Drug composition cytotoxic for pancreatic cancer cells (US)

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Recommended Citation
Disclosed herein are compositions comprising a drug combination that comprises ZD and S31-201, Das and S31-201, ZD and AG490, or Das and AG490. The disclosed drug combinations target two or more functional elements such as EGFR or Src and Stat3 or Jaks in pancreatic cancer cells. Also disclosed herein are methods of using the disclosed compositions to cytotoxically affect pancreatic cancer cells and methods of making the disclosed compositions.
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 PI3-C gamma. EMBO J. 9: 4375-4380.


(56) References Cited

OTHER PUBLICATIONS


* cited by examiner
FIG. 1
A

Panc-1

Stat3:Stat3\^\alpha

Lane 1 2 3 4 5 6 7 8 9 10 11 12

100 nM PD169 (h) - - - 0.5 1 24 - - - 0.5 1 24
100 nM Das (h) - - - - - - 0.5 1 24 0.5 1 24

ii)

Stat3:Stat3\^\alpha

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

100 nM PD169 (h) - 1 24 - - - - - 1 - - - - - - 24 - -
1 µM ZD (h) - - - - - - 1 24 - - - - - - - 24 - -
100 nM Das (h) - - - - - - 1 24 1 - - - - - - - 24
50 µM AG490 (h) - - - - - - - 1 24 - - - 24 24 24
50 µM AG879 (h) - - - - - - - 1 24 - - - - - - -

B

Colo-357

Stat3:Stat3\^\alpha

1 µM ZD (h) - 1 24 - -
100 µM Das (h) - - - 1 24

C

Panc-1

pStat3\rightarrow pStat3\rightarrow pStat3\rightarrow

FIG. 2
FIG. 3
FIG. 4

A
(i) Panc-1

(ii) Colo-357

B
(i) HPDEC

(ii) Panc-1

(iii) Colo-357
FIG. 5
FIG. 6
DRUG COMPOSITION CYTOTOXIC FOR
PANCREATIC CANCER CELLS

CROSS-REFERENCE TO RELATED
APPLICATIONS

This application is a national phase application of Interna­
tional Application No. PCT/US2009/0666079, which was filed
on 30 Nov. 2009, which claims priority to U.S. provisional
application Ser. No. 61/118,792, which was filed on 1 Dec.
2008, and to U.S. provisional application Ser. No. 61/249,
307, which was filed on 7 Oct. 2009, each application of
which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH OR DEVELOPMENT

The invention was made with government support under
ROI CA106439 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 develop­
ment and, more particularly, to a drug composition cytotox­
ic 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 may 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 activa­
tion of the EGFR protein, which has an incidence of 30-50%
of pancreatic cancer cases (1). Evidence indicates that the
hyperactive EGF/EGFR duo is important in the disease main­
tenance 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 induc­
tion 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) families (10). In contrast
to normal STAT signaling that is transient in accordance with
the requirements for normal biological processes, tumor cells
harbor aberrant Stat3 activation. Studies show that aberrant
Stat3 dysregulates cell growth and survival, promotes tumor
angiogenesis, cell migration and invasion, and induces tumor
immune tolerance (11-13).

De-regulated signal transduction provides the framework
for functional cooperativity and signaling cross-talk that
would not only support the malignant phenotype and the
disease progression, but also influence the drug responsive­
ness. Within the context of the concurrent activation of
EGFR, Src and Stat3 in pancreatic cancer, the potential for
cooperation between EGFR and Src kinases to induce aber­
rant Stat3 activation and to cooperate in support of the cancer
phenotype is a reasonable model to propose. Knowledge of
this functional relationship and the collective roles of
the proteins in supporting pancreatic cancer can facilitate the
design of effective, multiple-targeted therapy for disease. We
provide evidence that EGFR and Src promote constitutive
Stat3 activation, with a compensatory Stat3 activation mecha­
nism from Jaks, and together support the pancreatic cancer
phenotype. Importantly, our study identifies that the concur­
rent inhibition of aberrant Stat3 and EGFR or Src is more
effective in inducing antitumor cell response and pancreatic
tumor regression in xenografts.

SUMMARY OF THE INVENTION

With the foregoing in mind, the present invention advan­
tageously 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 combina­
tion contained therein is selected from ZD and S31-201, Das
and S31-201, ZD and AG490, Das and AG490, and combina­
tions thereof. Furthermore, the preferred cytotoxic composi­
tion is that wherein the drug combination inhibits said func­
tional 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, Gemicitabine.

