Drug composition cytotoxic for pancreatic cancer cells (US)

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Disclosed herein are compositions comprising a drug combination that comprises ZD and S31-201, or Das and S31-201, or ZD and AG490, or Das and AG490. The disclosed drug combinations target two or more functional elements such as EGFR, and/or Stat3. The disclosed drug combinations decreases the invasion of human pancreatic cancer cells in vitro, and/or promotes aberrant growth, survival, migration and invasion of pancreatic cancer cells. The disclosed drug combinations increases sensitivity of pancreatic cancer cells to the combination of gemcitabine compared with gemcitabine alone, and/or increases sensitivity of pancreatic cancer cells to gefitinib in combination with the disclosed drug combinations. The disclosed drug combinations exhibits activity and supports growth, survival, migration and invasion of pancreatic cancer cells. The disclosed drug combinations overcomes gefitinib resistance in human pancreatic cancer cells.
References Cited

OTHER PUBLICATIONS


Margolis B, et al. (1990) The tyrosine phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLCgamma. EMBO J. 9: 4375-4380.


References Cited

OTHER PUBLICATIONS


* cited by examiner
FIG. 1

A (i)  
Stat3:Stat3  
Panc-1

(ii)  
Stat5:Stat5  
K562

B (i)  
pY416Src  
EGFR

(ii)  
pY845EGFR  
EGFR

1 µM ZD (h)  
100 nM Das (h)

Panc-1  
Colo-357

C (i)  
Lane 1 2 3 4 5 6 7 8 9 10 11

pY1068EGFR  
EGFR

1 µM ZD (h)  
100 nM Das (h)

HPDEC  Panc-1  Colo-357

(ii)  
Lane 1 2 3 4 5 6 7 8 9 10 11

pY1086EGFR  
EGFR

1 µM ZD (h)  
100 nM Das (h)

HPDEC  Panc-1  Colo-357

(iii)  
Lane 1 2 3 4 5 6 7 8 9 10 11

pY1173EGFR  
EGFR

1 µM ZD (h)  
100 nM Das (h)

HPDEC  Panc-1  Colo-357
**FIG. 2**

**A**

Panc-1

i)

Stat3:Stat3

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<th>4</th>
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ii)

Stat3:Stat3

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**B**

Colo-357

Stat3:Stat3

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**C**

Panc-1

pStat3

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<th>Stat3</th>
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Con siRNA

EGFR siRNA

Src siRNA
**FIG. 3**

A (i) Panc-1
- Control
- ZD + S31-201

# of Cells (X10^3)

Days of treatment

(ii) Panc-1

ZD ± Das ± S31-201

# of Cells (X10^3)

Days of treatment

C (i) Panc-1
- ZD
- ZD + S31-201

FU (X10^5)

µM ZD

(ii) Panc-1
- Das
- Das + S31-201

FU (X10^5)

nM Das

(iii) S31-201
- S31-201 + Das

FU (X10^5)

µM S31-201

B (i) Panc-1
72 h

# of Cells (X10^3)

1 µM ZD
100 nM Das
50 µM AG490

(ii) Colo-357
72 h

# of Cells (X10^3)

1 µM ZD
100 nM Das
50 µM AG490

D (i) Panc-1
48 h

FU (X10^3)

1 µM Gem
1 µM ZD
100 nM Das
50 µM S31-201

(ii) Colo-357
48 h

FU (X10^3)

1 µM Gem
1 µM ZD
100 nM Das
50 µM S31-201

*** Statistical significance
A

(i) Panc-1

![Graph showing the number of colonies for Panc-1 cells with different treatments: 1 µM ZD, 100 nM Das, 50 µM S3I.](#)

(ii) Colo-357

![Graph showing the number of colonies for Colo-357 cells with different treatments: 1 µM ZD, 100 nM Das, 50 µM S3I.](#)

B

(i) HPDEC

![Graph showing the percentage of apoptosis for HPDEC cells with different treatments: 1 µM ZD, 100 nM Das, 50-100 µM S3I, 50 µM AG490.](#)

(ii) Panc-1

![Graph showing the percentage of apoptosis for Panc-1 cells with different treatments: 1 µM ZD, 100 nM Das, 50-100 µM S3I, 50 µM AG490.](#)

(iii) Colo-357

![Graph showing the percentage of apoptosis for Colo-357 cells with different treatments: 1 µM ZD, 100 nM Das, 50-100 µM S3I, 50 µM AG490.](#)

FIG. 4
FIG. 5
FIG. 6
The invention was made with government support under ROCA106439 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to the field of drug development and, more particularly, to a drug composition cytotoxic for pancreatic cancer cells.

