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Abstract

c-Src is a tyrosine kinase involved in tumor proliferation, migration, and angiogenesis and has been shown to modulate the cytotoxicity following cisplatin-induced DNA damages. c-Src is frequently activated in non–small cell lung cancer (NSCLC) tissues and cell lines, but no preclinical data regarding the effects of the novel potent Src inhibitor, dasatinib (BMS-354825), in the modulation of cisplatin resistance are currently available. The present study reports that treatment with dasatinib completely abrogated Src phosphorylation in the majority of the NSCLC cell lines tested ($n = 7$), with modest effects on cell proliferation and survival. In five cell lines, a higher cytotoxicity was observed delivering cisplatin in combination with dasatinib: the most evident effects were found in the squamous H520 cells due to the effective block of cisplatin-induced Src phosphorylation. Moreover, dasatinib treatment significantly blocked cisplatin-induced transcription of a panel of DNA repair and synthesis genes. In addition, a real-time PCR analysis done on tumor and matched normal lung specimens from 44 surgically resected NSCLC patients showed that Src transcripts are significantly upregulated in 23% of cases. In conclusion, Src-directed therapeutic strategies could interfere with cisplatin resistance, possibly allowing to reduce cisplatin doses, thus improving its efficacy. The data of this study support further clinical studies aimed to evaluate the efficacy of Src-inhibiting agents in combination with cisplatin in the treatment of NSCLC. [Mol Cancer Ther 2009;8(11):3066–74]

Introduction

For the systemic treatment of non–small cell lung cancer (NSCLC), platinum compounds represent the backbone of commonly used cytotoxic combination with or without targeted therapies. Platinum-based chemotherapy improves survival in any stage of NSCLC, with the exclusion of stage I, but its effect is limited and the choice of treatment in each patient is highly influenced by the trade off between therapeutic activity and toxicity (1, 2).

Src (pp60c-Src) is a tyrosine kinase involved in multiple fields of tumorigenesis including proliferation, migration, and angiogenesis, recently indicated as a promising therapeutic target in the treatment of solid tumors (3, 4). Src kinase is expressed and frequently activated in NSCLC especially in active smoker males with squamous cell carcinoma of the lung (5, 6). Retrospective studies in colon (7) and breast (8) cancers clearly indicated an association between Src activity and poor clinical prognosis. A previous study has shown that survival of cancer cell lines to cisplatin-induced DNA damages was mediated by the epidermal growth factor receptor (EGFR) pathway through the activation of Src kinase (9). Moreover, in adenocarcinoma cells, ectopic overexpression of oncogenic Src confers resistance to chemotherapeutic agents by altering the expression levels of genes involved in DNA repair pathways (10). Dasatinib (BMS-354825; Bristol Myers Squibb) is a potent inhibitor of Src kinase activity and has been recently approved for the use of chronic myelogenous and Philadelphia chromosome–positive acute lymphoblastic leukemia in patients resistant or intolerant to imatinib (11, 12). Dasatinib has shown promising results in preclinical studies conducted in solid tumors (13–16); in NSCLC, its *in vitro* efficacy has been recently selectively related to the presence of activating mutations of the EGFR (17). However, in the clinical practice, only a minority of NSCLC harbor EGFR mutations, and for these patients, a front-line treatment with EGFR tyrosine kinase-inhibiting agents may be considered (18), whereas, for the overwhelming majority of the NSCLC patients, platinum-based chemotherapy remains the treatment of choice.

Because both Src and EGFR are activated and overexpressed in lung cancer cell lines and tissues independently of the EGFR mutational status (6, 19), the aim of the present study was to test the effect of dasatinib in modulating the sensitivity to cisplatin in a panel of seven cell lines (all carrying wild-type EGFR) in terms of cell proliferation, apoptosis, and transcriptional regulation of a panel of DNA repair genes associated to cisplatin resistance. In addition, the level of Src expression was quantified by means of quantitative real-time PCR in a consecutive series of resected NSCLC