The invention herein disclosed also includes a method of
cytotoxically affecting (which could result in killing) pan­
creatic 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 AG490, and com­
binations thereof. A preferred method of the invention also includes contacting the
cells with a drug combination further comprising a nucleo­
side analog inhibitory for DNA replication, the nucleoside
analog preferably being Gemicitabine.

The invention additionally includes a method of making a
therapeutic medication cytotoxic for pancreatic cancer cells,
the method comprising preparing a pharmaceutically accep­
table 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, Gemicitabine.

BRIEF DESCRIPTION OF THE DRAWINGS

Some of the features, advantages, and benefits of the
present invention having been stated, others will become
apparent as the description proceeds when taken in conjunc­
tion with the accompanying drawings in which:

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 (i)
high-affinity sis-inducible element (hSIE) probe that binds
Stat3 and Stat1 or (ii) mammary gland factor element (MGFe) 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 Dasaatinib (Dus), 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) pY1068EGFR, (ii) pY1068EGERF and (iii) pY1173EGERF, 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) EMSA analysis of Stat3 DNA-binding activity in (A) Panc-1 or (B) Colo-357 cells treated or untreated with the pan ErbB inhibitor, PD169540 (PD169), ZD 1839 (ZD), Dasaatinib (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 invasion and suppresses c-Myc expression. (A) Effects of ZD1839 (ZD), Dasatinib (Das), 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 hereinafter with reference to the accompanying drawings, in which preferred embodiment of the invention are shown.
Small-Interfering RNA (siRNA) Transfection.

siRNA sequences for EGFR and Src were ordered from Dharmacon RNAi Technologies, Thermo Scientific (Lafayette, Colo.). Sequences used are: EGFR sense strand, 5'-GAAGGAAACUGAAUCAAAAUU-3', SEQ ID NO:3; EGFR antisense strand, 5'-UUGAAGAAUCAGUUCUUCUU-3', SEQ ID NO:4; control siRNA sense strand, 5'-AGUAUUAACCGUAAAGAUU-3', SEQ ID NO:5; and control siRNA antisense strand, 5'-UCUUAACCGUUGUAUUCUUU-3', SEQ ID NO:6. The c-Src SMARTpool siRNA reagent (NM-003417, 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 mM ZD1839 (Iressa) (reference 25), 100 nM Dasatinib (references 23 and 35), 50-100 μM S3I-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. S3I-201 is fully described in reference 30 (see below).

Colony Survival Assay.

Single-cell suspension of Panc-1 and Colo-357 cells were seeded in 6-cm dishes (500 cells per well) and assayed as previously reported (19), treated the next day with inhibitors for 48 h, and allowed to grow until large colonies were visible. 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 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

Ablent EGFR, Src and Stat3 in Pancreatic Cancer Lines.

Consistent with published reports (6, 7), Stat 3 activity, per DNA-binding with EMSA analysis in nuclear extract preparations is constitutive in Panc-1 and Colo-357, low in Mia-Paca-2, and undetectable in the normal human pancreatic duct epithelial cells (HPDEC), compared to aberrant levels in NIH3T3/v-Src (15) (FIG. 1A(i)). Per supershift analysis, the DNA-protein complex contains Stat3 (FIG. 1A(i), lane 3). By contrast, Stat5 activity is undetectable in pancreatic cancer cells (FIG. 1A(ii)), compared to aberrant levels in the K562 leukemic cells (16).

EGFR and c-Src are aberrant in many human cancers (2, 4).

Immunoblotting analysis showed a moderate pY416c-Src 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 motifs (22), pY1068EGFR (FIG. 1C(i), lanes 2 and 7), pY1086EGFR (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(i)-(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 levels (FIG. 1B(i)). By contrast, no detectable changes in pY416Src and pY845EGFR levels were induced by treatment with the pan-ErbB inhibitor, PD169340 (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 csiRNA), while EGFR knockdown by siRNA had minimal effect on pY416Src level (FIG. 1B(iv), EGFR siRNA). Scrabbed 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(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), lanes 3, 4, 8 and 9) or pY1086EGFR level (FIG. 1C(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, Das 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 aberrant Stat3 activation.

