underlying molecular mechanisms.

Material and Methods—We utilized a 3D culture system where spherules of human umbilical vein endothelial cells (HUVECs) embedded in a collagen scaffold were treated with escalating doses of human recombinant GLP-1 (50–2000nM) and the formation of new vessels was observed and quantified. Signaling inhibitors were utilized to identify molecular pathways through which GLP-1 promotes angiogenesis.

Results—We demonstrate that GLP-1 promotes angiogenesis in a dose-dependent manner. The maximum effect on angiogenesis was observed at a GLP-1 dose of 500nM, while increased angiogenesis occurred in response to doses ranging from 200nM to 1000nM. Pre-treatment of the system with Akt inhibitor IV, Bisindolylmaleimide (PKC inhibitor) and src inhibitor I resulted in a significant decrease of the GLP-1 induced angiogenesis.

Conclusions—This is the first study to demonstrate that GLP-1 promotes angiogenesis in a HUVEC three dimensional in vitro model. This effect requires pharmacological doses and is mediated through the Akt, PKC and src pathways.

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Keywords

GLP-1; Angiogenesis; src; PKC; Akt

Introduction

GLP-1 is a hormone released by the L-cells of the intestine following per os intake of glucose and other macronutrients and acting as an incretin, promoting insulin secretion and enhancing insulin sensitivity of the peripheral tissues (1). The appreciation of the significance of GLP-1's role in glucose homeostasis resulted in the development of Exenatide, the first GLP-1 receptor agonist that was approved in the U.S in June 2005, as an adjunct treatment for patients with type 2 diabetes who have failed to achieve glycemic control on established anti-diabetic monotherapy or combination therapy (2). Subsequently other GLP-1 agonists were developed such as Liraglutide, Lixisenatide and Taspoglutide (in phase III clinical trials since 2010) as well as medications inhibiting the major enzyme degrading GLP-1 [Dipeptidyl peptidase-4 (DPP-4) inhibitors] such as Sitagliptin, Saxagliptin and Linagliptin. GLP-1 not only improves glycemic control, but also leads to progressive weight reduction and sustained improvement in cardiovascular risk factors in patients with type 2 DM (2). Interestingly metformin inhibits DPP-4 activity, leading to increased levels of GLP-1 levels (3).

Interestingly, in addition to the beneficial effect of GLP-1 on cardiovascular risk profile, GLP-1 has also direct beneficial effects on the heart (4, 5) and vasculature (6-10). Although the underlying mechanisms for the cardio-protective function of GLP-1 remain largely unknown, there is emerging evidence from studies on diabetic foot wounds (11) and patients with critical limb ischemia (12) suggesting that GLP-1 could promote angiogenesis. There are no studies to date evaluating the effect of GLP-1 in angiogenesis in the heart. *In vitro* studies in human coronary arterial endothelial cells have demonstrated that GLP-1 and Exendin-4 stimulate proliferation of HCAECs via a GLP-1 receptor-dependent mechanism (5). However, there is no *in vitro* study to date, investigating the direct effect of GLP-1 on the process of angiogenesis, which, in addition to proliferation, involves other steps including collagen breakdown, endothelial cell migration and formation of tubules (13).

The purpose of this study was to evaluate the role of GLP-1 in angiogenesis *in vitro*. Given the fact that angiogenesis could also have some detrimental effects (such as diabetic retinopathy (14, 15), tumor growth and metastasis (16)), we attempted to identify not only whether GLP-1 has an effect on angiogenesis but also at what doses. For this purpose, we utilized a three dimensional assay of HUVEC spherules, embedded in a collagen matrix closely resembling the consistency of the basic membrane. We treated these cells with escalating doses of GLP-1 and characterized the optimal dose at which GLP-1 exerts its maximum angiogenic effect. Subsequently we performed experiments using signaling inhibitors to identify the molecular pathways that mediate GLP-1 induced angiogenesis in HUVECs.

