A Src-Abl kinase inhibitor, SKI-606, blocks breast cancer invasion, growth and metastasis *in vitro* and *in vivo.*

Houda Jallal

Experimental Medicine

McGill University, Montreal

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Table of contents

List of Figures

Introduction

Results

List of Tables

Introduction

List of Abbreviations

Abbreviation	Full term/ Meaning
ATP	Adenosine tri-phosphate
CML	Chronic myeloid leukaemia
CsK	Cellular Src kinase
DENV	Dengue virus
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FAK	Focal adhesion kinase
GFP	Green fluorescent protein
MAPK	Mitogen activated protein kinase
PI3K	Phosphatidylinositol-3-kinase
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonylflouride
PTHrP	Parathyroid hormone
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulphate
SH2	Region of Src homology 2
SH3	Region of Src homology 3
SH4	Region of Src homology 4
SFK	Src family kinase
RSV	Rous sarcoma virus
Ts	Temperature sensitive
uPA	Urokinase Plasminogen Activator
VEGF	Vascular endothelial growth factor

ABSTRACT

The central role of Src in the development of several malignancies including breast cancer and the accumulating evidence of its interaction with receptor tyrosine kinases (RTK), integrins and steroid receptors have identified it as an attractive therapeutic target. In the current study we have evaluated the effect of a Src/Abl kinase inhibitor SKI-606, on breast cancer growth, migration, invasion and metastasis. Treatment of human breast cancer cells MDA-MB-231 with SKI-606 caused a marked inhibition of cell proliferation, invasion and migration by inhibiting MAPK and Akt phosphorylation. For *in vivo* studies MDA-MB-231 cells transfected with the plasmid encoding green fluorescent protein (GFP) [MDA-MB-231-GFP] were inoculated into mammary fat pad of female BALB/c *nu/nu* mice. Once tumor volume reached 30-50 mm^3, animals were randomized and treated with vehicle alone or 150 mg/kg of SKI-606 by daily oral gavage. Experimental animals receiving SKI-606 developed tumors of significantly smaller volume (45-54%) as compared to control animals receiving vehicle alone. Analysis of lungs, liver and spleen of these animals showed a significant decrease in GFP positive tumor metastasis in animals receiving SKI-606 at a dose that was well tolerated. Western blot analysis and immunohistochemical analysis of primary tumors showed that these effects were due to the ability of SKI-606 to block tumor cell proliferation, angiogenesis, growth factors expression and inhibition of Src mediated signalling pathways *in vivo*. Together the results from these studies provide compelling evidence for the use of Src inhibitors as therapeutic agents for blocking breast cancer growth and metastasis.

RÉSUMÉ

La protéine Src joue un rôle clé dans le développement de plusieurs cancers, incluant celui du sein. Src interagit avec une variété de récepteurs et de molécules effectrices ce qui rend cette dernière une cible thérapeutique de choix. Dans cette étude nous avons évalué les effets de SKI-606, un inhibiteur des kinases Src et Abl, sur le cancer du sein. En effet, SKI-606 s'est avérait être efficace *in vitro* lors de tests de prolifération, migration et d'invasion, en plus d'abolir la phosphorylation de MAPK et d'Akt sur les cellules MDA-MB-231.

In vivo, les souris BALB/c *nu/nu* ont été inoculées dans la région mammaire avec les cellules MDA-MB-231 encodant le plasmide de la protéine GFP [MDA-MB-231-GFP]. Ces dernières ont été gavées avec 150 mg/kg de SKI-606. Par conséquent, les animaux ayant reçu SKI-606 ont développé des tumeurs de volume plus petit de 45 à 54% comparé au groupe contrôle.

L'analyse des sections de poumons, du foie et de la rate de ces animaux a démontré une diminution significative de tumeurs GFP positives. De plus, les analyses effectuées par Western blot et immunohistochimie des tumeurs primaires ont consolidés les résultats de l'étude où SKI-606 est apte à bloquer la prolifération, l'angiogenèse, ainsi que l'expression des facteurs de croissance. Ensemble, les résultats de cette étude prouvent que SKI-606 est un agent thérapeutique de choix pour bloquer le cancer du sein et les métastases.

INTRODUCTION

Context for the research Project

Gleevec, the Bcr-Abl inhibitor revolutionized the way to approach cancer treatment. Several Chronic Myeloid Leukemia (CML) patients benefited from this discovery[1]. Indeed Gleevec (also known as Imatinib), interferes with the aberrance created in the Philadelphia chromosome and hence keeps the number of leukocytes to a normal state[2]. Imatinib acts as a competitive inhibitor to the ATP binding site of Bcr-Abl and binds the inactive form of Abl kinase[3]. However some CML patients show resistance to Gleevec[4]. In order to circumvent this resistance, Whyte produced SKI-606 (Bosutinib) because of the close homology between Src and Abl. SKI-606, which is the third second generation of tyrosine kinase inhibitors, is a Src-Abl kinase inhibitor which gave good results in Philadelphia chromosome positive CML and acute lymphoid leukemia (ALL) in clinical trials[5] [6]. SKI-606 has been shown to be 200 fold more potent than Imatinib[6] . Other members of Src-Abl kinase inhibitors include Nilotinib, Dasatinib and INNO-406.

Unlike other Src-Abl inhibitors SKI-606 did not demonstrate any significant inhibition of c-kit and PDGFR. The advantage of this selectivity is the potential clinical benefit of reducing adverse events such as pleural effusions which have been attributed to the effect of other Src-Abl kinase inhibitors on PDGFR[7]. SKI-606 has already been evaluated in a phase I study in solid tumors and no pleural effusions were seen in that study. This study evaluates the role of SKI-606 in breast tumors.

Hypothesis

We postulated that the effect of Src kinase inhibition by SKI-606 on breast cancer growth would have similar effects *in vitro* and on tumor growth and metastasis *in vivo* as observed in non solid tumors and colorectal cancer cells, and potentially this inhibition would be the result of a down-regulation of signaling pathways.

Aim

The aim of this project is subdivided in two parts: the first part consists of assessing the efficacy of the Src-Abl kinase inhibitor, SKI-606, on highly invasive breast cancer cells, and investigating which pathways are affected. Secondly, *in vivo* studies were conducted in order to consolidate and compare with the findings *in vitro*.

Cancer

Cancer insight

Cancer is a major problem disease worldwide and it is the leading cause of morbidity and mortality in Canada. It is estimated that 153,100 new cases of cancer and 70,400 deaths from cancer will occur in Canada in 2006, a number that is estimated to surpass those of heart disease[8]. Cancer remains the bête-noire of medicine and overcoming this disease remains an enormous challenge.

Breast Cancer

Breast cancer is one of the leading causes of cancer deaths in women worldwide and its overall incidence appears to be rising. Of American women, 13% will be diagnosed with breast cancer in their lifetimes, making it the most common non-skin cancer among women[9]. It is also the most diagnosed cancer in Canadian women[10]. During their lifetimes, 1 in 8.9 Canadian women are expected to develop breast cancer and 1 in 27 women are expected to die from it[10]. Breast cancer has surpassed lung cancer as the leading cause of cancer death in women worldwide, accounting for more than 400,000 deaths per year[9].

The progression of breast cancer can be staged using the TNM classification according to the American Joint Committee on Cancer. This is based on primary tumor size (T), lymph node involvement (N) and metastatic disease (M).

This can also be broadly categorized into 5 different stages, Stage 0 – IV, with some of them having subsets according to the US National Cancer Institute. These stages are;

"Stage 0 (Ductal or Lobular Carcinoma-in-situ)

Stage I – the tumor is 2 cm or less and has not spread outside the breast.

Stage IIA - no tumor is found in the breast, but cancer is found in the axillary lymph nodes; or the tumor is 2 centimeters or smaller and has spread to the axillary lymph nodes; or the tumor is larger than 2 centimeters but not larger than 5 centimeters and has not spread to the axillary lymph nodes.

Stage IIB - larger than 2 centimeters but not larger than 5 centimeters and has spread to the axillary lymph nodes; or larger than 5 centimeters but has not spread to the axillary lymph nodes.

Stage IIIA - no tumor is found in the breast, but cancer is found in axillary lymph nodes that are attached to each other or to other structures; or the tumor is 5 centimeters or smaller and has spread to axillary lymph nodes that are attached to each other or to other structures; or the tumor is larger than 5 centimeters and has spread to axillary lymph nodes that may be attached to each other or to other structures.

Stage IIIB - has spread to tissues near the breast (the skin or chest wall, including the ribs and muscles in the chest); and may have spread to lymph nodes within the breast or under the arm.

Stage IIIC - has spread to lymph nodes beneath the collarbone and near the neck; and may have spread to lymph nodes within the breast or under the arm and to tissues near the breast. Stage IIIC breast cancer is divided into operable and inoperable stage IIIC. In operable stage IIIC, the cancer: is found in 10 or more of the lymph nodes under the arm; or is found in the lymph nodes beneath the collarbone and near the neck on the same side of the body as the breast with cancer; or is found in lymph nodes within the breast itself and in lymph nodes under the arm. In inoperable stage IIIC breast cancer, the cancer has spread to the lymph nodes above the collarbone and near the neck on the same side of the body as the breast with cancer.

Stage IV - the cancer has spread to other organs of the body, most often the bones, lungs, liver, or brain."[11]

The treatment of breast cancer largely depends on the stage of breast cancer in a patient. In patients with surgically resectable tumors, neo-adjuvant or adjuvant chemotherapy are usually administered, to either reduce the tumor load pre-operatively or reduce the likelihood of recurrence following surgical resection. In patients with non-resectable tumors, chemotherapy is sometimes used for palliation or symptomatic relief.

In the chemoprevention of breast cancer, retinoids, cyclooxygenase (COX) inhibitors, and selective estrogen receptor modulators (SERM) have been researched. In clinical trials, it has been demonstrated that treatment with tamoxifen reduced the risk of invasive breast cancer by 49%[9].

Aromatase inhibitors (AI) are also being used in the chemoprevention of breast cancer. Three third-generation aromatase inhibitors such as anastrozole, letrozole, exemestane are currently being used in adjuvant therapy for postmenopausal women with hormone receptor–positive breast cancer[9].

Trastuzumab is a chimerized mouse/human monoclonal antibody. It is part of a new group of cancer drugs which are based on antibody therapy. Trastuzumab targets the extracellular portion of the Her-2/neu membrane protein[9].

Potentially, the inhibition of Src by SKI-606 could have a double therapeutic effect in breast cancer as Src plays an important role in the estrogen receptor hormonal pathway as well as in tumor cell survival, proliferation and migration (see Fig. 4). So far, none of the above mentioned chemotherapy agents act on Src kinase directly, or have a dual effect on estrogen receptor pathway and Src kinase simultaneously. This could place SKI-606 in a special niche in the treatment of breast cancer.

The c-Src Proto-Oncogene

In 1910, Nobel Laureate Peyton Rous discovered from chickens a virus by injecting healthy chickens with a cell-free extract from a fibrosarcoma of a sick hen[12]. This finding was accepted with scepticism, as cancers were believed to be non-infectious. Several decades later, it had become readily acceptable that a range of viruses can induce tumours in suitable hosts. The Rous sarcoma virus (RSV) is the first cancer-causing retrovirus to be isolated. Howard Temin and Harry Rubin (1958) showed that chicken embryo fibroblasts transform following an RSV infection by altering their morphological structure to become spindle-shaped type of cells[13]. These studies were described in one of the first developed focus assays.

The subsequent isolation of temperature-sensitive (ts) mutants of Rous sarcoma virus was a major advancement towards the identification of the v-Src gene. At a permissive temperature of 30^0C the mutants were able to transform fibroblasts. Where as at 39^0C, a non-permissive temperature, the normal phenotype was observed and therefore the virus was unable to transform cells but replication was still occurring[14].