Both the pY1068EGFR and pY1086EGFR levels are binding sites for Stat3 (27, 28). Given the concurrent EGFR and Src activity 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, PD169340 (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 hour 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(ii), 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 pY416c-Src (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(ii), upper right panel). In control studies, immunoblotting analysis showed elevated pErk1/2/P2MAF 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(i), 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(ii), 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 (iv)), 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) 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 effects on Panc-1 and Colo-357 cells (FIGS. 3C(iii) 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 assay 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 anti-tumor 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 not statistically significant from the control, non-treated 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 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 ErbB2-dependent constitutive Stat3 activation in Mia-Paca-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 similarly has 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 insufficient to impact the disease. Present data that simultaneously inhibit Stat3 and Src induced greater antitumor cell effects and a higher sensitization to Gemcitabine provides a strong support for the opinion that Stat3 may cooperate with EGFR and Src to support the malignant phenotype. Indeed, the inhibition of Stat3 seemed to sensitize pancreatic cancer cells to the antitumor cell effects of ZD and Das. Multiple targeting of Stat3 and EGFR or Src therefore has the potential to induce a greater antitumor efficacy. This is supported by our present data that concurrent treatment with the Stat3 inhibitor, S3I-201 and ZD or Das induced greater regression of xenografts of Colo-357 than treatment with either inhibitor alone. Such a multiple-targeted therapy has received a strong interest in recent times, particularly given the dismal results in certain cases of molecular targeted monotherapy, such as anti-EGFR monotherapy (40, 41).

Thus, a combined Gemcitabine and Erlotinib (EGFR TK inhibitor) therapy has recently been approved for patients with locally advanced/metastatic pancreatic cancer (42, 43), although we note by our data that the inhibition of Stat3 and EGFR or Src together induces a higher Gemcitabine sensitivity than inhibition of EGFR alone. The enhanced antitumor effects due combined Stat3 and EGFR or Src inhibitors may in part be due stronger repression of the expression of c-Myc oncogene. Altogether, present study provides support for a multiple-modality therapeutic approach and lays the foundation for concurrent targeting of aberrant Stat3 and EGFR or Src as a more effective approach for achieving an enhanced antitumor therapeutic efficacy in pancreatic cancer.

Accordingly, in the drawings and specification there have been disclosed typical preferred embodiments of the invention and although specific terms may have been employed, the terms are used in a descriptive sense only and not for purposes of limitation. The invention has been described in considerable detail with specific reference to these illustrated embodiments. It will be apparent, however, that various modifications and changes can be made within the spirit and scope of the invention as described in the foregoing specification and as defined in the appended claims.

References


That which is claimed:

1. A pharmaceutical composition comprising:
   a drug combination selected from gefitinib (ZD-1839 or ZD) and 74859 (S31-201), Das and S31-201, ZD and tyrosinase AG 490 (AG490), and dasatinib (Das) and AG490 in a pharmaceutically acceptable carrier.

2. The cytotoxic pharmaceutical composition of claim 1, further comprising gemcitabine.

3. A method of cytotoxicity 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 S31-201, Das and S31-201, ZD and AG490, and Das and AG490.

4. The method of claim 3, wherein two or more functional elements in pancreatic cancer cells comprise one of EGFR or Src and one of Stat3 or Jaks.

5. The method of claim 3, wherein the pharmaceutical composition further comprises gemcitabine.

6. The method of claim 3, wherein the drug combination is ZD and S31-201 and the two or more functional elements in pancreatic cancer cells comprise EGFR and Stat3, wherein the drug combination is Das and S31-201 and the two or more functional elements in pancreatic cancer cells comprise Src and Stat3, wherein the drug combination is ZD and AG490 and the two or more functional elements in pancreatic cancer cells comprise EGFR and Jaks, or wherein the drug combination is Das and AG490 and the two or more functional elements in pancreatic cancer cells comprise Src and Jaks.

7. The method of claim 3, wherein the administration of a pharmaceutical composition suppresses c-myc expression in pancreatic cancer cells.

8. The method of claim 3, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

9. A method of making a pharmaceutical composition, comprising:
   mixing a pharmaceutically acceptable carrier and a drug combination selected from ZD and S31-201, Das and S31-201, ZD and AG490, and Das and AG490.

10. The method of claim 9, wherein the pharmaceutical composition further comprises gemcitabine.

* * * * *
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