Materials & Methods

HUVEC 3-D angiogenesis assay

In order to screen for the effect of GLP-1 on angiogenesis *in vitro*, we utilized a novel three-dimensional HUVECs angiogenesis assay (Promo cell, C13010, Heidelberg, Germany). In this assay, endothelial beads, consisting of 400–500 cells each, were embedded in a collagen matrix that closely resembles the properties of the basal membrane. We treated the cells with escalating doses of GLP-1 (namely 50, 100, 200, 500, 1000, 2000nM; GLP-1 (7-36), amide;

CAT#:46-1-13B; American Peptide Company, Sunnyvale, CA) and VEGF (20 ng/mL, as a positive control) for 48 hours at 37°C and 5% CO₂. These doses were selected based on previous published data on the effect of GLP-1 on proliferation of human endothelial cells (11). As a negative control, we utilized the same culture media that we used to dissolve GLP-1 and VEGF, provided by the assay manufacturer. After treating the cells, we analyzed the assay under a phase contrast microscope at ×10 magnification, and expressed the angiogenic effect of the various treatments as cumulative sprout length in pixels. To analyze the pictures taken from the microscope, we used Image J software (NIH, NIMH, and Bethesda, MD, USA).

HUVEC 3-D angiogenesis inhibitor experiment

In order to evaluate for potential signaling pathways that mediate GLP-1 induced angiogenesis, we repeated the above experiment after pre-treating the cells with various signaling inhibitors. The inhibitors that we used were: AG490 1uM (STAT-3 inhibitor), Compound C 20uM (AMPK inhibitor), PD98059 10uM (ERK ½ inhibitor), SB203580 25uM (JNK inhibitor), SP600125 20uM (P38 inhibitor), AKT Inhibitor IV 1uM, Bisindolylmaleimide 1uM (PKC inhibitor), FAK inhibitor 14 30uM, Src inhibitor I 10uM. All inhibitors were dissolved in the assay's culture media. Cells were pretreated with the above signaling inhibitors for 1 hour at 37°C and 5% CO₂. The supernatant was then removed, cells were washed with PBS and culture media and subsequently incubated with 500nM of GLP-1 for 48 hours. A positive and a negative control were also used, similar to the previous experiment, as well as a non-pre-treated 500nM GLP-1 control. FAK inhibitor 14 was used as an inhibitor control, since pretreatment with this was expected to arrest all angiogenic processes (17). The cumulative length of the sprouts was measured as described above. Since the inhibitor experiment was performed in two different 8-well plates, each plate had its own negative, positive and non-pre-treated GLP-1 500nM controls, to control for between-assay variability.

GLP-1 Stability in the supernatant

To demonstrate that GLP-1 was not degraded or metabolized during the experiment, we measured GLP-1 levels in the supernatant during the above experiments. A small amount of the supernatant was collected at 0, 24 and 48 hours after the incubation. GLP-1 levels in the supernatant were measured using a competitive Enzyme-linked immunosorbent assay (Biovendor, Candler, NC).

Statistical Analysis

The angiogenic effect of GLP-1 was quantified as the cumulative length of the spurs. Differences of the cumulative length between two groups are expressed as means of the difference ± standard error of the mean differences. Comparison between different groups was performed using one-way analysis of variance. Post-hoc comparisons between pairs of groups were performed with t-tests using the Fisher's least significant difference correction for multiple comparisons. Multiple comparisons between the negative control group and all different treatments were performed with the Holm-Sidak method. All p-values are two sided and the alpha criterion was set to 0.05. Statistical analysis was performed using Stata v.12 (Stata Corp, College Station, TX).

Results

HUVEC 3-D angiogenesis assay (Figure 1)