Nobel Laureates Michael Bishop and Harold Varmus discovered in 1976, the cellular counterpart of v-*Src* by using a cDNA probe that happened to hybridize to normal avian DNA[15]. The term 'proto-oncogene' for the cloned c-Src was then coined; as v-*Src* was already called an 'oncogene' [16] [17] [18]. It is worth mentioning the discovery of how c-Src was tyrosine but not threonine phosphorylated. In 1979, Tony Hunter omitted to prepare fresh pH buffer and used and old stock of pH 1.9 for the electrophoretic separation of

phosphoamino-acids of polyomavirus middle T antigen. Following autoradiography of 32p labelled immunoprecipitates of middle T protein, he noticed an unusual spot that did not coincide with phosphoserine and phosphothreonine internal markers. After several repeats of the experiment it was later confirmed that this hydroxyamino acid is phosphotyrosine which resolves at a higher pH[19 20]. The Src protein was then the first tyrosine kinase to be discovered. As a result of these studies, several cellular oncogenes were identified.

c-Src versus v-Src

The c-Src and v-Src genes are highly conserved in general. Several point mutations can be found within the v-Src gene. Moreover, the v-Src lacks the introns that are present in c-Src. The Rous sarcoma virus, like all viruses in general, is composed of three genes (gag, pol and env). These genes are necessary for the replication of the virus, as well as its encapsulation for survival. A fourth gene is also present, (v-Src) and encodes for the protein responsible for transformation[21 23].

At the protein level, both c-Src and v-Src are myristoylated at Glycine 2 in the amino terminus. A unique region lies between the modular domain SH4 and SH3[22]. The unique region functions as modulator of specific interactions between the proteins and Src. The SH2 domain is also shared by the two proteins, however, the negative regulatory tyrosine Y527 is found only in c-Src. The SH2 domain is separated from the tyrosine kinase

domain by a linker region[23]. The C-terminus of the c-Src protein contains amino acids that have been deleted in the v-Src protein[24].

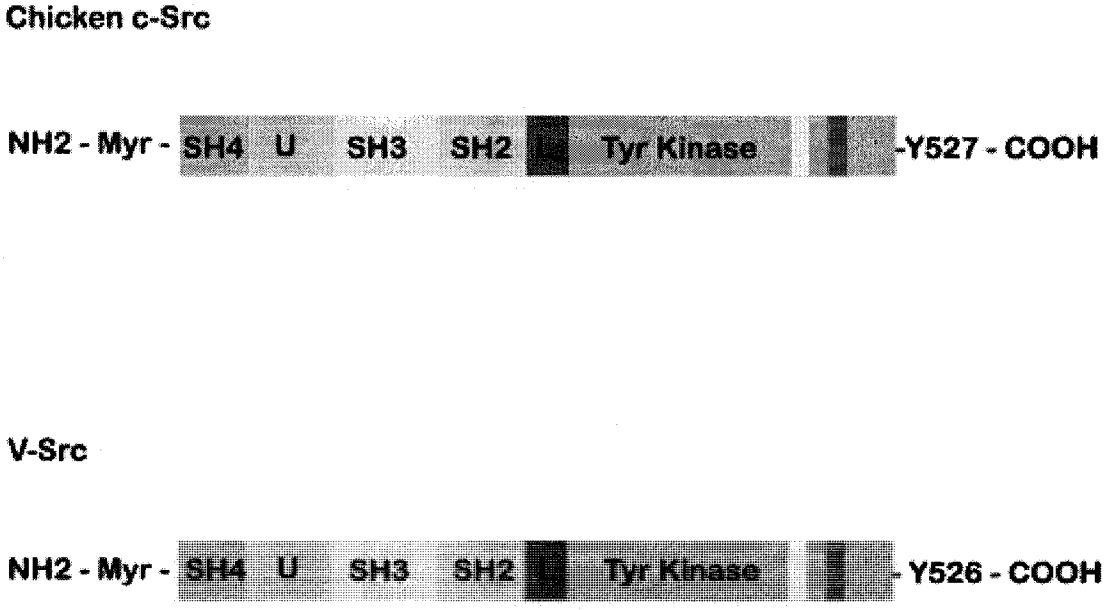


Figure 1: Comparison of c-Src and v-Src proteins. At the amino terminus both c-Src and v-Src are myristoylated (Myr) at Glycine 2. A unique region (U) lies between the modular domain SH4 and SH3. SH2 domain is also shared by the two proteins, however the negative regulatory tyrosine Y527 is found only in c-Src's tyrosine kinase domain (TK). The SH2 domain is separated from the tyrosine kinase domain by a linker region. The C-terminus of the c-Src protein contains amino acids that have been deleted in the v-Src protein.

Src Family Tyrosine Kinases

Src belongs to a non-receptor tyrosine kinase family that comprises of nine members in vertebrates[25]. The members of the Src family can be divided into 2 classes: those with a broad range of expression, such as Fyn, Lyn, Src and Yes, and those with expression patterns restricted primarily to hematopoetic cells like Hack, Balk, Lck and Fgr (Table 2). Src Family members share common structures as described in Table 1. They also act as signalling molecules similar to Src[23].

Domain	**Function**
Amino terminus	Anchoring to cell membranes
Unique domain	Modulator of specific interactions between proteins.
Src-homology domain 3 (SH3)	Binding to proline-rich ligands
Src-homology domain 2 (SH2)	Binding to phosphorylated tyrosine
Tyrosine kinase domain (TK)	Enzymatic activity
Activation loop	Role in regulation, harbors the autophosphorylation site
C-terminal tail	Harbors the negative regulatory tyrosine

Table 1: Common structures shared between Src-family members.

Adapted from Ref.[26]

Table 2: Src Family members, their expression pattern

Expression	Member	Knock-out
Broad	Src	Osteopetrosis
	Yes	None observed
	Fyn	Abnormal hippocampal development
	Lyn	Impaired B-cell function
Myeloid leukocytes	Hck	None observed
B-lymphocytes	Blk	None observed
T-lymphocytes	Lck	Impaired T-cell development
Myeloid leukocytes	Fgr	None observed

Adapted from Ref[27 28]

Structure of Src Family Tyrosine Kinases

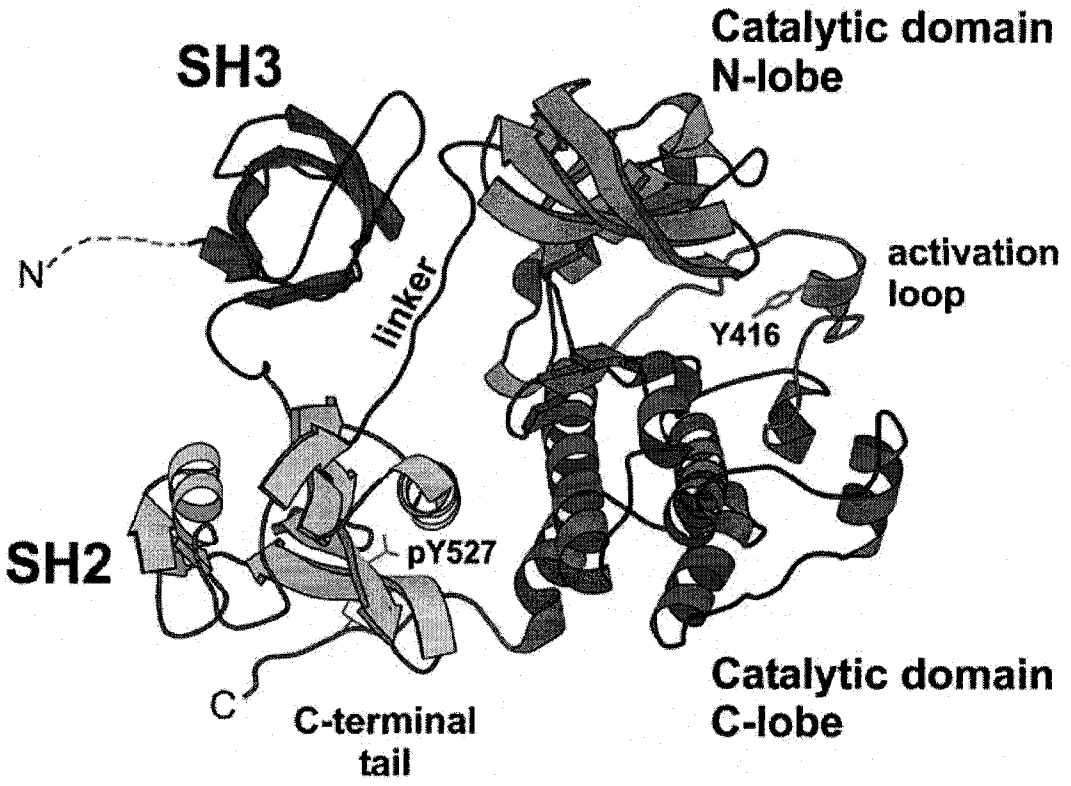


Figure 2: Crystal structure of interaction of c-Src domains

Illustrated in this high-resolution crystal structure model of chicken c-Src are: the SH2 domain, the SH3 domain, the small and large lobes of the catalytic domain, the linker region and the C-terminal 'tail' sequences. pY527 represents down-regulation of Src activity, whereas Y416 represents autophosphorylation and positive regulation[29]. (Figure from ref[30].)

Regulation of Src activation

Src kinase domain, which harbors the kinase active site, plays an important part in Src regulation along with the SH2 and SH3 domains. Indeed, X-ray crystallography (Figure 2) studies demonstrated a ''closed'' conformation in the structure of Src, hence, Src can convert from an inactive to an active state[31]. This regulation occurs through two major phosphorylation sites on Src, tyrosine 416 and tyrosine 527 as illustrated in Figure 3[21 25].

Negative regulation of c-Src occurs mainly on the critical tyrosine 527 for the chicken c-Src and tyrosine 530 for its human homologue. When tyrosine 527 or 530 is phosphorylated, Src becomes inactive as the phosphorylated site interacts with the SH2 domain. This interaction renders Src to fold in a closed conformation which makes it inaccessible and inactive[32 33]. Negative regulation of Src can also happen through various proteins such as CSK kinase which phosphorylates tyrosine 527. Dephosphorylation of tyrosine 527 by SHP-1 increases Src kinase activity[34].

The mechanism involved in the activation of Src is achieved by auto-phosphorylation of tyrosine 416, which is then displaced from the binding pocket. Tyrosine 416 in the activated state permits the substrates to gain access. Protein interactions are also other ways of regulating Src. Indeed, Src adopts an ''open'' active conformation as growth factor receptors bind to its SH2 domain[35].

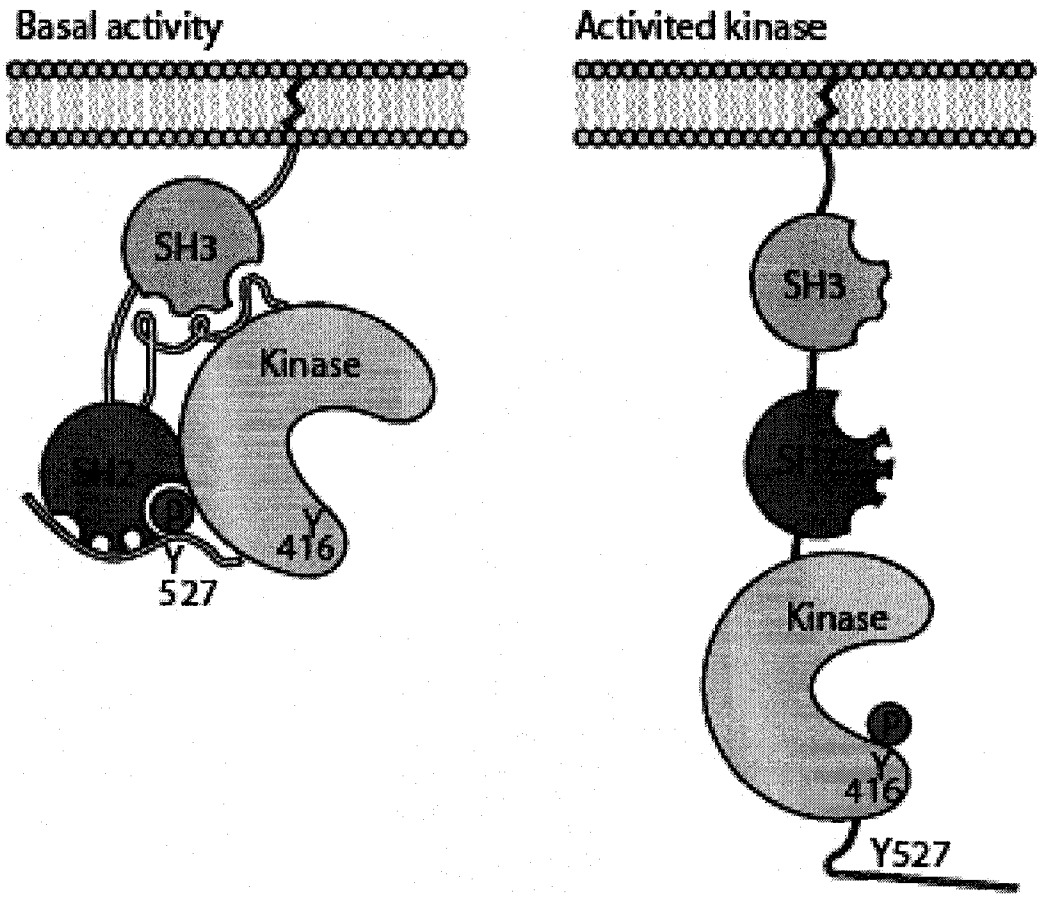


Figure3: Schematic diagram of regulation of c-Src's activity.