BACKGROUND OF THE INVENTION

Pancreatic cancer is a lethal disease with a poor prognosis and a mortality rate nearly the same as the rate of incidence. Moreover, the disease remains poorly understood. Multiple signal transduction proteins are activated during pancreatic ductal cell carcinogenesis, some may be secondary events, while many others might have critical roles and collectively contribute to the maintenance and the progression of the disease and its responsiveness to therapy. One of the major molecular abnormalities is the overexpression and/or activation of the EGFR protein, which has an incidence of 30-50% of pancreatic cancer cases (1). Evidence indicates that the hyperactive EGFR/EGFR duo is important in the disease maintenance and progression (2). Similarly, the overexpression of the c-Src tyrosine kinase occurs in a large percentage of pancreatic adenocarcinoma and is observed to augment EGFR activities during tumorigenesis (3, 4). The over-activity of Src family kinases leads to deregulation of tumor cell growth and survival, disruption of cell-to-cell contacts, and the promotion of migration and invasiveness, and the induction of tumor angiogenesis (4, 5).

Another molecular abnormality is the aberrant activation of Stat3, a member of the Signal Transducer and Activator of Transcription (STAT) family of cytoplasmic transcription factors, which has also been detected in pancreatic tumors and tumor cell lines and been implicated in the disease (6-9). Stat3, as are the other Stats, requires extrinsic tyrosine phosphorylation to become activated and this is induced by growth factor receptors and cytoplasmic tyrosine kinases, such as Src and Janus kinase (Jaks). The method of the invention also includes contacting the cells with a drug combination further comprising a nucleoside analog preferably being Gemcitabine.

SUMMARY OF THE INVENTION

With the foregoing in mind, the present invention advantageously provides a cytotoxic composition containing a drug combination targeting two or more functional elements in pancreatic cancer cells, the functional elements comprising EGFR or Src and Stat3 or Jaks. A preferred embodiment of the cytotoxic composition is one wherein the drug combination contained therein is selected from ZD and S31-201, Das and S31-201, ZD and AG490, Das and AG490, and combinations thereof. Furthermore, the preferred cytotoxic composition is that wherein the drug combination inhibits said functional elements at substantially the same time. The preferred composition of the present invention may also comprise a nucleoside analog inhibitory for DNA replication, for example, Gemcitabine.

The invention herein disclosed also includes a method of cytotoxically affecting (which could result in killing) pancreatic cancer cells, the method comprising contacting the cells with a drug combination which inhibits two or more cellular functional elements, the functional elements including EGFR or Src and Stat3 or Jaks. The method of the invention also includes an embodiment wherein the drug combination is selected from ZD and S31-201, Das and S31-201, ZD and AG490, Das and AG490, and combinations thereof. A preferred method of the invention also includes contacting the cells with a drug combination further comprising a nucleoside analog inhibitory for DNA replication, for example, Gemcitabine.

The invention additionally includes a method of making a therapeutic medication cytotoxic for pancreatic cancer cells, the method comprising preparing a pharmaceutically acceptable composition containing a drug combination selected from ZD and S31-201, Das and S31-201, ZD and AG490, Das and AG490, and combinations thereof. The method of making the medication preferably also includes an embodiment wherein the drug combination further comprises a nucleoside analog inhibitory for DNA replication, for example, Gemcitabine.

FIG. 1 shows EMSA and immunoblotting analyses of Stat3, Src and EGFR activities for effects of inhibitors. (A) EMSA analysis of STAT DNA-binding activity using (hSIE) probe that binds to high-affinity sis-inducible element (hSIE) probe that binds to
Stat3 and Stat1 or (ii) mammary gland factor element (MGFε) probe that binds Stat1 or Stat3; and (B) and C) Immunoblotting analysis of whole-cell lysates from cells (B) (i) untreated or (ii) treated with ZD 1839 (ZD), or Dasatinib (Das), or transfected with or without (iii) Src siRNA, (iv) EGFR siRNA, or scrambled siRNA control (con) and probing for pY416c-Src (pY416Src), Src, pY845EGFR, and EGFR; and (C) untreated or treated with ZD or Das and probing for (i) pY1086EGFR, (ii) pY1086EGFR and (iii) pY1173EGFR, and EGFR. Positions of STAT:DNA complexes in gel are shown. *Supershifts were performed with antibodies specifically recognizing either Stat1 (a-Stat1), Stat3 (a-Stat3), or Stat5 (a-Stat5a or a-Stat5b); asterisk indicates position of supershifted complexes. Data are consistent with those obtained from 4 independent experiments.