We evaluated 19-34 beads in each treatment group (negative control, GLP-1 50, 100, 200, 500, 1000, 2000nM and VEGF 20 ng/ml). Beads that were close to the edges of the scaffold

gel and / or where truncated by shear stress were excluded from the analysis. 200, 500 and 1000 nM of GLP-1 increased the cumulative lengths of the sprouts statistically significantly compared to the negative control (Difference of the means: 420.98 ± 321.63 pixels, $p=0.04$; 731.18 ± 289.91 pixels, $p<0.01$; 541.80 ± 310.96 pixels, $p<0.01$ accordingly). Doses of GLP-1 at 50 and 100nM were not able to trigger an angiogenic response (difference of the means compared to the negative control: 46.42 ± 302.5 pixels, $p=0.76$; 144.55 ± 335.52 pixels, $p=0.64$ accordingly). Interestingly there was a biphasic response to GLP-1 induced angiogenesis with the maximum effect observed at 500nM (difference of means GLP-1 500nM vs. GLP-1 200nM: 310.20 ± 289.91 , $p=0.04$; GLP-1 500nM vs. GLP-1 1000nM: 189.38 ± 278.02 , $p=0.18$), while 2000nM of GLP-1 were not able to trigger a significant angiogenic response (difference of means compared to negative control: 241.29 ± 288.22 pixels, $p=0.1$). The positive control (difference of means compared to negative control: 998.0 ± 335.52 , $p<0.001$) confirmed the validity of the assay.

HUVEC 3-D angiogenesis inhibitor experiment (Figure 2)

We evaluated 14-35 beads in each treatment group (negative control, non-pretreated GLP-1 500nM, AG490, Compound C, PD98059, SB203580, SP600125, AKT Inhibitor IV, Bisindolylmaleimide, FAK inhibitor 14, Src inhibitor I pre-treated and VEGF 20 ng/mL). Similar with above beads that were close to the edges of the scaffold gel and / or where truncated by shear stresses were excluded from the analysis. Sprouts cumulative length in the AG490, Compound C, PD98059, SB203580, and SP600125 pre-treated cultures was not significantly different from the non-pretreated, GLP-1 500nM treated cells (all p -values >0.05). Interestingly AKT Inhibitor IV, Bisindolylmaleimide and Src inhibitor I pre-treatment resulted in decreased cumulative sprout length compared to the nonpretreated spherules (differences of the means: -1461.52 ± 230.43 , $p<0.001$; -829.29 ± 224.55 , $p<0.001$; -1431.12 ± 227.38 , $p<0.001$ accordingly). This signifies that blockage of the Akt, PKC, and Src pathways inhibits GLP-1 induced angiogenesis. As expected FAK inhibitor 14 completely inhibited any angiogenic process (differences of the means compared to the negative control: -2757.48 ± 237.36 , $p<0.001$). Sprout cumulative length was significantly increased in the positive control group confirming the validity of the assay. As an indication of internal reproducibility of the study, the non-pretreated, GLP-1 500nM treated spherules had a positive angiogenic response compared to negative control.

GLP-1 Stability in the supernatant

The optical density in the competitive GLP-1 ELISA, performed on the supernatant of the angiogenesis assays, did not change significantly between 0 and 24 hours of treatment (OD 0.111 ± 0.016 AU vs. 0.106 ± 0.007 AU, $p=0.92$) or between 0 and 48 hours (OD 0.111 ± 0.016 AU vs. 0.132 ± 0.02 AU, $p=0.051$, data not shown). This means that the levels of GLP-1 in the supernatant remained stable during the time of the experiment and thus degradation or metabolism of the molecule is unlikely. Consequently the spherules remained exposed to a constant concentration of GLP-1 during the experiment.

Discussion

In this study we demonstrate that GLP-1 can promote angiogenesis in an *in vitro* model of HUVECs. In addition the results of our signaling inhibitor experiments suggest that GLP-1 induced angiogenesis, is mediated through the PI3K/Akt, PKA and Src pathways. These findings are novel since no study to date has evaluated the direct effect of GLP-1 on angiogenesis *in-vitro*. In more detail, we demonstrated that the angiogenic response of HUVECs to GLP-1 stimulation is biphasic: GLP-1 promotes angiogenesis at doses ranging between 200 and 1000nM, with the maximal effect observed at 500nM. Interestingly 2000nM did not trigger any significant angiogenic response. The cause of this dose-response