The left panel depicts the inactive 'closed' conformation of Src, in which tyrosine 527 (avian c-Src) interacts with the SH2 domain, positioning the SH3 domain to interact with the linker between the SH2 and catalytic domains. The right panel illustrates the open or active conformation. Figure from ref[21], adapted from ref[25].

Role of Src in Signalling

Src plays a role in several signalling pathways that are involved in cell proliferation and survival[24]. Additionally, the ability of Src to promote tumor cell invasion can lead to the development of tumor metastasis[36]. Collectively Src plays a major role in regulating important mechanisms of specific receptor pathways where their activation can influence the biological activities of the tumor cell [25].

In the vascular endothelial growth factor (VEGF) signalling pathway, phosphorylation of VE-cadherin on tyrosine 685 by Src plays a pivotal role in regulating VE-cadherin adhesive activity[37]. In addition, Src has been shown to regulate PI3K and Akt pathways[38]. Several growth factors and proteases including parathyroid hormone-related peptide (PTHrP) and urokinase (uPA) can serve as downstream targets for Src kinase[39 40]. Previous studies have demonstrated the role of PTHrP in tumor cell proliferation, invasion, metastasis and the development of hypercalcemia of malignancy in several common cancers including breast cancer[40 41 42 43]. It is important to note that elevated PTHrP production, regulated by several oncogenes like Ras, Tpr-Met and Src, can activate osteoclastic bone resorption which leads to increased bone metastasis[41 42 43]. uPA, production of which is activated by Src, has been shown to play a major role in the invasion, growth and metastasis of several malignancies including breast cancer due to its ability to break down various components of the extracellular matrix[44 45 46]. Src has also been shown to interact with integrins which are responsible for interaction between tumor cells and their surrounding stroma, which can alter tumor cell growth, differentiation, and migration[47 48]. Integrins can in turn activate focal adhesion kinase (FAK) and

extracellular matrix proteins. Autophosphorylation of FAK at tyrosine 397 enables the recruitment of Src family tyrosine kinases by engaging their SH2 domains[49 50 51]. Src can phosphorylate additional sites in FAK leading to the recruitment of the Grb-2 adapter molecule and in turn activation of signalling pathways, such as the mitogen activated protein kinase (MAPK) pathway[52].

Due to the important role of Src in tumor progression, studies were carried out to evaluate the role of inhibition of Src in reducing tumor growth[53]. Duxbury et al. have used the well characterized pyrazolopyrimidines (PP2) to show their ability to decrease pancreatic tumor growth and metastasis in an orthotopic xenograft model of pancreatic cancer[54]. The purine analogue AP23846 can inhibit members of the Src kinase family, but has not been advanced for clinical development. Nonetheless, both interleukin-8 (IL-8) and VEGF expression were inhibited by AP23846 in human solid tumor cell lines, lending further experimental support to a role for Src kinase in tumor growth and angiogenesis[55]. IL-8 signalling is of significance for the anti-tumor activity of AP23846 as IL-8 is also an important mediator of angiogenesis along with other angiogenic factors or receptors[55]. SKI-606 was recently shown to be active in colon tumor xenograft models in nude mice[56 57]. This compound is also an Abl kinase inhibitor and is active in chronic myelogenous leukemia models *in vivo*[58]. Recently, Collucia et al. also demonstrated that SKI-606 decreases growth and motility of colorectal cancer cells via inhibition of Src mediated tyrosine phosphorylation of β-catenin[59]. Nam et al. showed that Dasatinib (BMS-354825), another Src-Abl inhibitor, blocks the kinase activities of Lyn, Src and FAK signalling in human prostate cancer cells[60]. Collectively, these studies have demonstrated the role of the Src oncogene in several malignancies. In the current study we have

evaluated the effect of Src kinase inhibition using SKI-606 on breast cancer growth, invasion and migration *in vitro* and on tumor growth and metastasis *in vivo*.

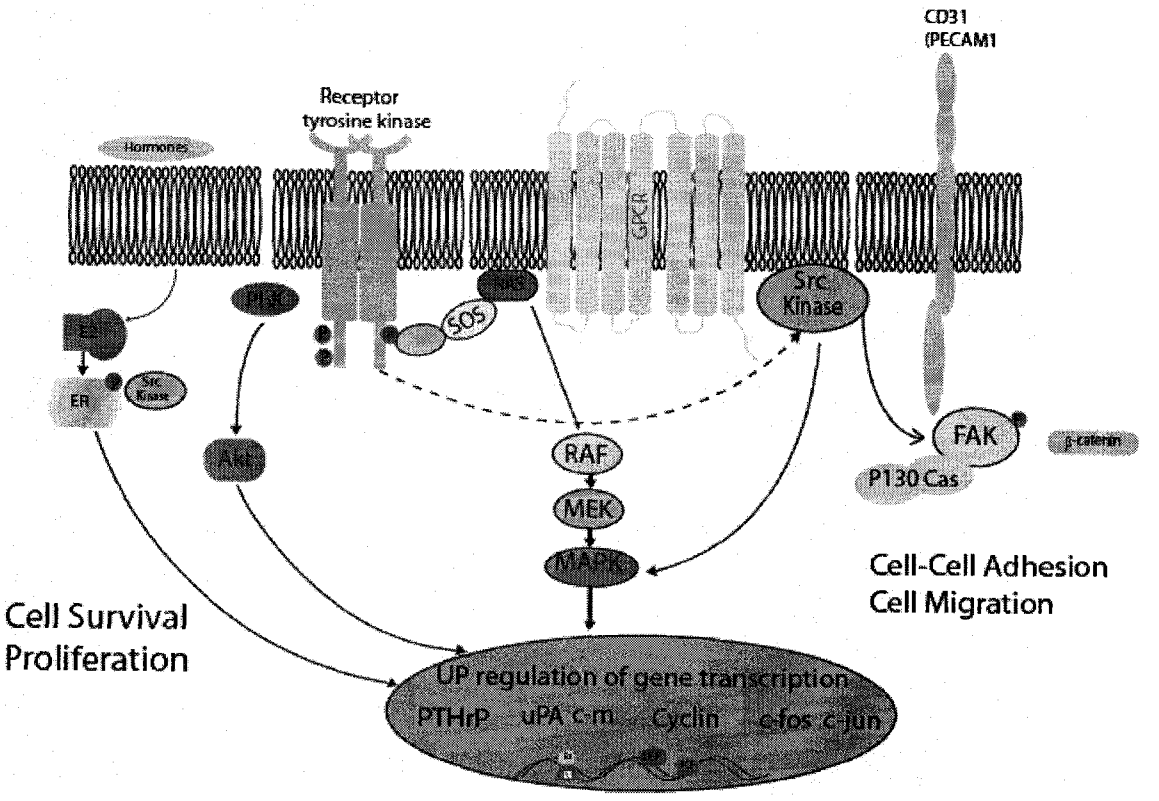


Figure 4: Signalling cascade mediated by c-Src

Src can be activated by various transmembrane receptors. Specific examples shown here are the receptor tyrosine-kinase receptor family, G-protein-coupled receptor and integrin receptor such as the CD31 PECAM1. Src is activated by dephosphorylation of Y527 as well as binding effector molecules to the SH2 and/or SH3 domains. In the case of an integrin, the SH2 ligand is a phosphorylated tyrosine residue by the autophosphorylated focal adhesion kinase (FAK). Src also phosphorylates substrates in the cytoplasm. The subtrates illustrated here are Ras-mitogen-activated-protein-kinase (Ras-MAPK) pathway and phospatidylinositol-3-kinase-Akt (PI3K-Akt) pathway. Src also plays an important role in the regulation of the estrogen receptor hormonal pathway. The end-result of the signaling pathways is the up-regulation of gene transcription leading to cell survival, cell proliferation and cell migration.

SKI-606 compound

SKI-606 (or Bosutinib), is a 7-alkoxy-3-quinoline-carbonitrile. Its main activity is to act as dual inhibitor of Src and Abl kinases. This compound has been previously tested in both a Src kinase assay, as well as cell proliferation assays. The most potent compound had an IC_{50} of 0.78 nM in Src's enzyme assay as well as an IC_{50} of 15 nM in Src transfected cells. SKI-606 was challenged against several protein tyrosine kinases such as AKT and the Epidermal Growth Factor Receptor (EGFR) where the IC_{50} values were respectively greater than 1μM and 230nM. In a cell proliferation assay performed on rat fibroblast cells over-expressing Lck, the IC_{50} calculated then was of 20nM, which indicates that this compound is highly potent against Src Family Kinase (SFK) but with more specificity to Src.

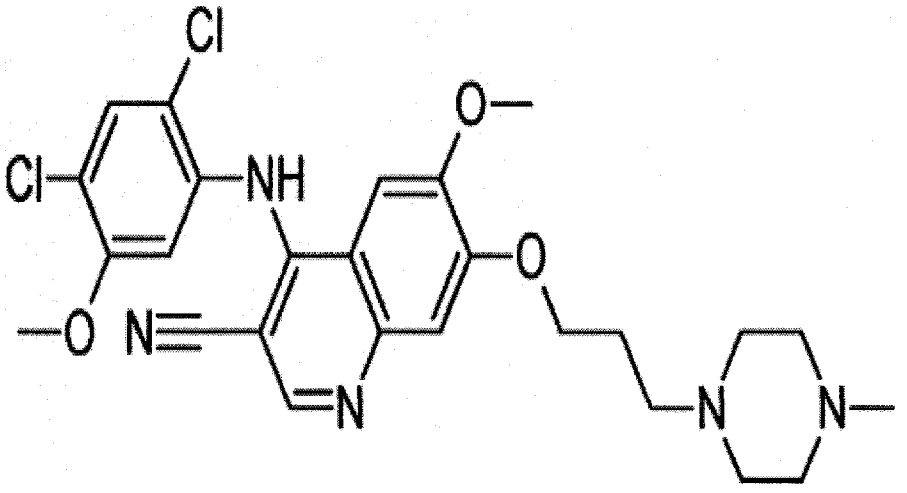


Figure 5: SKI-606, Bosutinib, a 7 alkoxy-3-quinoline-carbonitrile.

Src and Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is defined as the transformation of an epithelial to a mesenchymal phenotype. EMT has been described as a distinct process by Greenburg and Hay in 1982[61]. It has been found recently that Src is one of the key regulators in this process. It is also known that several signalling pathways that occur in EMT in normal development are also associated with tumor progression[62]. Src is implied in this process through its effects on E-cadherin and β-Catenin.

Cadherin-mediated cell-cell contacts are one of the most important aspects of epithelial cells. The expression of E-cadherin is controlled by Src. However, in EMT, the epithelial cells lose these cadherin-mediated cell-cell contacts. As the cells have decreased stability of cell-cell contacts and decreased formation of adherent junctions, they are free to migrate and are less adhesive. In colon cancer, it has been shown that the over expression of Src down-regulates E-cadherin and that PP2 and SKI-606 reverse this effect [59 63]. This down-regulation of E-cadherin is seen as a negative prognostic indicator in cancer and is linked to metastatic disease.

Although the function of β-catenin in EMT is still unclear, its role in tumorigenesis is affected by both E-cadherin and c-Src. As E-cadherin is down-regulated during EMT, it is no longer able to bind to cytoplasmic β-catenin, and hence β-catenin translocates to the cell nucleus where it acts as a transcriptional activator, inducing the transcription factors T-cell factor 4 and lymphoid enhancer factor[64]. Src also elevates the expression of β-

catenin, contributing to its nuclear accumulation[65]. In another study, it has been shown that SKI-606 brings about relocalization of β-catenin to E-cadherin and prevents its nuclear translocation, decreasing cell growth and motility. c-Src was found to be in control of β-catenin function as either a cell adhesive promoter or transcriptional activator[59].