FIG. 2 depicts EMSA and immunoblotting analyses for effects of inhibitors on Stat3. (A and B) EMSA analysis of Stat3 DNA-binding activity in (A) Panc-1 or (B) Colo-357 cells treated or untreated with the pan ErbB inhibitor, PD169350 (PD169), ZD 1839 (ZD), Dasatinib (Das), the Jak inhibitor, AG490, the ErbB2-selective inhibitor, AG879, or inhibitor combinations for the indicated times, or (C) immunoblotting analysis of whole-cell lysates from Panc-1 cells transfected with EGFR siRNA, Src siRNA, or scrambled siRNA (control) and probing for pstat3 or Stat3. *Supershift analysis. Data are consistent with those obtained from 3 independent experiments.

FIG. 3 presents data of cell viability studies for effects of inhibitors. (A and B) Trypan blue exclusion/phase-contrast microscopy for viable Panc-1 or Colo-357 cells following treatment for 0-96 h with inhibitor 1 µM ZD, 100 nM Das, 50 µM S3I-201, Jak inhibitor, AG490, or combinations; (C and D) CyQuant cell proliferation assay for viability of Panc-1 (C, left panel, and D(i)) or Colo-357 cells (C, right panel and D(ii)) for effects of 48-h treatments with the designated concentrations of ZD, Das, S3I-201, Gemcitabine (Gem) alone and in combinations. Values, mean and S.D., n = 4 experiments each in triplicates. p values, *=<0.05, **=<0.01, and ***=<0.001.

FIG. 4 shows colony survival and apoptosis studies for effects of inhibitors. (A) Number of colonies emerging from cells in culture (500 per 6 cm dish) untreated or treated once with ZD1839 (ZD), Dasatinib (Das), S3I-201 (S3I), or combinations and allowed to culture; or (B) Annexin V binding/ Flow Cytometry analysis of normal HPDEC, Panc-1 or Colo-357 cells treated or untreated with inhibitors or combinations. Values, mean and S.D., n = 4 experiments each in triplicates. p values, *=<0.05, **=<0.01, and ***=<0.001.

FIG. 5 presents the concurrent inhibition of Stat3 and EGFR or Src inhibits migration and suppression of c-Myc expression. (A) Effects of ZD1839 (ZD), Dasatinib (Das), and/or S3I-201 (S3I) on migration and invasion; (B) Immunoblotting analysis of whole-cell lysates for c-Myc and b-Actin expression in Panc-1 cells. Values, mean and S.D., n=3-4 experiments each in triplicates. p values, *=<0.05, **=<0.01, and ***=<0.001.

FIG. 6 is a line graph showing progression of tumor volume under the different therapies; concurrent inhibition of Stat3 and EGFR or Src induces human pancreatic tumor growth inhibition in xenografts.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

The present invention will now be described more fully hereafter with reference to the accompanying drawings, in which preferred embodiments of the invention are shown.

**Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. Any publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including any definitions, will control. In addition, the materials, methods and examples given are illustrative in nature only and not intended to be limiting. Accordingly, this invention may be embodied in many different forms and should not be construed as limited to the illustrated embodiments set forth herein. Rather, these illustrated embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.**

**Materials and Methods**

Cells and Reagents.

v-Src-transformed mouse fibroblasts (NIH3T3/v-Src), human pancreatic cancer (Panc-1) and leukemic (K562) lines have been described (14-16). The human pancreatic cancer lines, Colo-357 and Mia-PaCa-2 were kind gifts from Drs. Lancaster and Mokenges (Moffitt Cancer Center). The immortalized human pancreatic duct epithelial cell (HPDEC) line was obtained from Dr. Tsao, OCI, UHN-PMH, Toronto (17). Except for HPDEC grown in Keratinocyte-SFM media supplemented with 0.2 ng EGF, 30 µg/ml bovine pituitary extract and containing antinmyel, and K562 line in RPMI 1640 containing 10% heat-inactivated FBS and 100 units/ml penicillin-streptomycin, all other cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% iron-supplemented bovine calf serum and 100 units/ml penicillin-streptomycin. Recombinant human EGF (hEGF) from Creative Biolabs, Port Jefferson Station, N.Y.); Gemcitabine is from Ely Lilly (Indianapolis, Ind.).

Nuclear Extract Preparation and Gel Shift Assays.

Nuclear extract preparation and DNA-binding with electrophoretic mobility shift assay (EMSA) were carried out, as previously reported (14, 15). The 32P-labeled oligonucleotide probes used were hSIE (high affinity sis-inducible element from hefos gene, m67 variant), 5'AGCCTATTTCCGGTAAATCCCTA; (SEQ ID NO:1) that binds Stat1 and Stat3 (Wagner et al., 1990) and the MGFε (mammary gland factor element from the bovine β-casein gene promoter, 5'AGATTCTAGGATTCAA; (SEQ ID NO:2) that binds Stat1 and Stat5 (Gouilleux et al., 1995; Seidel et al., 1995).