relationship remains to be elucidated. Mechanisms that could explain these observations include that increased concentrations of GLP-1 may bind to and activate receptors other than the GLP-1 receptor, which in turn may activate anti-proliferative and anti-angiogenic signaling pathways and/ or GLP1 receptors with a different affinity for GLP-1 that may exist on relevant cell types. Alternatively, concentrations of GLP-1 higher than 1000 nM may be resulting in downregulation of the putative receptors in a true endocrine manner and thus may be attenuating GLP-1 signaling. Since our study was not designed to investigate this issue the above hypotheses need to be evaluated in future studies. Of note, native GLP-1 concentrations range between 0.4 to 1.4 pM in the fasting and 10 to 12 pM in the postprandial state (18), while its concentrations can reach up to 2000pM after administration of pharmacologic doses of GLP-1(19), and 10.6 nmol/L in response to lowest dose of Liraglutide (0.6 ng/ml) used in the clinic (20). These circulating levels are significantly lower compared to the doses that we observed angiogenesis in our in-vitro model but levels achieved locally at the vasculature could be higher and need to be studied in the future. In a similar study utilizing human coronary artery endothelial cells (HCAECs), 100nM of GLP-1 increased DNA synthesis and endothelial proliferation (11). In the same study, Exendin-4, which is a stable GLP-1 analogue with similar potency but higher efficacy than native GLP-1 (21), had similar results with the latter on promoting cell proliferation, while co-administration of Exendin-4 and GLP-1 did not have any additive effect (11). The latter is the only publication available to date investigating the in vitro effect of GLP-1 and a GLP-1 analogue on endothelial cells. Although the investigators demonstrate an increase in proliferation they do not investigate its effect on angiogenesis which is a process that, in addition to proliferation, involves other steps including collagen breakdown, endothelial cell migration and tubule formation (13). In this study we used a 3-dimensional model of spherules of HUVECs that enabled us to directly observe the net angiogenic effect of GLP-1 treatment. The effects of GLP-1 analogues such as Liraglutide, which reach higher levels in the circulation and could be more potent than GLP-1 per se, need to be studied by future studies.

The fact that GLP-1 could promote angiogenesis has also been suggested through animal and human studies. Studies in a murine model of critical limb ischemia demonstrated that sitagliptin increases circulating GLP-1 levels, leading to increased levels of circulating endothelial progenitor cells, upregulation of tissue expression of endothelial NOS (eNOS) and ultimately enhanced angiogenesis (12). Studies in humans are in agreement with our in vitro observations. Vildagliptin increases the levels of GLP-1 in wound lysates, resulting in increased levels of VEGF and HIF-1 α after 3 months of treatment and ultimately leading to increased capillary density after 12 months of treatment (22).

In our study, we also demonstrate that inhibition of Akt, Src or PKC lead to inhibition of GLP-1 induced angiogenesis, implying that the angiogenic effects of GLP-1 are, in part, mediated through these pathways. These results are consistent with previous studies demonstrating that GLP-1 promotes proliferation in HCAECs through an Akt and eNOS dependent manner (11). Akt has been shown to have a pivotal role in angiogenesis, by directly promoting endothelial cell proliferation and NO-induced cell migration, through phosphorylation of eNOS (23). Src is a well described upstream activator of eNOS (24) that promotes endothelial proliferation, regulates the microvascular epithelial phenotype and ultimately leads to enhanced angiogenesis (25, 26). Src kinase is also essential for VEGF-induced angiogenesis (27). eNOS has a central role in promoting ischemia-induced angiogenesis (28, 29) and similar to src has a pivotal role in VEGF-induced angiogenesis (30). Interestingly there is sufficient evidence to support that Dysregulation of the VEGF and angiopoietins system has also been shown to contribute to the pathogenesis of diabetic nephropathy (31). We also demonstrated that inhibition of PKC leads in inhibition of GLP-1 induced angiogenesis. Of note, there is no study to date evaluating the effects of GLP-1 on

PKC signaling on endothelial cells. In different studies, activation of PKC has been demonstrated to be associated with promotion of angiogenesis through VEGF upregulation but no study to date has evaluated the association of this pathway with the angiogenic activity of GLP-1 (32).