Anti-Cancer Drug Resistance and Src

Anti-cancer drug resistance has been linked with Src activation and this has been shown in the literature, ranging from pancreatic to ovarian cancer cell lines. There are various pathways which lead to chemoresistance in different cancer cell lines.

In gemcitabine-resistant pancreatic cancer cell lines, it has been shown that increasing gemcitabine resistance is associated with higher Src activation. Moreover, it has been demonstrated that when PP2 was introduced to gemticabine-resistant pancreatic cancer cell lines *in vitro*, this chemoresistance is reversed[54]. This was further validated by producing an siRNA against c-Src which then increased the sensitivity of several different pancreatic cell lines to gemcitabine[66].

In breast cancer cells, an increase in Src activation was seen in tamoxifen resistant cell lines. They also behaved more aggressively with increased invasion and migration. However, when a Src-Abl kinase inhibitor, AZD0530, was introduced this resulted in decreased Src activity as well as tumor invasion and migration[67].

In ovarian cancer cells, it has been shown that inhibition of Src by PP2 enhanced their sensitivity to paclitaxel. Interestingly, Src inhibition in chemoresistant ovarian cancer cell lines made them become sensitive to paclitaxel and cisplatin[68].

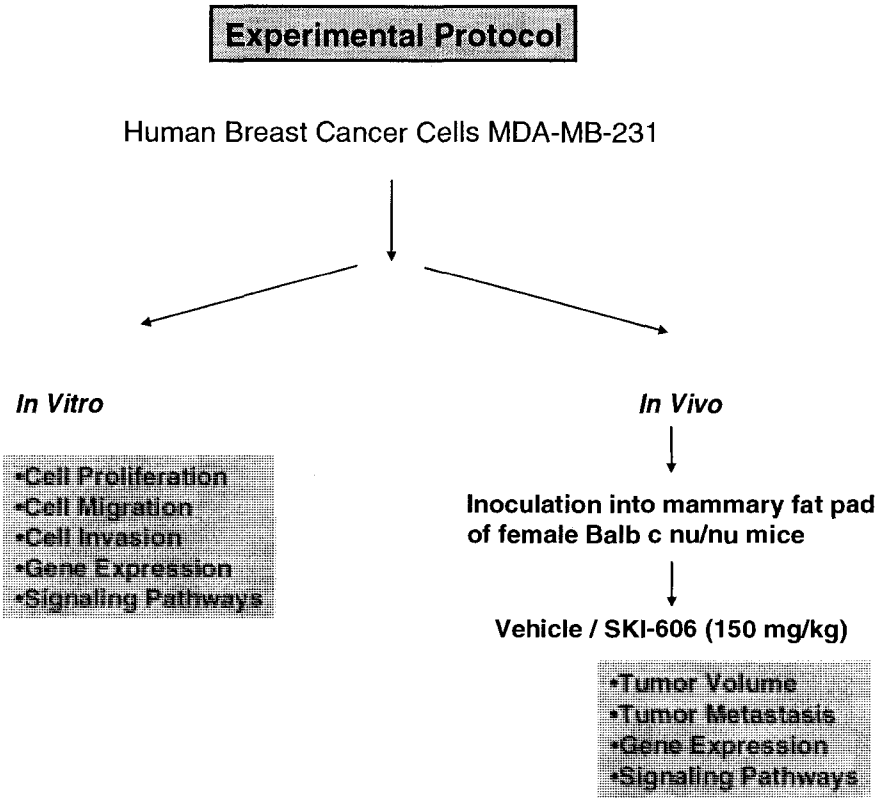


Figure 6: Schematic diagram of the experimental protocol.

MATERIALS AND METHODS

Cells and Cell Culture

Human MDA-MB-231 breast cancer adenocarcinoma cells were obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). Cells stably transfected with green fluorescent protein (MDA-MB-231-GFP) were prepared and maintained in culture as previously described[69 70].

In vitro Cell Assay and Western Blotting

MDA-MB-231 cells were plated in triplicate in 6-well plates in the presence of 2% FBS with vehicle alone or different concentrations (0.1-1 μM) of SKI-606 [71 72 73]. Triplicate wells were trypsinized and counted using a Coulter Counter on alternate days (model ZF, Coulter Electronics, Harpenden, Hertfordshire, UK). Cell culture medium was replenished every second day[72 74].

For cell migration assays, MDA-MB-231 cells (3 x 10^5 per well) were plated in a 6 well plate. Approximately 48 hours later, when the cells were 100% confluent, the monolayer was scratched using a 1 mL pipette tip. Media and non-adherent cells were aspirated, the adherent cells washed once and new media containing various concentrations of SKI-606 (0.1–100 μM) was added. Cells were observed under the microscope at different times and the inhibition of migration was assessed when the wound in the control was closed.

Tumor cell invasive capacity was examined as previously described using two-compartment Boyden chambers (Transwell; Costar, Cambridge, MA) and basement membrane Matrigel (Becton Dickinson, Bedford, MA). The 8 μm pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter) and analyzed 5 x 10^4 cells in each chamber as described[71 72]. The filters were then fixed for 30 min in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature, washed with phosphate- buffered saline, stained with 1.5% toluidine blue and mounted onto glass slides. The invading cells were then examined and counted in 10 randomly selected fields under a light microscope at x400 magnification. The average number of invading cells was then calculated.

For Western blotting, MDA-MB-231 (1 ×10^6) cells were plated in 100 mm Petri dishes for 24 h. Cells were then washed with cold PBS and lysed with 200 μl of cold lysis buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.2, 0.2 mM sodium vanadate, 1% PMSF, 0.2% aprotinin). Samples were kept on ice for 20 min, then spun at 13000 x *g* for 20 min and the protein concentrations of the supernatants were determined. Cell lysates were resolved on 10% SDS–PAGE and protein transferred onto nitrocellulose membranes. Expression of β-tubulin as control was determined using anti- β-tubulin (Neomarker, BD BioSciences, Mississauga, ON, Canada).

Animal Protocols

For xenograft studies, 5-week-old (15-20g) female BALB/c *nu.nu* mice (Charles River, St. Constant, QC) were used[71 72]. Prior to inoculation, MDA-MB-231-GFP cells grown in serum containing culture medium were washed with Hank's balanced buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (5 x 10^5 cells/mouse) were resuspended in 100 µl of Matrigel (Becton Dickinson Labware, Mississauga ON, Canada) and saline mixture (20% Matrigel) and injected into the mammary fat pad of mice subcutaneously (s.c.). All animals (n=8 for each group) were numbered and kept separately in a temperature-controlled room on a 12 h/12 h light/dark schedule with food and water *ad libitum*. Tumors were allowed to grow to 30-50 mm^3. At this stage, animals were randomized and treated with vehicle alone or different doses of SKI-606 (150 mg/kg/day) by oral gavage five days per week for 5 weeks. Tumor volumes were determined from calliper measurements obtained weekly. At the end of the study, animals were sacrificed and their lung, liver, spleen as well as the primary tumors were removed for further analysis. Lung, liver and spleen were sliced to 1-mm thick slices of fresh tissue for direct examination under the fluorescent microscope for the presence of GFP-expressing tumor foci. The number of GFP-expressing tumor foci per field of examination was counted from 10 random sites of five different slides for each organ and calculated and graphed the average for each group. Total proteins from the primary tumors were extracted for western blot analysis to examine levels of Src, pSrc, pAKT, pMAPK in these tumors. All animal protocols were in accordance with and approved by the institutional review board.

Immunohistochemistry

Immunohistochemical reactions were carried out as previously described using the avidin-biotin-peroxidase complex method[75 76 77]. Briefly, paraffin-embedded tumor samples were cut into 5 μm thick sections. The sections were deparaffinized in xylene, rehydrated through a series of ethanol to water, and then treated with 1% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min to block non-specific binding, before incubation with the primary antibody. The antibodies used were a Polyclonal antiserum against PTHrP (1–34) from rabbit[77], at 1:100, monoclonal antibodies against E-cadherin (1:100) and CD31 (1:50) from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; rabbit polyclonal antibody against pAkt (1:50) from Cell Signalling Technology, Beverly, MA, USA; Monoclonal antibody against Ki-67 (1:100) from DAKO, Carpinteria, CA, USA; monoclonal antibody against uPA (1:50) from American Diagnostica In., Stanford, CT, USA) overnight at 4°C (39-41). Biotinylated goat anti-mouse or goat anti-mouse antibodies (Vector Laboratories Inc., Burlingame, CA, USA) were used as the secondary antibodies at 1:200 for 30 min at room temperature. The slides were treated with Vectastain ABC-AP kit (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:200 for 30 min at room temperature. The signals were visualized with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich) containing 1 mM levamisole for 10–15 min. The sections were then lightly counterstained with hematoxylin (Fisher Scientific, Ltd., Nepean, ON, Canada) and mounted with Kaiser's glycerol jelly. All sections were washed three times, 10 min each,

with Tris buffer (pH 7.6) after each step. Negative controls included substitution of the primary antibody with PBS.

Computer-assisted image analysis

The immunostaining of all antibodies was quantitatively analyzed by using a computer-assisted image analysis system as previously described[78 79]. Briefly, images of stained sections were captured with a Leica digital camera and processed using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis Corporation, Nashville, TN, USA). The threshold was set by determining the positive staining of control cells and was used to automatically analyze all recorded images of all samples that were stained in the same session under identical conditions. The area of immunostained regions in each microscopic field was calculated automatically by the software. Pixel counts of the immunoreaction product were calculated automatically and were given as total density of the integrated immunostaining over a given area of the well. This reflects the relative amount of proteins detected by the antibodies on cells.

Statistical Analysis

Results are expressed as the mean ± SEM of at least triplicate determinations and statistical comparisons are based on Student's *t* test, ANOVA and analysis of variance. A probability value of <0.05 was considered to be significant.

RESULTS

Effects of SKI-606 on cell proliferation, invasion and migration.

SKI-606 is a potent dual Src-Abl kinase inhibitor that was shown to have anti-proliferative effects on CML cells; however the efficacy of SKI-606 in breast cancer is still unknown. Therefore we determined the effect of SKI-606 on the growth of mesenchymal like human breast cancer cells MDA-MB-231. Treatment of these breast cancer cells with SKI-606 caused a dose dependent inhibition in tumor cell proliferation (Figure 1A). Furthermore, treatment of MDA-MB-231 cells with 0.1-1.0 μM of SKI-606 for 2 hours caused a morphological change from a spindle-like phenotype to an epithelial and condensed shape (data not shown). The effect of SKI-606 on tumor cell invasive capacity was evaluated by Matrigel invasion assay. The results of these studies show that SKI-606 inhibits the invasive capacity of MBA-MB-231 cells following an invasion time of 16 hours, in a dose dependent manner (Figure 1B).

To assess whether SKI-606 affects cell migration, a wound healing assay was performed. Following treatment with various concentrations of SKI-606 for 4 hours, cells were allowed to migrate into the denuded area for 16, 24 and 48 hours. At the highest dose of 1.0 μM only there was a significant dose and time dependent decrease in MDA-MB-231 cell migration. It also important to note that the effect of migration is not affected by

changes in proliferation since SKI-606 did not inhibit proliferation until after 48 h. (Figure 2).

SKI-606 inhibits kinase activity of Src and its downstream signalling pathways *in vitro*.

Tyrosine 416 in the kinase domain plays a critical role in the regulation of Src TK activity. MDA-MB-231 cells were treated with 1.0 μM of SKI-606 for 2 hours, a dose and time that are most effective in inhibiting cell proliferation, invasion and migration as shown in Figure 1 and 2. At the end of this incubation period, cell lysates were subjected to Western blot analysis to determine Src kinase activity. These studies showed that SKI-606 can inhibit kinase activity as measured by auto-phosphorylation of tyrosine 416 in these human breast cancer cells (Figure 3A).