**SDS-PAGE/Western Blot Analysis.**

Western blotting analysis was performed as previously described (15, 18). Primary antibodies used were anti-Stat3 (C20) (Santa Cruz, Santa Cruz, Calif.), anti-pY845EGFR (Upstate Biotech, Millipore, Billerica, Mass.), and antibodies against pY705Stat3, Stat3, pY1086EGFR, pY1173EGFR, EGFR, pY416Src, Src, c-Myc, and β-Actin from Cell Signaling (Danvers, Mass.).

**Inhibitors**

The inhibitors used herein are known to the art. ZD or ZD1839 (Iressa™) is also referred to as Gefitinib (CAS No. 184475-32-2). ZD1839 has the molecular formula C22H24ClFN4O3. ZD has the formula: ZD.
Small-Interfering RNA (siRNA) Transfection.

See Reference 24 and 25. Das or Dasatinib (Sprycel™) is also referred to as BMS-354825 (CAS No. 302962-49-8). Das has the molecular formula C_{22}H_{26}ClN_{7}O_{2}S. Das has the formula:

See Reference 23, 29, and 35. AG490 is also referred to as Tyrophostin AG490 (CAS No. 133550-30-8). AG490 has the molecular formula C_{17}H_{14}N_{2}O_{3}. AG490 has the formula:

See Reference 14.

Small-Interfering RNA (siRNA) Transfection.

siRNA sequences for EGFR and Src were ordered from Dharmacon RNAi Technologies, Thermo Scientific (Lafayette, Colo.). Sequences used were: EGFR sense strand, 5'-GAAAGGAACUGAAUUCAAUU-3', SEQ ID NO:3; EGFR antisense strand, 5'-UUUGAAUUCAGUUUCUU-3', SEQ ID NO:4; control siRNA sense strand, 5'-AGUAUAUCAACGGUAAGAUU-3', SEQ ID NO:5; and control siRNA antisense strand, 5'-UCUUUAACCNGUUGAUUACUUU-3', SEQ ID NO:6. The c-Src SMARTpool siRNA reagent (NM-005417, Catalog #M-003175-01-05) was used for Src. Transfection into cells was performed using 20 nM of EGFR siRNA or 25 nM of Src siRNA and 8 µl Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, Calif.) in OPTI-MEM culture medium (GIBCO, Invitrogen). Cell Proliferation Viability Assay and Annexin V Binding and Flow Cytometry.

Proliferating cells in 6-well or 96-well plates were treated once with 0.1-1 nM ZD1839 (Iressa) (reference 25), 100 nM Dasatinib (references 23 and 35), 50-100 µM S31-201, 1 µM Gemcitabine (reference 43), or combinations of inhibitors for up to 96 h. Viable cells were counted by trypan blue exclusion/phase contrast microscopy or assessed by CyQuant cell viability assay, according to manufacturer’s (Invitrogen) instructions, or cells were processed for Annexin V binding (BD Biosciences) with flow cytometry for apoptosis. S31-201 is fully described in reference 30 (see below).

Colonies were stained with crystal violet for 4 h and counted under phase-contrast microscope.

Cell Migration and Matrigel Invasion Assays.

Cell migration and invasion experiments were carried out and quantified as previously described (20), using Bio-Coat migration chambers (Becton Dickinson, Franklin, N.J.) of 24-well companion plates with cell culture inserts containing 8 µm pore size filters, according to the manufacturer’s protocol.

Statistical Analysis.

Statistical analysis was performed on mean values using GraphPad Prism GraphPad Software, Inc. (La Jolla, Calif.). The significance of differences between groups was determined by paired t-test at p <0.05*, <0.01**, and <0.001***. Results

Ablative c-Src activity is fully described in reference 25.

Immunoblotting analysis showed a moderate pY1068EGFR level in Mia-Paca-2, but enhanced levels in Panc-1 and Colo-357 cells similar to levels in NIH3T3/v-Src (FIG. 1A(i)). By contrast, Stat5 activity is detectable in pancreatic cancer cells (FIG. 1A(ii)), compared to aberrant levels in the K562 leukemic cells (16).

EGFR and c-Src are abberant in many human cancers (2, 4). Immunoblotting analysis further showed elevated levels of the EGFR autophosphorylation motif (22) pY845EGFR (FIG. 1C(i), lanes 2 and 7), and pY1173EGFR (FIG. 1C(ii), lanes 2 and 7) in Panc-1 and Colo-357, compared to basal levels of same in NIH3T3/v-Src (FIG. 1C(iii), lane 1).

Statistical analysis was performed on mean values using GraphPad Prism GraphPad Software, Inc. (La Jolla, Calif.). The significance of differences between groups was determined by paired t-test at p <0.05*, <0.01**, and <0.001***. Results

Ablative c-Src activity is fully described in reference 25.