Understanding the molecular biology underlying GLP-1 induced angiogenesis is very important since enhanced angiogenesis could be one of the mechanisms through which GLP-1 exerts its pleiotropic, direct cardioprotective effect. Both the myocardial myocytes and the endothelial cells express functional GLP-1 receptors (33). In a murine model of myocardial infarction Liraglutide resulted in a significantly improved survival compared to metformin, independently of weight loss, for the same level of glycemic control in both euglycemic and diabetic mice (34, 35). The latter was primarily mediated through upregulation of pro-survival pathways (34, 35). In translational, proof-of-concept human studies, GLP-1 infusion resulted in a significantly improved LVEF and regional wall motion score indexes in both diabetic and non-diabetic patients with left ventricular (LV) dysfunction after myocardial injury and angioplasty (4). This improvement persisted for several weeks after discontinuation of the GLP-1 infusions (4). Parallel to this, longer term GLP-1 administration improved parameters of LV function, functional status, and quality of life in both diabetic and nondiabetic subjects with congestive heart failure (5). Furthermore in large-scale database studies with hard outcome data derived from pharmaceutical and insurance claims Exenatide treated patients were less likely to have myocardial infarction, ischemic stroke, or coronary revascularization intervention (36). There is emerging evidence that targeting the GLP-1 pathway leads to improved endothelial function. Diabetes is associated with impaired flow mediated dilatation (FMD) and endothelial dysfunction (37), while lifestyle modifications with a low fat diet and daily exercise can improve it (38). A single GLP-1 infusion increases post-ischemic (FMD) in both euglycemic and diabetic patients with coronary artery disease (39). Similarly, treatment with Exenatide for 16 weeks increases FMD more than glimepiride in patients with type 2 diabetes (6), while Liraglutide, has beneficial effects on endothelial function, by improving markers of endothelial dysfunction (7, 8) and preventing endothelial cell glucotoxicity through reduction of the hyperglycemia-induced ER-stress (9).

There is emerging evidence that augmentation of the GLP-1 pathway could also have some beneficial effects in addition to the cardiovascular. Studies in animal models have yielded that GLP-1 could have a protective effect in diabetic nephropathy by reducing mesangial reactive oxygen formation and MCP-1 expression in response to advanced glycosylation products exposure (40) and by reducing albuminuria (41). In addition, GLP-1 has a beneficiary role on pancreatic beta cell physiology by reduce glucolipotoxicity induced apoptosis through activation of the PI3K pathway (42) and by up-regulating GLUT-2 expression, ultimately leading to enhanced insulin secretion (43, 44).

Interestingly GLP-1 levels are increased after bariatric surgery and this has been proposed as a potential mechanism of bariatric surgery induced improvement of insulin resistance. In patients that have undergone Roux-en-Y gastric bypass, an increase in the post-prandial GLP-1 levels has been reported to occur as soon as two days after the procedure (45) and has been documented to remain elevated up to three years after the procedure (46). Similarly, in patients undergoing Biliopancreatic Diversion post prandial GLP-1 levels increase 1 week after the procedure (45). Patients with Jejuno-ileal bypass have documented improved GLP-1 responses up to 20 years after the procedure (46). Enhanced incretin secretion is also observed in patients that undergo different types of gastric banding procedures but the increment is smaller compared to the above gastric bypass procedures (47). The mechanisms behind these observations remain largely unknown but one of the most appealing hypotheses is that direct exposure of distal ileus to nutrients leads to

increased secretion of GLP-1. This is based on animal model studies, demonstrating that transposition of the ileus, without bypass, increases plasma levels of GLP-1 (48).