In order to confirm the loss of Src activity we examined the effect of Src inhibition on downstream signalling proteins such as MAPK and AKT. Results shown in Figure 3B show that SKI-606, through Src inhibition, acts as an inhibitor of these signalling pathways, findings which are consistent with the anti-invasive effect demonstrated in Figure 1B. In contrast, SKI-606 had no significant effect on autophosphorylation of FAK at tyrosine 397.

Role of SKI-606 in MDA-MB-231 tumor growth and metastasis *in vivo*.

Previously we had generated MDA-MB-231 cells stably transfected with a plasmid containing cDNA encoding green fluorescent protein (GFP). These MDA-MB-231-GFP cells, which have similar cell proliferation and invasive capacity as the wild type cells, allow for the determination of the number and size of microscopic GFP positive tumor cells in various organs (lungs, liver and spleen)[71 72]. In order to test the effect of SKI-606 on tumor growth and metastasis, MDA-MB-231-GFP cells were inoculated into the mammary fat pad of female BALB/c *nu.nu* mice. SKI-105, a less soluble analogue of SKI-606, was also used in the experiment to act as a blinded control. Once the tumor volume reached 30-50 mm^3, animals (n= 8 for each group) were randomized and treated with vehicle alone, SKI-606 (150 mg/kg) or SKI-105 (150 mg/kg) by daily oral gavage five days per week for 4 weeks. Tumor volumes were determined by caliper measurements obtained weekly. The control group of animals treated with vehicle alone showed a progressive increase in their tumor volume; however experimental animals treated with SKI-606 developed tumors of significantly smaller volume ($P < 0.05$) throughout the course of these studies (Figure 4 A). The statistical analysis comparing the three groups is described in the Appendix.

To determine the efficacy of SKI-606 treatment on the ability of MDA-231-GFP cells to metastasize *in vivo*, at the end of these studies control and experimental animals were sacrificed and various organs (lung, liver, spleen) were removed and evaluated for the presence of GFP positive tumor cells. Using fluorescent microscopy for the analysis of slices of fresh tissue from lung, liver and spleen we were able to show the presence of

GFP-expressing tumor foci recognized by their green fluorescence. Results clearly demonstrate that animals inoculated with MDA-MB-231-GFP cells and treated with SKI-606 developed microscopic tumor metastases of significantly smaller number and size as compared to control animals receiving vehicle alone (Figure 4B).

Effects of SKI-606 on Src mediated signalling pathway *in vivo*.

In order to investigate the impact of Src inhibition on signalling pathways *in vivo*, MDA-MB-231-GFP tumors taken from 4 different mice treated with the control vehicle and 4 different mice treated SKI-606 were collected at the time of necropsy and snap frozen in liquid nitrogen. Frozen tumors were grinded in liquid nitrogen. The mice were treated with SKI-606 at a concentration of 150 mg/kg via oral gavage following inoculation of MDA-MB-231-GFP tumor cells in the mammary fat pad. Consistent with the results of our *in vitro* studies, SKI-606 completely abolished phosphorylation of pSrc, pAkt and pMAPK signalling proteins (Figure 5).

Effects of SKI-606 on gene expression, tumor cell proliferation, angiogenesis and survival *in vivo*.

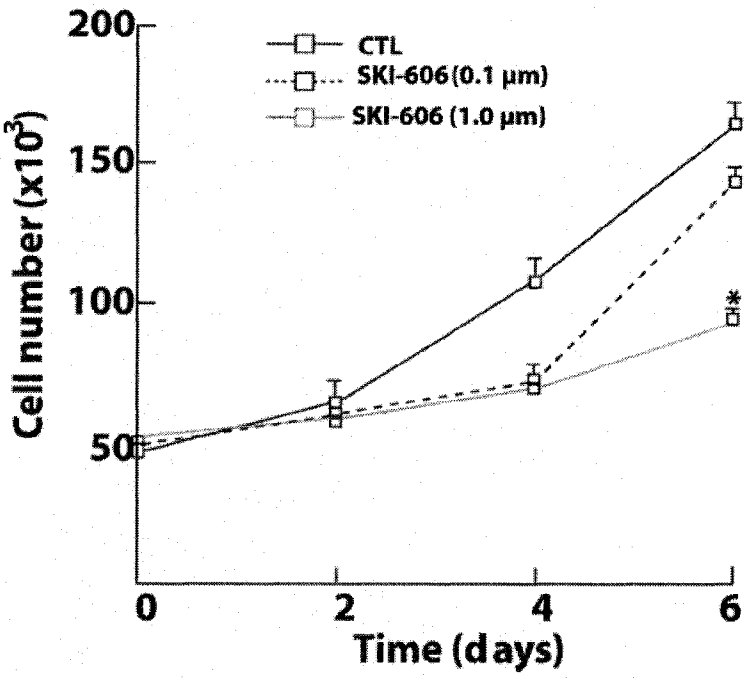
Src can regulate multiple signalling pathways and gene expression. Control and experimental tumors were evaluated for the expression of genes involved in tumor progression. Immunohistochemical analysis of primary tumors treated with SKI-606 showed a significant decrease in PTHrP and uPA expression, genes which are known to promote breast cancer progression. In contrast, treatment with SKI-606 showed increased expression of E-Cadherin which suggests an ability of SKI-606 to increase tumor cell-cell adhesion (Figure 6). Furthermore, as shown previously, SKI-606 inhibits phosphorylation of Akt through the PIK3 pathway which results in decreased Akt activity. In addition, we evaluated the effect of SKI-606 on MDA-MB-231 proliferation and neovascularization using antibodies against Ki67 and CD31, which are markers of cell proliferation and angiogenesis respectively. These studies showed a significant decrease in the levels of Ki67 and CD31 in tumors from experimental animals treated with SKI-606 (Figure 6).

SKI-606 mediates transition from a fibroblastic to an epithelial phenotype on MDA-MB-231 cell.

The mesenchymal phenotype of the aggressive breast cancer cells MDA-MB-231 was used as a cell system, to investigate whether treatment with SKI-606 would lead to a morphological reversion. Incubation of these cells with 0.1μM SKI-606 for 2 hours, caused phenotypic changes in reverse of a typical EMT process. We also examined MDA-MB-231 cells treated with DMSO and no changes were observed. (Figure 7)

Figure 1

A


200
150
100
50
Cell number (x10³)
CTL
SKI-606 (0.1 µm)
SKI-606 (1.0 µm)
*
0
2
4
6
Time (days)

B

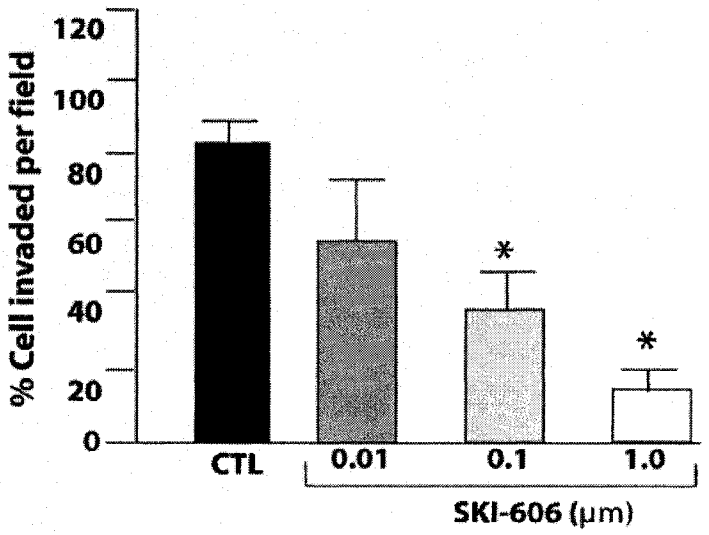
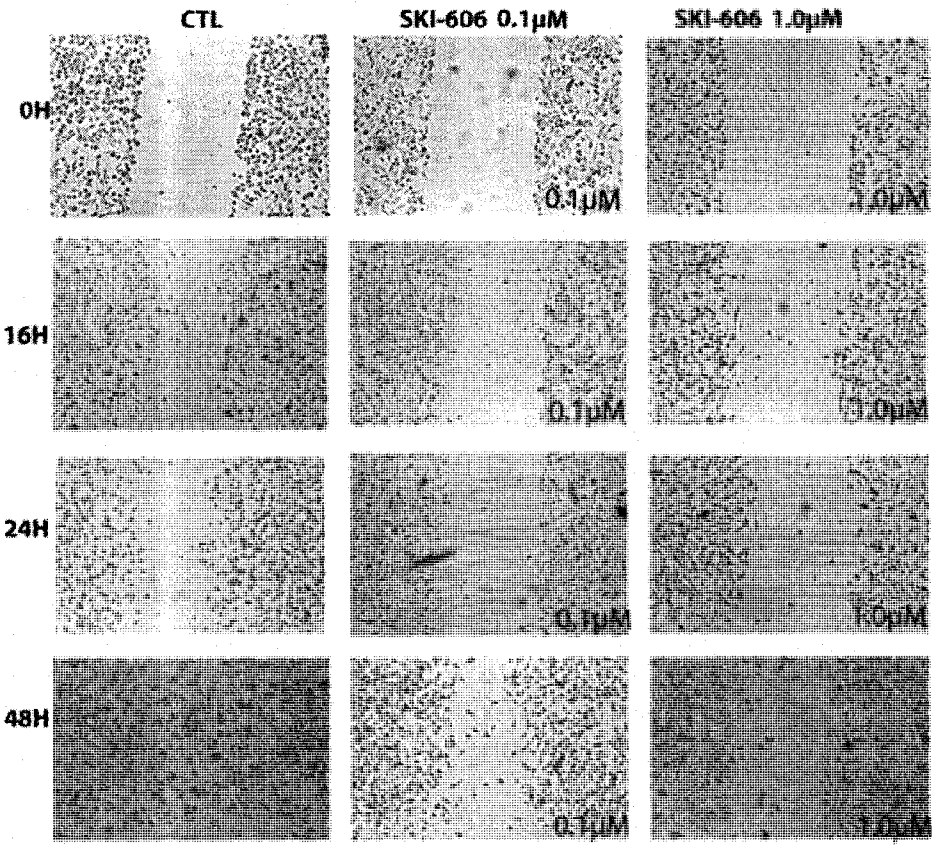

120
100
80
60
40
20
0
% Cell invaded per field
*
*
CTL
0.01
0.1
1.0
SKI-606 (µm)

Figure 1: Effect of SKI-606 on MDA-MB-231 cell growth and invasion

(Panel A): MDA-MB-231 cells were seeded at a density of 5 x 10^4 cells/well in 6-well plates and treated with different doses (0.1 μM and 1.0 μM) of SKI-606. DMSO treated control (CTL) and experimental cells were trypsinized and counted with a Coulter counter as described in "Materials and Methods". Changes in cell number at various time points after treatment are shown in the graph. Results are the mean ± SEM of three such experiments.

(Panel B): MDA-MB-231 cells were grown in culture as described in "Materials and Methods." The number of cells migrating to the lower aspect of the Boyden chamber filter 16h after treatment with SKI-606 (0.01, 0.1 and 1.0 μM) were counted. Results are the mean ± SEM of three such experiments. Significant inhibition in cell invasion from control cells is represented by an asterisk ($P < 0.05$).

Figure2


CTL
SKI-606 0.1μM
SKI-606 1.0μM
0H
16H
24H
48H
0.1μM
1.0μM

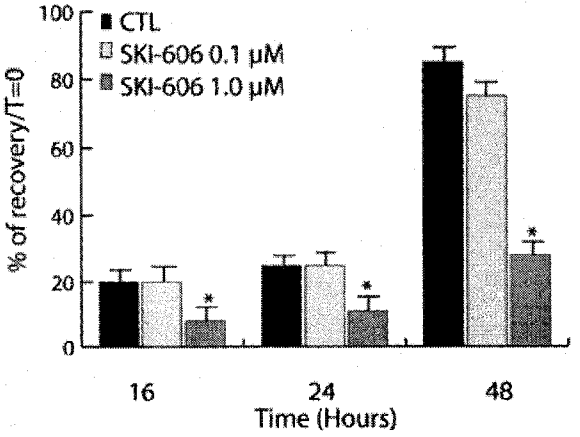

CTL
SKI-606 0.1 μM
SKI-606 1.0 μM
% of recovery/T=0
100
80
60
40
20
0
16
24
48
Time (Hours)
*

Figure 2: Effect of SKI-606 on MDA-MB-231 cell migration

MDA-MB-231 cells were treated with SKI-606 (0.1 μM and 1.0 μM) or vehicle alone as control (CTL) for 4 hours followed by assessment of cell migration at various time points using wound-healing based assay. Representative experimental cells from control and experimental groups were photographed and change in cell migration was quantified as described in "Materials and Methods". Results are the mean ± SEM of three such experiments. Significant difference in cell migration from control is shown by an asterisk ($P<0.05$).