Immunoblotting analysis showed a moderate pY1068Src level in Mia-Paca-2, but enhanced levels in Panc-1 and Colo-357 cells similar to levels in NIH3T3/v-Src, compared to low levels in HPDEC (FIG. 1B(i), upper panel). The elevated pY416Src levels parallel enhanced levels of the Src-sensitive pY845EGFR motif (21) in Panc-1 and Colo-357 cells, compared to low levels of same in HPDEC (FIG. 1B(i), lower panel). Total Src or EGFR protein remained unchanged.

Immunoblotting analysis further showed elevated levels of the EGFR autophosphorylation motif (22) pY1068EGFR (FIG. 1C(i), lanes 2 and 7), pY1173EGFR (FIG. 1C(ii), lanes 2 and 7), and pY1173EGFR (FIG. 1C(iii), lanes 2 and 7) in Panc-1 and Colo-357, compared to basal levels of same in HPDEC (FIG. 1C(iii), lane 1).

Functional Integration of EGFR and Src in Pancreatic Cancer Cells.

We next examined the functional relationship between the activated EGFR and Src. Immunoblotting analysis showed
treatment of cells with Dasatinib (Das) inhibited Src activity (pY416Src) (23) and induced an early (1 h) and a sustained (24 h) decrease in pY845EGFR (FIG. 1B(i)). By contrast, no detectable changes in pY416Src and pY845EGFR levels were induced by treatment with the pan-ErbB inhibitor, PD168395 (PD169) (24) (data not shown) or the selective EGFR inhibitor, ZD 1839 (ZD, Iressa) (25) (FIG. 1B(ii)). In confirmation, siRNA knockdown of c-Src abrogated pY845EGFR levels (FIG. 1B(iii), Src siRNA), while EGFR knockdown by siRNA had minimal effect on pY416Src level (FIG. 1B(iv), EGFR siRNA). Scrambled siRNA has no effect (FIG. 1B(iii) and (iv), con siRNA). Thus, elevated pY845EGFR levels in pancreatic cancer cells are sensitive to Src activity.

Immunoblotting analysis further showed that treatment of Panc-1 and Colo-357 cells with ZD diminished pY1173EGFR levels (FIG. 1C(i)(iii), lanes 3, 4, 8 and 9) by as early as 1 h and up to 24 h, with no effect on pY1068EGFR (FIG. 1C(i)(i), lanes 3, 4, 8 and 9) or pY1086EGFR level (FIG. 1C(i)(ii), lanes 3, 4, 8 and 9), suggesting that EGFR kinase is essential for the induction of pY1173EGFR levels, but not pY1068EGFR or pY1086EGFR. By contrast, treatment decreased pY1068EGFR and pY1086EGFR levels (FIG. 1C(i) and (ii), lanes 5, 6, 10 and 11), with minimal effect on pYEGFR1173 (FIG. 1C(iii), lanes 5, 6, 10 and 11). Both EGFR and Src Promote Ablant Stat3 Activation.

Both the pY1068EGFR and pY1086EGFR levels are binding sites for Stat3 (27, 28). Given the concurrent EGFR and Src activation in Panc-1 and Colo-357 cells, we sought to define the regulation of aberrant Stat3 activation. By in vitro DNA-binding assay with EMSA analysis of nuclear extract preparations, we observe an early repression (in the first 30 min to 1 h of treatment) of constitutively-active Stat3 by the pan-ErbB inhibitor, PD168395 (PD169), the ErbB2-selective inhibitor, AG879 (7), ZD, or Das (FIG. 2A(i)), lanes 4, 5, 7, and 8, and (ii), lanes 2, 4, 6, and 11, and FIG. 2B, 1 h), or by a combined PD169 and Das (FIG. 2A(ii), lanes 10 and 11, and (ii), lane 8). However, the Stat3 activity in Panc-1 cells consistently rebounded following 24 h treatments with Das, ZD, or PD169 (FIGS. 2A(i) and (ii), 24 h), even though EGFR or Src activity remained inhibited (FIGS. 1B and 1C, 24 h). Twenty-four h treatment with the AG879 moderately inhibited Stat3 activity (FIG. 2A(ii), lane 12), which we speculate may be due to its widespread activity as a pan-ErbB inhibitor. By contrast, treatment with the Jak inhibitor, AG490 for 1 h had no effect on constitutive Stat3 activity, but surprisingly abolished Stat3 activity at 24 h treatment (FIG. 2A(i), lanes 9 and 10). Moreover, combined treatment with AG490 and ZD, Das or PD169 for 24 h similarly abolished constitutively-active Stat3 (FIG. 2A(ii), lanes 14, 15, and 16). In Colo-357, Stat3 activity was inhibited by both ZD and Das, with the effects more striking for Dasatinib (FIG. 2B). These findings together reveal a pattern of constitutive Stat3 activation in pancreatic cancer cells that is mediated by both EGFR and Src, and a compensatory, Jak-dependent secondary Stat3 activity. A similar pattern of Stat3 activation has been observed in head and neck squamous carcinoma, mesothelioma, squamous cell skin carcinoma, and non-small cell lung cancer cell lines following the inhibition of Src (29). In further support, the siRNA knockdown of EGFR (EGFR siRNA) or Src (Src siRNA) led to pStat3 suppression, as assayed by immunoblotting analysis (FIG. 2C). Scrambled siRNA (con) has no effect. Immunoblotting analysis also shows that EGFR stimulation induces pY705Stat3, pY1068EGFR, pY1173EGFR, pY845EGFR and pY416Src (Supplemental FIG. S1(i)-(iii), lane 4) over and above constitutive levels in Panc-1 cells, in a manner that is similar to the induction of}