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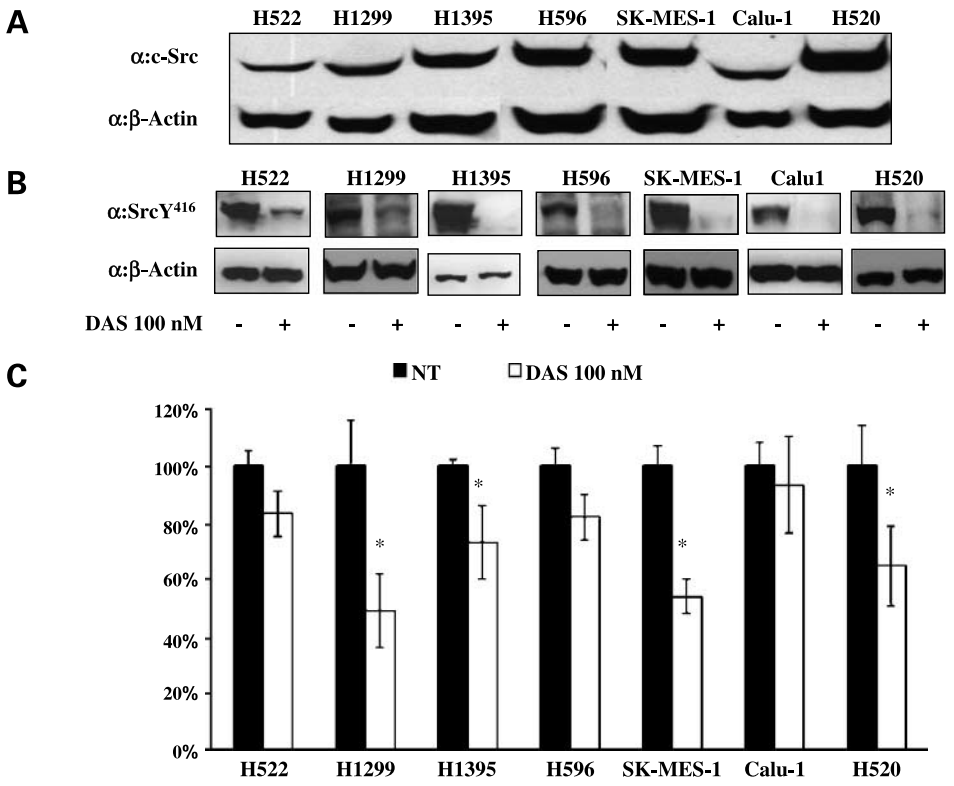

Figure 1. Whole-cell lysates obtained from all cell lines were subjected to Western blot analysis with (**A**) pan-Src (total Src) antibody and (**B**) phospho^{Y416}-Src antibody in the presence (+) or absence (−) of 100 nmol/L dasatinib for 24 h. β-Actin antibody was used as a loading control. **C,** cell proliferation assay in NSCLC cell lines in response to 100 nmol/L dasatinib (*DAS*) for 48 h. *Bars,* SD of at least eight replicates. *Asterisk,* significant difference of treated compared with untreated controls (*NT*).

and in the corresponding normal lung tissues to determine potential deregulation of Src mRNA transcript and its association with clinicopathologic characteristics.

Materials and Methods

Cell Lines, Cultures, and Drugs

Seven human NSCLC cell lines (three squamous cell carcinomas: Calu-1, H520, and SK-MES-1; one adenosquamous: H596; two adenocarcinomas: H522 and H1395; and one large-cell carcinoma: H1299) were purchased from the American Type Culture Collection. All cell lines carried wild-type EGFR. Cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, penicillin (25 units/mL), and streptomycin (25 μg/mL; all from Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37°C.

Dasatinib and imatinib mesylate (STI-571; Novartis) were prepared as 20 mmol/L and dissolved in DMSO (Sigma-Aldrich). Dasatinib was used in all experimental settings at a dose of 100 nmol/L, as reported previously (16), and the concentration of DMSO used to deliver dasatinib had no effect on cell proliferation (data not shown). Original stock solutions of cisplatin (*cis*-diammine-dichloroplatinum; Pfizer) at a concentration of 0.5 mg/mL were stored at 4°C and freshly dissolved in culture medium before use.

Cell Viability Assay

Cells were plated in 96-well plates for 24 h and then treated with increasing concentrations of cisplatin (range, 1-50 μmol/L) for 48 h in the presence or absence of dasatinib 100 nmol/L. A MTT (Sigma-Aldrich) assay was done according to the manufacturer's instructions. Absorbance was measured at 590 nm with a microplate reader.

Apoptosis Assay

Cells were seeded in 6-well plates at appropriate density and then treated for 48 h with 5, 10, or 20 μmol/L cisplatin in the presence or absence of 100 nmol/L dasatinib. Cells were then harvested and stained with Annexin V and propidium iodide and then analyzed on a cytofluorimeter by FACScan (BD Biosciences). Propidium iodide–positive cells were considered as necrotic, Annexin V–positive/propidium iodide–negative as apoptotic cells, and the double-negative cells as alive. All untreated controls ranged from 5% to 10% of apoptotic cells. Results are expressed as ratios between the percentage of apoptotic/nonapoptotic cells.