Figure3:

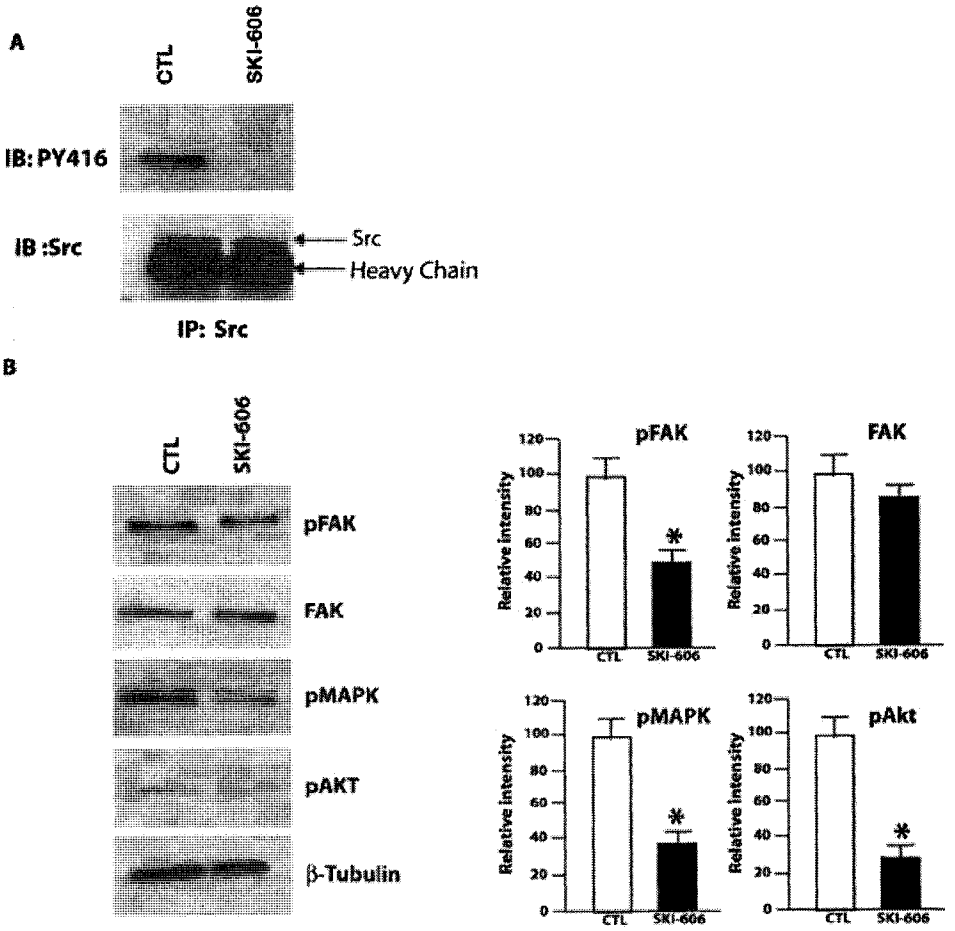

A
CTL
SKI-606
IB: PY416
IB :Src
Src
Heavy Chain
IP: Src
B
CTL
SKI-606
pFAK
FAK
pMAPK
pAKT
β-Tubulin
pFAK
FAK
pMAPK
pAkt
Relative intensity
120
100
80
60
40
20
0
CTL
SKI-606
*

Figure 3: Effect of SKI-606 on tyrosine phosphorylation of signalling molecules *in vitro*

(Panel A): On the left side of the blot, MDA-MB-231 cells were treated with the DMSO as control. On the right side of the blot, MDA-MB-231 cells were treated with 1.0μM SKI-606 for 2 hours followed by immunoprecipitation with Src antibody and Western blot analysis for Src-Y416.

(Panel B): MDA-MB-231 cells were harvested at different time points, 0 and 2 hours, following treatment with 1.0μM of SKI-606. This was labelled as CTL and SKI-606 respectively in the panel. Western blot analysis was carried out with 40 μg of total protein loaded in each lane and immunoblotted with antibodies against FAK, phosphotyrosine (pFAK 397), pMAPK and pAkt. Anti-β-tubulin antibody was used as loading control (CTL). Levels of pFAK, FAK, pMAPK and pAkt were quantified by densitometric scanning and expressed as relative density to their respective control lysate. Results are the mean ± SEM of three such experiments. Significant changes in pMAPK and pAkt production are represented by an asterisk ($P<0.05$).

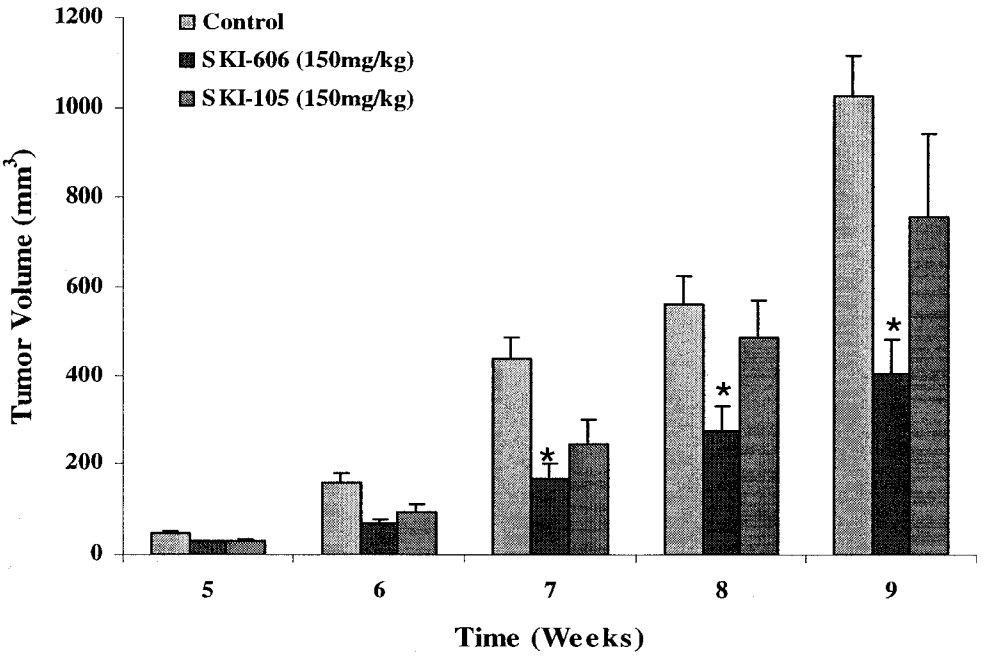


Figure 4: Effect of SKI-606 on MDA-MB-231-GFP Tumor Growth.

(Panel A): MDA-MB-231-GFP cells (5 x 10^5) were inoculated in mammary fat pad of female BALBc *nu/nu* mice. Once the tumor volume reached 30-50 mm^3, 24 animals (n=8 per group) were randomized and treated with vehicle alone as control (CTL) or 150 mg/kg of SKI-606 (SKI-606 dissolved in 0.5% methyl cellulose/0.4% Tween 80) or SKI-105 five days per week for 4 weeks by daily oral gavage. Tumor volumes were determined by calliper measurements obtained weekly as described in "Materials and Methods". Significant difference in tumor volume was observed only for SKI-606 versus the control group where P= 0.0040. This is denoted by an asterisk above the relevant bar in the graph.

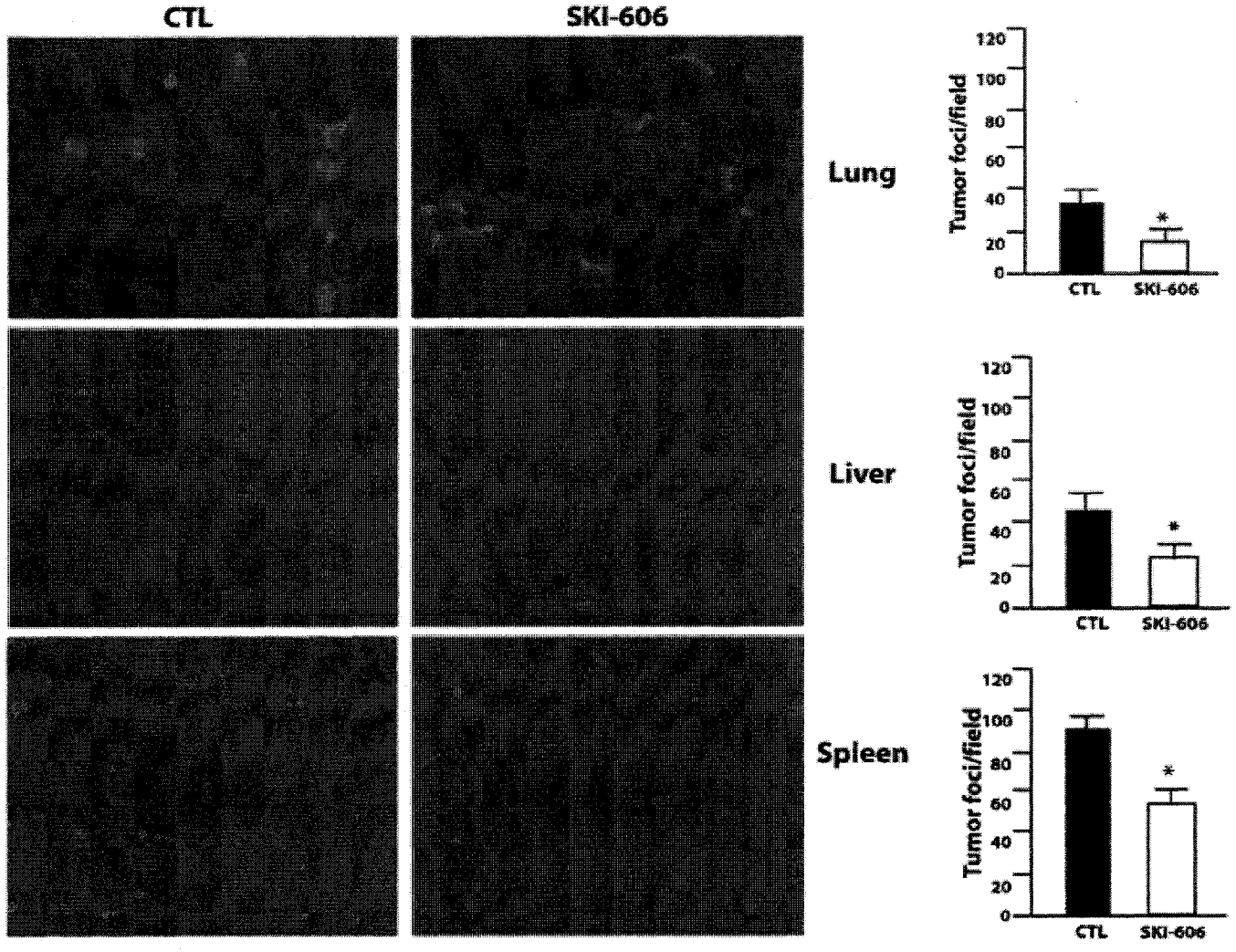

CTL
SKI-606
Lung
Liver
Spleen
Tumor foci/field
120
100
80
60
40
20
0
*
CTL
SKI-606

Figure 4B: In order to determine the effect of SKI-606 on tumor metastasis, at the end of these studies, control (CTL) and experimental animals treated with SKI-606 were sacrificed. Various organs (lungs, liver and spleen) were removed and evaluated for the presence of metastatic tumor cells. The number of tumor foci in 10 random fields per slide, five slides per organ was counted to determine the average number of tumor foci in each organ as described in "Materials and Methods". Results are the mean ± SEM of at least 10 animals in each group in two separate experiments. Significant differences in tumor volume and tumor foci are represented by an asterisk ($P < 0.05$).