same in response to the stimulation of normal HPDEC (Supplemental FIG. S1, lane 2), except for pY1068EGFR levels in Panc-1 (FIG. S1(iii), upper right panel). In control studies, immunoblotting analysis showed elevated pErk1/2/pErk2MAPK and pAkt in Panc-1 and Colo-357 cells compared to normal HPDEC, neither of which was significantly affected by treatment with ZD or Das (data not shown). Inhibition of Stat3 Sensitizes Pancreatic Cancer Cells In Vitro to EGFR and Src Inhibitors.

Given the preceding data on the inter-relation between EGFR, Src and Stat3 activation, we investigated the biological implications and the therapeutic potential of a combinatorial approach. Dasatinib and ZD were used at 100 nM and 0.1-1 µM, respectively, as in literature reports (23, 24), while the Stat3 inhibitor, S3I-201 was used at the sub-optimum, 50 µM, or at the 100 µM required to inhibit Stat3 activation (30). Viable cell count by trypan blue exclusion/phase-contrast microscopy showed that treatment with 1 µM ZD, 100 nM Das, or 50 µM S3I-201 alone minimally affected cell viability by 24 h (FIG. 3A, Day 1). By contrast, treatment for 48 to 96 h with or Das or S3I-201 alone progressively decreased cell viability, while treatment for the same period with ZD showed minimal effect (FIG. 3A), except at 96 h when the number of viable Panc-1 cells decreased (FIG. 3A(i), ZD, Day 4). Comparatively, the combined inhibition of Stat3 (by S3I-201) and EGFR (by ZD) or Src (by Das) or the combined treatment with AG490 (Jaks inhibitor) and ZD or Das induced greater losses of viability at 48-96 h (FIGS. 3A and B). The effects on cell viability as captured by trypan blue exclusion were confirmed by the CyQuant cell proliferation/viability assay. Unlike 24 h treatment duration that showed minimal effect on viability (FIG. 3A), CyQuant assay showed that 48-h treatment with each inhibitor alone decreased viable cell numbers (quantified as fluorescent unit, FU) in a dose-dependent manner (FIG. 3C, ZD, Das and S3I-201). We infer from the graphs that treatment with 1 µM ZD for 48 h has minimal effect on cell viability (FIGS. 3C(i) and (ii)), as observed by the trypan blue exclusion assay (FIG. 3A). However, the observed effects of single agents were significantly weaker compared to the concurrent treatment with a Stat3 inhibitor and an inhibitor of EGFR or Src. Results show that the treatment with S3I-201 increased the sensitivity of Panc-1 and Colo-357 cells to ZD and Das, shifting the dose-response curves to the left (FIG. 3C, ZD+S3I-201, and Das+S3I-201). Concurrent treatment with S3I-201 significantly decreased the IC50 values as follows: 17 to 0.4 µM, and 100 to 6 nM, respectively, for ZD and Das against Panc-1 viability (FIG. 3C(i)(i) and (ii)); and 6.5 to 2.4 µM, and 90 to 8 nM, respectively for ZD and Das against Colo-357 viability (FIGS. 3C(iv) and (v)). For the impact of ZD and Das on the sensitivity to S3I-201, CyQuant cell viability assay showed that Das, but not ZD increased the sensitivity of both cell lines to S3I-201, decreasing its IC50 from 40 to 15 µM, and from 45 to 20 µM, respectively, for Panc-1 and Colo-357 cells (FIGS. 3C(ii) and (iv)). Thus, treatment with S3I-201 sensitized cells to ZD and Das, while treatment with Das, but not ZD similarly sensitized cells to S3I-201.