This study has several strengths: This is the first study to date that demonstrates that GLP-1 has a pro-angiogenic potential *in vitro* and points to molecular pathways through which the latter is mediated. The assay used for this purpose is well-validated and the scaffold in which the HUVECs were embedded closely resembles the consistency and properties of the vascular base membrane. There is strong internal validity in the data, since the pro-angiogenic effect of GLP-1 was replicated in multiple assays and the data presented herein are consistent with and build upon the pre-existing literature. One limitation of our study is the fact that it does not include direct signaling experiments; the conclusions are drawn through signaling inhibitor experiments. In addition, we utilized specifically HUVECs, and thus the results of this study may not necessarily be generalizable to other sub-types of endothelial cells. The cellular micro-environment in which we performed the experiments might be different from the one present in diabetics where poor diabetic control is associated with higher VEGF levels (49). The doses at which we observed angiogenesis *in vitro*, are significantly higher compared to the physiologic and pharmacologic doses measured in the plasma, suggesting that future translational research aiming to investigate the role of GLP-1 in *in vivo* angiogenesis models should be evaluating GLP-1 levels, not only in the plasma, but also in the target-tissue specific level. Finally, comparative evaluation, *in vitro* and possibly *in vivo*, of GLP-1 with GLP-1 analogues such as Liraglutide and Exendrine needs to be performed since molecular modifications in the latter may confer altered angiogenesis related potential.

To summarize, this is the first study demonstrating that GLP-1 has a pro-angiogenic action on HUVECs *in vitro* and that the Akt, Src and PKC signaling pathways may be playing a pivotal role in this process. Elucidation of the mechanisms that mediate the pleiotropic cardioprotective effects of GLP-1 is essential, especially in the era of clinical research investigating broader indications for medications targeting the GLP-1 pathway. The pro-angiogenic capacity of GLP-1 might be one of the links between GLP-1 augmentation and favorable cardiovascular outcomes. Although the former could provide some benefit in clinical situations such as diabetic foot healing, angiogenesis promotion might also result in adverse outcomes such as promotion of tumor expansion and/or metastasis or promotion of proliferative retinopathy in diabetics. Although the concentrations that GLP-1 promotes angiogenesis are significantly higher than the physiological circulating GLP-1 levels, little is known about the *in situ* GLP-1 concentrations in different tissues at rest and during periods of injury / recovery. Further research aiming at providing a deeper understanding of the molecular principles underlying GLP-1 induced angiogenesis and related translational potential in different clinical settings is thus warranted.

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KNA designed the study, performed experiments and statistical analysis and wrote the manuscript. JPC contributed to the conduct of the experiments and reviewed versions of the manuscript. CSM designed the study and oversaw the entire study design, conduction and the manuscript writing.

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Abbreviations

GLP-1	Glucagon-Like Peptide 1
HCAECs	Human Coronary Artery Endothelial Cells
HUVECs	Human Umbilical Vein Endothelial Cells
VEGF	Vascular Endothelial Growth Factor
STAT-3	Signal transducer and activator of transcription 3
AMPK	5' adenosine monophosphate (5' AMP) -activated protein kinase
FAK	Focal Adhesion Kinase
ELISA	Enzyme Linked Immunosorbent Assay
OD	Optical Density
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
NOS	Nitric Oxide Synthase
eNOS	Endothelial Nitric Oxide Synthase
PKC	Protein Kinase C
LVEF	Left Ventricular Ejection Fraction
FMD	Flow-Mediated Dilatation

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Figure 1.

A. Angiogenic effect of GLP-1 expressed as cumulative length of spurs in pixels. Error bars represent standard errors of the means. Star (*) represents statistically significant difference from the negative control. Negative CTRL: negative control; Positive CTRL: positive control (VEGF 20ng/mL); B. Phase contrast microscope images of HUVEC spherules treated with GLP-1 500nM; C. NC: Phase contrast microscope images of HUVEC spherules treated negative control; D. PC: Phase contrast microscope pictures of HUVEC spherules treated with positive control.

Figure 2.

Angiogenic effect of GLP-1 expressed as cumulative length of spurs in pixels and inhibitor pre-treatment. Error bars represent standard errors of the means. Columns with the same letter are not statistically different between each other. NC: negative control, PC: positive control, GLP-1: non-pretreated 500nM GLP-1 treated cells, AG490: STAT-3 inhibitor, Compound-C: AMPK inhibitor, PD98059: erk inhibitor, SB203580: jnk inhibitor, SP600125: p38 inhibitor, AKT-INH-4: Akt inhibitor 4, Bisindolylmaleimide: PKC inhibitor; FAK-INH-14: focal adhesion kinase inhibitor 14; SRC-INH-1: src inhibitor 1.