Figure 5

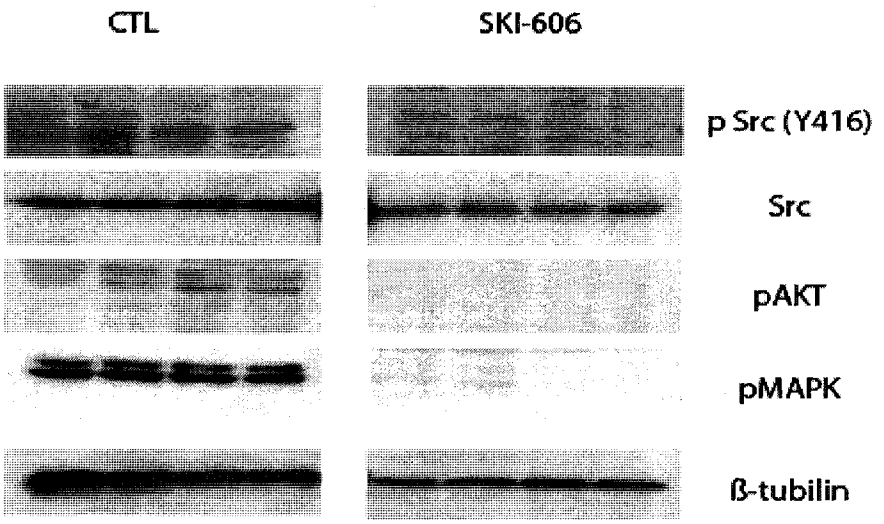


Figure 5: Effect of SKI-606 on signalling molecules *in vivo*.

MDA-MB-231-GFP cells (5 x 10^5) were implanted in mammary fat pad of female BALBc *nu/nu* mice. Once the tumor volume reached 30-50 mm^3, animals were randomized and treated with vehicle alone as control (CTL) or 150 mg/kg of SKI-606 by daily oral gavage. At the end of this treatment tumors were removed and protein lysates were subjected to Western blot analysis using antibodies against phosphotyrosine Akt and MAPK (pAkt, pMAPK). Protein lysates were also immunoblotted using antibodies against Src and phosphotyrosine Src 416. Membranes were stripped and re-blotted for β-tubulin as control for protein loading as described in "Materials and Methods". Representative blots from three such experiments are shown. In this experiment, tumors from 4 experimental and 4 control animals were used as some of the experimental

tumours were too small to process. This resulted in the slight decrease of β- tubulin in the experimental column (SKI-606) compared to the control column (CTL).

Figure 6

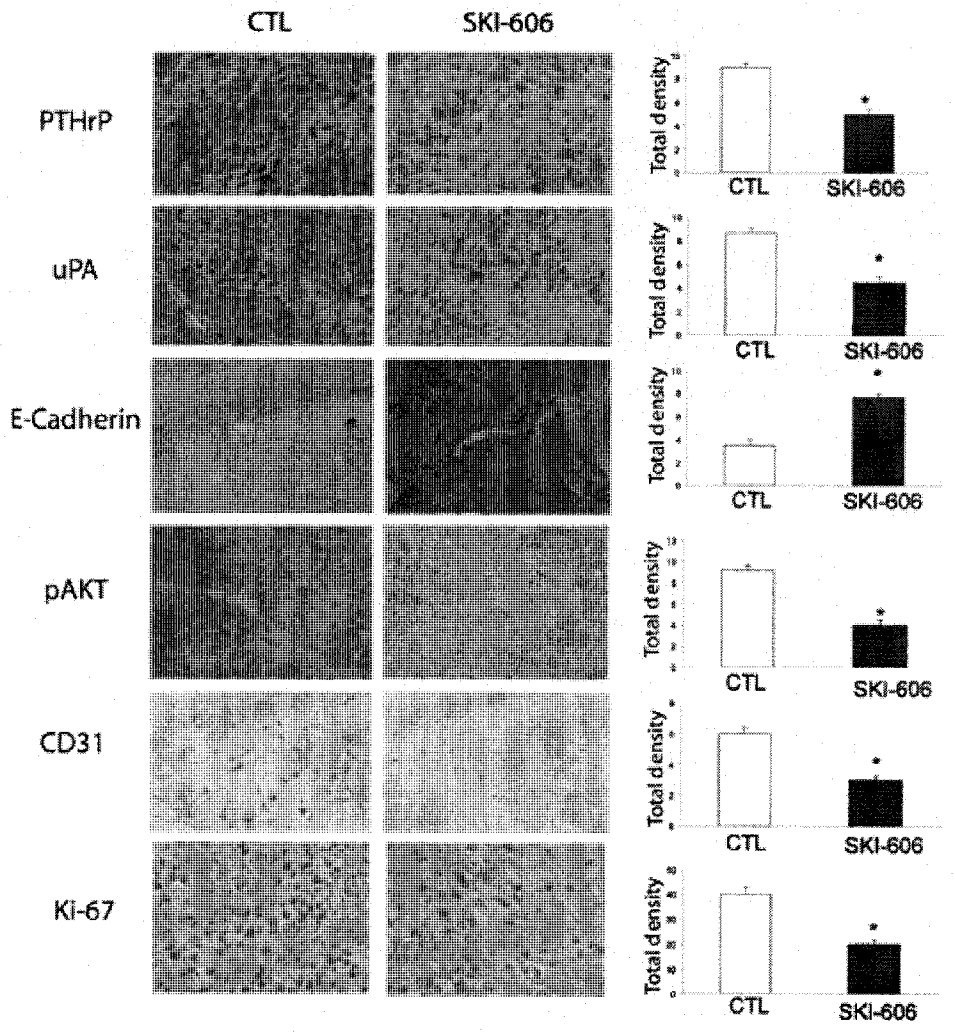

CTL
SKI-606
PTHrP
uPA
E-Cadherin
pAKT
CD31
Ki-67
Total density
CTL
SKI-606

Figure 6: Effect of SKI-606 on gene expression, signalling pathways, cell proliferation and angiogenesis in MDA-MB-231-GFP tumors.

Female BALBc *nu/nu* mice were inoculated in the mammary fat pad with MDA-MB-231-GFP cells. Following treatment with vehicle alone as control (CTL) or SKI-606, animals were sacrificed. Primary tumors were removed and subjected to immunohistochemical analysis as to determine the expression PTHrP, uPA, E-cadherin, pAkt, Ki67 and CD31 as described in "Material and Methods". Quantitative analysis was carried out in 10 high power fields (x400) in control and experimental sections. Positive staining is indicated red. Results are representative of the mean ± SEM of six animals in each group from two separate experiments. Significant difference from control is shown by asterisks in right hand panels ($P<0.05$).

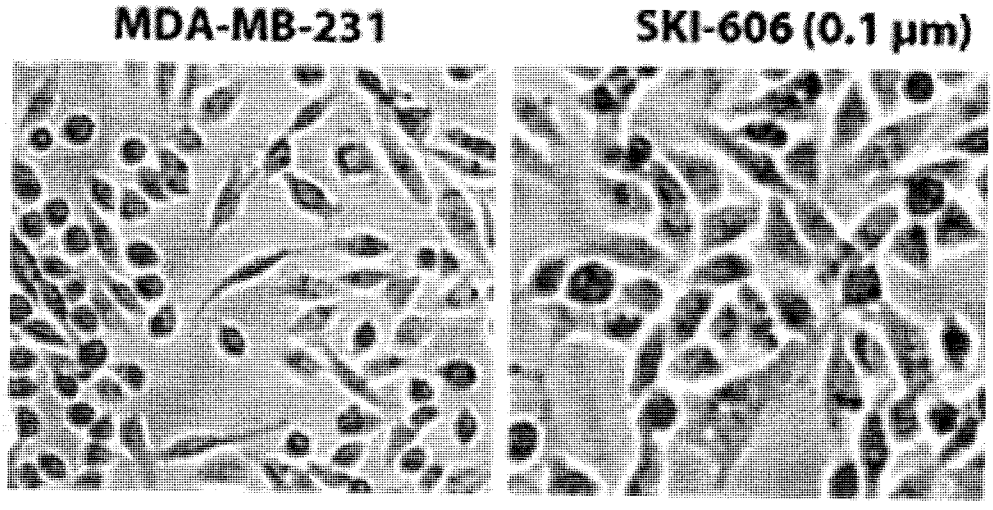


Figure 7: Effect of SKI-606 on MDA-MB-231 transition to an epithelial phenotype. The left panel depicts MDA-MB-231 cells pre-treated with DMSO. In the right panel cells were treated with 0.1 µM of SKI-606 for 2 hours and observed by phase contrast microscopy. Photographs of living cells were taken using a 10x magnification. Most of the cells treated with SKI-606 showed reversion to an epithelial phenotype as shown in the right panel.

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DISCUSSION

SKI-606 belongs to a group of ATP-competitive inhibitors. ATP-competitive inhibitors can be divided into two groups, the 2-phenylaminopyrimidine-based compounds and the Src-Abl inhibitors. Imatinib, Nilotinib (AMN107) and INNO-406 (NS-187) belong to the former subclass of inhibitors, while Dasatinib and SKI-606 are classified as Src-Abl inhibitors[78]. Nilotinib was derived from the lead compound Imatinib through replacements of its N-methylpiperazine group. Nilotinib was found to be approximately 30 times more potent than Imatinib *in vitro* against Bcr-Abl[7]. Similar to Imatinib, Nilotinib binds only to the inactive configuration of Bcr-Abl and inhibits the kinase activity of most Bcr-Abl mutants. INNO-406 (NS-187) is at least 10 times more potent than Imatinib against Bcr-Abl and is also effective against Imatinib-resistant Abl mutants[7]. The structure of Dasatinib is not related to Imatinib. Dasatinib binds to both the inactive and active configurations of Bcr-Abl and is 100 to 300 times more potent inhibitor of Bcr-Abl kinase activity than Imatinib[7].

In October 17, 1973, Dr. R. Calleau at M. D. Anderson Cancer Center obtained some cancer cells from a pleural effusion of a 51-year-old white female. These highly invasive and metastatic cancer cell lines were then called MDA-MB-231[79]. The cells are ER-negative, E-cadherin negative and highly invasive in *in vitro* assays[80]. As the cancer cells were taken from a patient with Stage IV breast cancer, the use of MDA-MB-231 in this study should be representative of late stage breast cancer. This is useful to fully study the effects of SKI-606 on tumor cell proliferation, invasion and migration.

SKI-606 is effective at inhibiting Src kinase which results in the inhibition of various signalling pathways such as MAPK and Akt at micromolar concentrations. This effect was tested on mesenchymal-like breast cancer cells MDA-MB-231 which exhibit high invasive capacity. SKI-606 showed anti-proliferative effects and inhibited cell migration and invasion which directly correlate with a significant (45-54%) reduction in tumor volume in experimental animals (Figure 4A).

As Src plays an important role in many signalling pathways[81] it would be interesting to examine whether SKI-606 combined with another ''small molecule'' drug (for e.g. Tarceva) can abolish aberrant signals by reducing cell proliferation and hence the percentage of inhibition of tumours. Furthermore, MDA-MB-231 cell invasive capacity was also diminished by SKI-606 in a dose dependent manner (Figure 1B).

Cell migration is necessary in many physiological processes such as tissue development[82], wound healing[83] and tumor metastasis[84]. Several studies suggested that Src might be involved in cell migration[85] [86]. Indeed, in Src -/- mice fibroblasts, locomotion is reduced as compared to rates displayed by wild-type fibroblasts[87]. Consistent with those findings, SKI-606-mediated inhibition of Src activity impeded cell migration after 2 hours of treatment (Figure 2). These findings correlate with the reduction of uPA levels which is associated with inhibition of cell adhesion and migration.

In inhibiting cell migration, SKI-606 demonstrated to be a highly effective compound. Indeed, significant decrease in migration was shown at a concentration of 0.1 μM after 16 hours of treatment. At a concentration of 1.0 μM, the effect on the highly invasive MDA-MB-231 cells by SKI-606 was even more potent. Aphidicolin, which is commonly used during cell migration assays,[88] was not used during this experiment. Aphidicolin, a tetracyclic diterpene with antiviral and anti-mitotic properties, is known to be a reversible inhibitor of eukaryotic nuclear DNA replication[89]. Nonetheless, SKI-606 reduced cell migration of MDA-MB-231 prior to their cellular division. Therefore, we suggest that this effect is due to a cellular migration as opposed to an inhibition of cellular proliferation.