Given the clinical implications of these findings, we extended these studies to examine the effect of EGFR Src and Stat3 pathway on the response to Gemcitabine, the anti-metabolite agent used in the treatment of pancreatic cancer. CyQuant cell proliferation/viability studies showed that inhibition of EGFR, Src or Stat3 sensitized Panc-1 and Colo-357 cells to Gemcitabine (FIG. 3D). More importantly, the combined inhibition of Stat3 and EGFR or Src induced a higher sensitization of cells to Gemcitabine than that induced by the inhibition of any one alone (FIG. 3D).
As known to the skilled, Gemcitabine is a nucleoside analog of cytidine which interferes with DNA replication, arresting tumor growth and resulting in apoptosis of the cell. Gemcitabine is also known to bind to the active site of the enzyme ribonucleotide reductase (RNR) to irreversibly inactive the enzyme, thus interfering with the cell’s ability to produce deoxyribonucleotides necessary for DNA replication and repair. This also leads to apoptosis. As noted above, the combined inhibition of Stat3 and EGFR or Src induces a higher sensitization of cells to Gemcitabine, creating another possibility for combination therapy of tumors.

To further explore the sensitization potential of inhibition of aberrant Stat3, we performed colony survival assay (19). Results show that inhibition of Src (by Das) or Stat3 (by S3I-201 (S3I)), but not EGFR inhibition (by ZD) resulted in reduced colony numbers (Fig. 4A). More importantly, the concurrent inhibition of Stat3 and EGFR or Src resulted in much lower colony numbers (Fig. 4A), consistent with the much greater loss of viable cells due to the combined inhibition of Stat3 and EGFR or Src (Fig. 3). To extend these studies, we performed Annexin V binding/flow cytometric analysis for apoptosis. Higher percentages of Panc-1 and Colo-357 cells undergoing apoptosis were observed for concurrent inhibition of Stat3 and EGFR or Src than for the inhibition of any one signaling molecule alone (Fig. 4B(i) and (iii)). Similar results were obtained for the concurrent treatments with AG490 and ZD or Das (Fig. 4B(ii) and (iii)). By contrast, similar treatments of normal HPDECs showed no significant apoptosis (Fig. 4B(i)) with the combination treatments. Thus, we establish that pancreatic cancer cells have higher sensitivity to concurrent inhibition of Stat3 and EGFR or Src than to the inhibition of a single entity. EGFR, Src and Stat3 Together Promote Pancreatic Cancer Cell Migration and Invasion.

Aberrantly-active Src and Stat3 have both been implicated in tumor cell motility, migration, invasion and metastasis (4, 23). In vitro matrigel migration confirmed that inhibition of Src or Stat3 alone suppresses migration and invasion (Fig. 5A). However, concurrent inhibition of Stat3 and EGFR or Src for 24-h has a stronger effect on Colo-357 migration and Panc-1 invasion, except for Src inhibition, which showed a similar effect on Panc-1 migration (Fig. 5A). At the 24-h treatment when these studies were done, there is no significant effect on cell viability (Fig. 3). These findings are further evidence that pancreatic cancer lines are more sensitive to concurrent inhibition of Stat3 and Src or EGFR. EGFR, Src and Stat3 Module Regulates c-Myc Over-Expression in Pancreatic Cancer Cells.

For insight into the underlying molecular mechanisms by which the EGFR, Src and Stat3 pathway may support the cancer phenotype, we studied the regulation of key cancer-relevant genes. We show that c-Myc is over-expressed in pancreatic cancer lines compared to normal HPDEC (Fig. 5B). Furthermore, the concurrent inhibition of Stat3 and EGFR or Src consistently repressed c-Myc expression. These findings suggest a functional synergy between EGFR, Src and Stat3 in inducing c-Myc expression in the context of pancreatic cancer phenotype and that the stronger repression of c-Myc expression contributes to the antitumor cell effects of and the increased sensitivity of pancreatic cancer lines to concurrent Stat3 and EGFR or Src inhibition.

Inhibition of Tumor Growth by Combination Treatment

Concurrent inhibition of Stat3 and EGFR or Src induces human pancreatic tumor growth inhibition in xenografts. Subcutaneous xenografts of Colo-357, a metastatic pancreatic adenocarcinoma line were used to study the therapeutic implication of the Stat3, EGFR and Src inter-relationships and to evaluate the in vivo antitumor effects of concurrent inhibition of Stat3 and EGFR or Src. Data showed that in general, xenografts of Colo-357 cells showed low responsiveness to treatment with inhibitor of EGFR, Src or Stat3 alone, although, as the therapy progressed, those tumors treated with only one inhibitor alone appeared to show reduced growth, which was statistically not significant from the control, untreated tumors (Fig. 6). By contrast, tumors from mice treated with combined S3I-201 and Das or S3I-201 and ZD consistently showed reduced growth and smaller tumor sizes throughout the entire study (Fig. 6). Thus, the residual tumor volumes (sizes) for tumors in mice treated with combination inhibitors were significantly different (p<0.05) from tumor volumes for tumors in control mice at days 20 and upwards post treatment. These in vivo antitumor effects of combination treatment with inhibitors of S3I-201 and Das or S3I-201 and ZD are consistent with the in vitro antitumor cell data and together indicate that aberrant Stat3 cooperates with hyperactive EGFR or Src to sustain human pancreatic cancer.