Western Immunoblot Analysis

After treatments, cells were lysed in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP-40, 10 mmol/L glycerol, 5 mmol/L EDTA, and 0.5% sodium deoxycholate and the lysates were treated with a mixture of protease and tyrosine phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate). Proteins were separated on 8% SDS-PAGE and blotted onto polyvinylidene fluoride membrane (Hybond-P; Amersham). Filters were incubated with the following monoclonal antibodies against β-actin (Santa Cruz Biotechnology), pan-Src, and phospho^{Y416}-Src (Cell Signaling). Detection was done using enhanced chemiluminescence kit (Pierce).

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RNA Isolation and cDNA Synthesis from Cells and Fresh Snap-Frozen NSCLC Specimens

Total RNA was isolated from lung specimens using the RNeasy 96 kit implemented on Biorobot 8000 (Qiagen) according to the manufacturer's instructions. RNA was extracted and retrotranscribed to cDNA as described previously (20). Total RNA was isolated from cell lines with Qiazol lysis reagent (Qiagen) according to the manufacturer's instructions.

Quantitative Real-time PCR

Relative cDNA quantitation for each gene was done by a fluorescence-based real-time detection method (ABI PRISM HT7900; Applied Biosystems). The sequences of the primers and probes used for excision repair cross-complementing 1 (ERCC1), ribonucleotide reductase subunit M1 (RRM1), topoisomerase IIα (TopoII-α), thymidylate synthase (TS), polymerase η (Pol η), and β-actin (reference gene) have been published previously (20–22). E2F transcription factor 1 (E2F1) primers and probe were designed according to the Ref. Seq. NM_005225 and sequences were as follows: forward 5′-CTCCAAGCCGTGGACTCTT-3′, reverse 5′-ACATCGATCGGGCCTTGT-3′, and probe 5′-CGGAGAACTTTCAGATCTCCCTTAAGAGCA-3′. Src primers and probe were designed according to the Ref. Seq. NM_005417 and sequences were forward 5′-CCTCGTGCGAGAAAGTGAG-3′, reverse 5′-TGGCGTTGTCGAAGTCAG-3′, and probe 5′-CCACGAAAGGTGCCTACTGCCTCTC-3′. All primers and probes were intron-spanning to avoid genomic DNA contamination and validation process was done as described previously (23). Primers and probe for breast cancer 1 (BRCA1) gene were purchased as "Assay-on-Demand" (Applied Biosystems). PCR mixture and cycling conditions were as described previously (20).

Patients and Samples

Fresh snap-frozen surgical specimens of both tumor and corresponding normal lung tissues of 44 NSCLC patients completely resected between 2003 and 2004 at the San Luigi Hospital were consecutively collected. Patients' characteristics were as follows: median age of 63 years (range, 41-82), 86% were male, 15 had squamous cell carcinoma, 28 adenocarcinoma, and 1 large cell carcinoma, 84% had postsurgical stage I or II, and median survival of 56 months, with a minimum follow-up time of 4 years. None of the patients received preoperative or postoperative treatments with chemotherapy and/or radiotherapy mainly based on the negative outcomes of a large phase III study done at that time in Europe (24). All cases were reviewed and classified according to the WHO classification by one of the investigators (M.P.). All patients carried wild-type EGFR gene as reported previously (19). The study was approved by the institutional review board of our University Hospital.