There are several methods to assess migration. In a study conducted by Yang et al (2006)[90], cells were plated on fibronectin coated dishes for 12 hours followed by a scratch removing a thin strip of cells from the dish. Whether the use of fibronectin would change the migration results with SKI-606 in this experiment would be something to investigate in future studies. In the same study Yang et al 2006 conducted another type of migration assay in order to assess the motility of a single melanoma cell. In this experiment blue fluorescent beads are plated on a fibronectin coated 96 well plates. The GFP-transfected melanoma cells are then added on plates coated with beads and incubated at 37°C at different time points. Images are then taken under an inverted fluorescent microscope. This assay is efficient in terms of looking at migrating cells and calculating the migration area.

Phosphorylation of tyrosine 416 in the kinase domain is a critical activation step in the regulation of Src tyrosine kinase activity[25 91 92]. Src phosphorylation at tyrosine 416 was significantly reduced in MDA-MB-231 tumors from animals treated with SKI-606. Furthermore we showed that blockade of Src results in decreased expression of both PTHrP and uPA which are known to promote the growth and metastasis of several common cancers including breast cancer[93 94 95]. Additionally the ability of SKI-606 to inhibit tumor cell proliferation and angiogenesis as shown in Figure 6 plays a significant role in its anti-tumor and anti-metastatic effects.

In addition, as Src regulates many cellular events through phosphorylation of multiple substrates[96], we investigated whether downstream signalling pathways would be altered through inactivation of Src. Our data clearly show a decrease in phosphorylation of MAPK and AKT pathway *in vitro and in vivo.*

More recently, using a xenograft model of breast cancer metastasis, Src tyrosine kinase activity was shown to be associated with tumor cell colonization in bone[97]. Furthermore Src tyrosine kinase stimulated the production of PTHrP at transcriptional levels in MDA-MB-231, which in turn can activate bone resorption by osteoclasts, leading to the progression of bone metastases[96]. In this study we examined metastasis in lung, liver and spleen. Our results show that in the organs of treated animals, the number of MDA-MB-231-GFP cells is reduced as compared to the organs of control animals.

Another interesting effect of SKI-606 observed during this study is the transition from mesenchymal MDA-MB-231 cells to epithelial cells (Figure 7). Certainly, upon addition of SKI-606 for 2 hours at a concentration of 1.0uM, a reverse EMT effect was observed. The role of Src in EMT is well documented through its effects on E-cadherin[98]. When epithelial cells lose their cadherin-mediated cell-cell contacts, they become less adhesive and thus, free to migrate leading to tumor metastasis. We have thus shown that SKI-606 potentially has the ability to reverse EMT which would be useful to prevent the spread of metastasis if used as an anti-cancer agent.

The anti-tumor effect of SKI-606 on MDA-MB-231 was also demonstrated in other studies of solid tumors such as colorectal tumor xenografts where it was effective against HT29, Colo205, HCT116 and DLD1 colorectal cancer cell lines[56]. SKI-606 has also been shown to be highly effective against CML in mice as well as humans. A once daily oral administration of SKI-606 at 100 mg/kg for 5 days caused complete regression of large K562 (CML) xenografts in nude mice[99]. Most recently, SKI-606 was part of a clinical trial involving patients with Philadelphia chromosome positive CML or ALL who were resistant to Imatinib. SKI-606 was shown to be highly effective where 84% of patients with Imatinib-resistance showed complete hematologic response and 52% showed major cytogenetic response[100].

In conclusion, we show in this study that SKI-606 inhibits metastasis and breast tumor growth in a xenograft mouse model. It is important to note that E-cadherin levels were increased in SKI-606 treated tumor. This is in accordance to the recent study where Nam

et al demonstrated that PP2 is also capable of restoring E-cadherin/β-catenin in various tumor cell lines[60]. Collectively results from these studies have provided compelling evidence for continued evaluation of the efficacy of SKI-606 in breast cancer.

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Appendix

The figures below describe the statistical analysis using ANOVA tests comparing tumor volumes in three different groups of eight mice. The first group was treated with SKI-606, another with SKI-105 (a less soluble analogue of SKI-606 provided by Whyte) and the third is a control group treated with the common vehicle (0.5% methyl cellulose/ 0.4% Tween 80).

When comparing the tumor volume of SKI-105 mice with the control group, the P value was equal to 0.2349 which is not significant. However, the P value between SKI-606 mice and SKI-105 mice was 0.0986 which is not statistically significant (taking $p \leq 0.05$ as statistically significant).

The Mixed Procedure

Model Information

Data Set	WORK.UN
Dependent Variable	outcome
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Group	2	0 1
Time	5	5 6 7 8 9
R	7	1 2 3 4 5 6 7

Dimensions

Covariance Parameters	2
Columns in X	7
Columns in Z	7
Subjects	1
Max Obs Per Subject	70

Number of Observations

Number of Observations Read	70
Number of Observations Used	70
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	943.11288387	
1	1	935.15274402	0.00000000

Convergence criteria met.

Groupe control (=0) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Group	2	0 2
Time	5	5 6 7 8 9
R	7	1 2 3 4 5 6 7

Number of Observations Read	70
Number of Observations Used	70

Groupe control (=0) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

Dependent Variable: outcome outcome

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	7584442.79	689494.80	5.10	<.0001
Error	58	7833759.43	135064.82		
Corrected Total	69	15418202.22			

R-Square	Coeff Var	Root MSE	outcome Mean
0.491915	98.54724	367.5117	372.9294

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Group	1	194583.194	194583.194	1.44	0.2349
Time	4	6254625.075	1563656.269	11.58	<.0001
R	6	1135234.517	189205.753	1.40	0.2299

Groupe control (=0) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

t Tests (LSD) for outcome

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	135064.8
Critical Value of t	2.00172
Least Significant Difference	175.86

Comparisons significant at the 0.05 level are indicated by ***.

Group Comparison	Difference Between Means	95% Confidence Limits	
0 - 2	105.45	-70.41	281.30
2 - 0	-105.45	-281.30	70.41

Groupe control (=0) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

t Tests (LSD) for outcome

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	135064.6
Critical Value of t	2.00172
Least Significant Difference	278.05

Comparisons significant at the 0.05 level are indicated by ***.

Time Comparison	Difference Between Means	95% Confidence Limits		
9 - 8	447.5	169.4	725.5	***
9 - 7	538.2	260.2	816.3	***
9 - 6	758.3	480.2	1036.3	***
9 - 5	857.6	579.6	1135.7	***
8 - 9	-447.5	-725.5	-169.4	***
8 - 7	90.8	-187.3	368.8	
8 - 6	310.8	32.8	588.9	***
8 - 5	410.2	132.1	688.2	***
7 - 9	-538.2	-816.3	-260.2	***
7 - 8	-90.8	-368.8	187.3	
7 - 6	220.0	-58.0	498.1	
7 - 5	319.4	41.3	597.4	***
6 - 9	-758.3	-1036.3	-480.2	***
6 - 8	-310.8	-588.9	-32.8	***
6 - 7	-220.0	-498.1	58.0	
6 - 5	99.3	-178.7	377.4	
5 - 9	-857.6	-1135.7	-579.6	***
5 - 8	-410.2	-688.2	-132.1	***
5 - 7	-319.4	-597.4	-41.3	***
5 - 6	-99.3	-377.4	178.7	

The Mixed Procedure

Model Information

Data Set	WORK.DEUX
Dependent Variable	outcome
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Group	2	0 2
Time	5	5 6 7 8 9
R	7	1 2 3 4 5 6 7

Dimensions

Covariance Parameters	2
Columns in X	7
Columns in Z	7
Subjects	1
Max Obs Per Subject	70

Number of Observations

Number of Observations Read	70
Number of Observations Used	70
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	956.10728296	
1	1	955.76874612	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
R	5414.09
Residual	135065

Fit Statistics

-2 Res Log Likelihood	955.8
AIC (smaller is better)	959.8
AICC (smaller is better)	960.0
BIC (smaller is better)	959.7

Solution for Fixed Effects

Effect	Group	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	0		945.97	111.13	58	8.51	<.0001
Group	2		840.52	111.13	58	7.56	<.0001
Time		5	-857.62	138.91	58	-6.17	<.0001
Time		6	-758.28	138.91	58	-5.46	<.0001
Time		7	-538.24	138.91	58	-3.87	0.0003
Time		8	-447.45	138.91	58	-3.22	0.0021
Time		9	0	.	.	.	.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	1	58	1.44	0.2349
Time	4	58	11.58	<.0001

> 0.05

Groupe SKI-606 (150MG/KG) (=1) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Group	2	1 2
Time	5	5 6 7 8 9
R	7	1 2 3 4 5 6 7

Number of Observations Read	70
Number of Observations Used	70

Groupe SKI-606 (150MG/KG) (=1) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

Dependent Variable: outcome outcome

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	3540832.848	321893.895	4.47	<.0001
Error	58	4176239.099	72004.122		
Corrected Total	69	7717071.947			

R-Square	Coeff Var	Root MSE	outcome Mean
0.458831	100.7402	268.3358	266.3643

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Group	1	202925.114	202925.114	2.82	0.0986
Time	4	2611378.687	652844.672	9.07	<.0001
R	6	726529.046	121088.174	1.68	0.1418

Groupe SKI-606 (150MG/KG) (=1) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

t Tests (LSD) for outcome

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	72004.12
Critical Value of t	2.00172
Least Significant Difference	128.4

Comparisons significant at the 0.05 level are indicated by ***.

Group Comparison	Difference Between Means	95% Confidence Limits	
2 - 1	107.68	-20.72	236.08
1 - 2	-107.68	-236.08	20.72

Groupe SKI-606 (150MG/KG) (=1) Vs Groupe SKI-105 (150MG/KG) (=2)

The ANOVA Procedure

t Tests (LSD) for outcome

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	72004.12
Critical Value of t	2.00172
Least Significant Difference	203.02

Comparisons significant at the 0.05 level are indicated by ***.

Time Comparison	Difference Between Means	95% Confidence Limits		
9 - 8	223.0	20.0	426.0	***
9 - 7	325.0	121.9	528.0	***
9 - 6	477.1	274.1	680.1	***
9 - 5	544.1	341.1	747.1	***
8 - 9	-223.0	-426.0	-20.0	***
8 - 7	102.0	-101.0	305.0	
8 - 6	254.1	51.1	457.1	***
8 - 5	321.1	118.1	524.1	***
7 - 9	-325.0	-528.0	-121.9	***
7 - 8	-102.0	-305.0	101.0	
7 - 6	152.1	-50.9	355.1	
7 - 5	219.1	16.1	422.1	***
6 - 9	-477.1	-680.1	-274.1	***
6 - 8	-254.1	-457.1	-51.1	***
6 - 7	-152.1	-355.1	50.9	
6 - 5	67.0	-136.0	270.0	
5 - 9	-544.1	-747.1	-341.1	***
5 - 8	-321.1	-524.1	-118.1	***
5 - 7	-219.1	-422.1	-16.1	***
5 - 6	-67.0	-270.0	136.0	

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
R	4908.41
Residual	72004

Fit Statistics

-2 Res Log Likelihood	916.6
AIC (smaller is better)	920.6
AICC (smaller is better)	920.8
BIC (smaller is better)	920.5

Solution for Fixed Effects

Effect	Group	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Group	1		526.34	82.9035	58	6.35	<.0001
Group	2		634.03	82.9035	58	7.65	<.0001
Time		5	-544.08	101.42	58	-5.36	<.0001
Time		6	-477.07	101.42	58	-4.70	<.0001
Time		7	-324.96	101.42	58	-3.20	0.0022
Time		8	-222.99	101.42	58	-2.20	0.0319
Time		9	0	.	.	.	.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Group	1	58	2.82	0.0986
Time	4	58	9.07	<.0001

The Mixed Procedure

Model Information

Data Set	WORK.TROIS
Dependent Variable	outcome
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Group	2	1 2
Time	5	5 6 7 8 9
R	7	1 2 3 4 5 6 7

Dimensions

Covariance Parameters	2
Columns in X	7
Columns in Z	7
Subjects	1
Max Obs Per Subject	70

Number of Observations

Number of Observations Read	70
Number of Observations Used	70
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	917.45297645	
1	1	916.60702723	0.00000000

Convergence criteria met.