Discussion

Within the context of aberrations in the EGFR, Src and Stat3 pathway in pancreatic cancer, present study reveals a strong role for Src in promoting aberrant EGFR activation by not only inducing the phosphorylation of Y845EGFR motif (31), but also promoting the induction of pY1068EGFR and pY1086EGFR motifs. These Src-promoted events will greatly influence the status of EGFR in pancreatic cancer cells. A role for EGFR in aberrant Stat3 activation in cancer cells has previously been reported in other tumor cells, including head and neck squamous cell carcinoma and breast cancer (26, 32). Present study extends those findings to pancreatic cancer and show that EGFR is key in facilitating aberrant Stat3 activation. Moreover, the pY1068EGFR and pY1086EGFR induction by Src is likely to have significant impact on the activation of Stat3, given that these two motifs are essential sites for the binding of Stat3 to EGFR in order to promote its phosphorylation and activation (27, 28). Furthermore, Src may not only facilitate Stat3 activation via the induction of those two Tyr motifs of EGFR, but it can also directly phosphorylate Stat3, as has been previously reported in other systems (18). It is therefore consistent that both hyperactive EGFR and Src promote baseline constitutive Stat3 activation in pancreatic cancer, as revealed by our study.

The present study is also in agreement with an earlier report of ErbB-2 dependent constitutive Stat3 activation in MiaPaca-2 and UK Pan-1 cells (7) and another study that showed that the full induction of Stat3 activation by ErbB2 required both Src and Jaks (33). Our findings indicate that Jaks contribute to the maintenance of constitutive activation in revealing a Jak-dependent compensatory mechanism of Stat3 activation upon inhibition of EGFR and Src. Given that Jaks inhibition did not abolish aberrant Stat3 at the earliest time point, we deduce that this family of cytoplasmic tyrosine kinases may not be the predominant mediators of the baseline aberrant Stat3. Thus, in pancreatic cancer cells, a two-phase model of activation of Stat3 signaling emerges composed of an EGFR- and Src-dependent baseline, constitutive Stat3 induction, and an induced Stat3 activation that is dependent on Jaks. The observed secondary induction of Stat3 activation via Jaks has similarly been reported in head and neck squamous cell carcinoma line (29) and could be due to growth-stimulatory factors released from tumor cells (34), which in turn would induce the activation of Jaks and thereby promote Stat3 activation.

EGFR, Src and Stat3 has each independently been established to have critical roles in malignant transformation (6, 14, 23, 26, 35), while their collective roles in promoting...
tumorigenesis have not been explored. While the inhibition of the activity of each of the three proteins induced antitumor cell response to some degree, data presented here strongly indicate that the multiple targeting of Stat3 and EGFR or Src together has a higher potential to inhibit growth, viability, survival, malignant transformation, and migration and invasion in vitro.

Significantly, hyperactivation of the EGFR signaling has been deemed a prognostic indicator of low survival among pancreatic cancer patients (36-38). Also, there is evidence to indicate that the concurrence with aberrant Src signaling potentiates the effects of aberrant EGFR and induces biological synergy (3, 21, 39). Given the potential collective roles of Stat3, EGFR and Src in promoting and supporting pancreatic cancer, the inhibition of any single entity alone is unlikely to be sufficient to impact the disease. Present data that simultaneous inhibition of the Stat3, EGFR and Src in promoting and supporting pancreatic cancer have not been explored. While the inhibition of the activity of each of the three proteins induced antitumor cell response to some degree, data presented here strongly indicate that the multiple targeting of Stat3 and EGFR or Src together has a higher potential to inhibit growth, viability, survival, malignant transformation, and migration and invasion in vitro.

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That which is claimed:

1. A pharmaceutical composition comprising:

   - A drug combination selected from gefitinib (ZD-1839 or ZD) and 74859 (S3I-201), Das and S3I-201, ZD and tyrphostin AG 490 (AG490), and dasatinib (Das) and AG490 in a pharmaceutically acceptable carrier.

   - A method of cytotoxically affecting pancreatic cancer cells in a host in need thereof, comprising:

     - contacting said pancreatic cells with a pharmaceutical composition comprising a drug combination that targets two or more cellular functional elements in pancreatic cancer cells selected from ZD and S3I-201, Das and S3I-201, ZD and AG490, and Das and AG490.


It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

The first or sole Notice should read --

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 142 days.

Signed and Sealed this
Twenty-ninth Day of September, 2015

Michelle K. Lee
Director of the United States Patent and Trademark Office