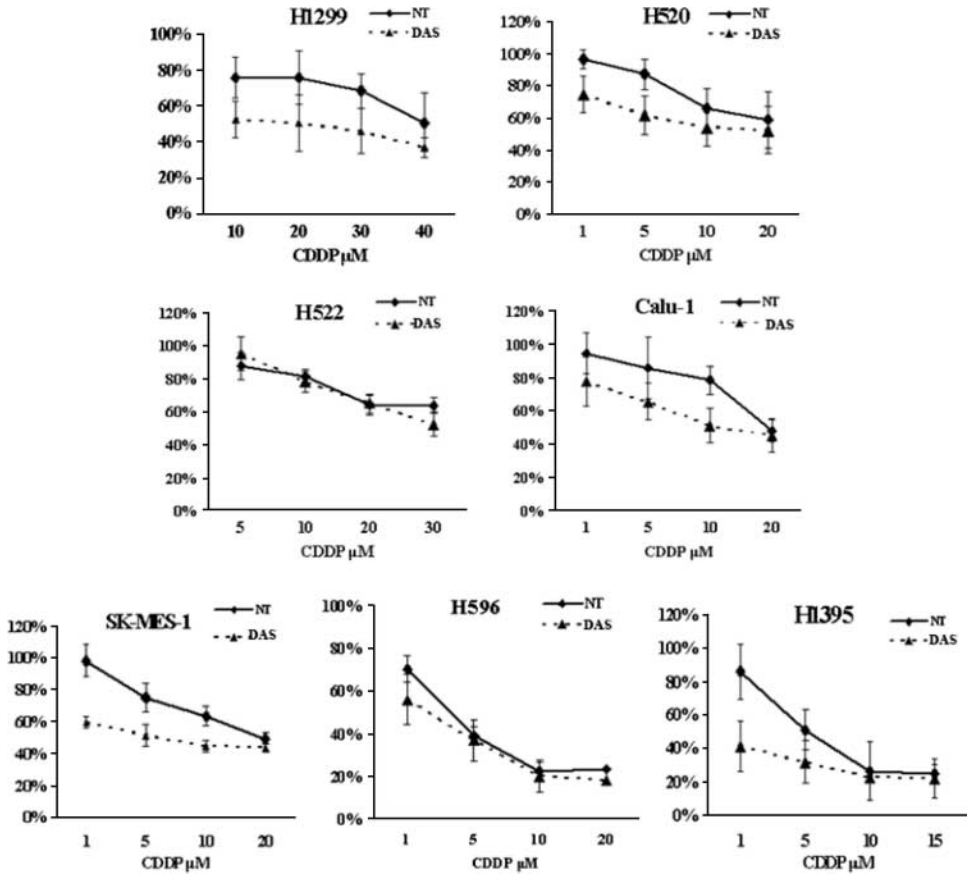

Figure 2. Evaluation of cell viability after combined drug treatment measured by MTT assay. Increasing dose of cisplatin (*CDDP*) alone (*continuous line*) or in combination with 100 nmol/L dasatinib (*dashed line*) was administered in NSCLC cell lines for 48 h. *Bars,* SD values of at least eight replicates of MTT measurements.

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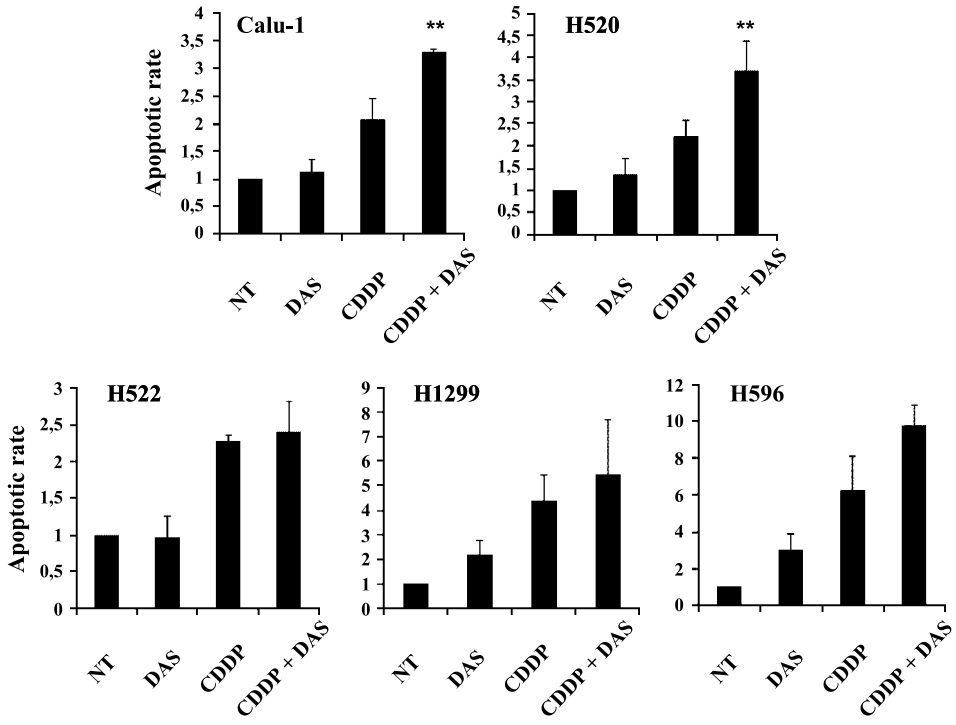

Figure 3. Evaluation of apoptosis rate measured by Annexin V/propidium iodide staining. NSCLC cell lines were treated for 48 h with cisplatin, dasatinib, and cisplatin/dasatinib combination. Cisplatin concentrations were 5 μmol/L for H596, 10 μmol/L for Calu-1, H520, and H522, and 30 μmol/L for H1299. *Asterisk,* significantly higher apoptotic rate in dasatinib/cisplatin-treated cells compared with those treated with cisplatin alone.

Statistical Analyses

In cell line experiments, to assess the drug effects on cell proliferation, the *t* test was done using the GraphPad software 5.0. In Western blot experiments, integrated densitometry data were obtained by the ImageJ software. In the transcriptional regulation assay, ΔCt values and SDs were calculated according to User's Bulletin #2 (Applied Biosystems). To test differential Src mRNA expression between tumors and corresponding normal tissues, the ΔΔCt method was used and significant overexpression was defined when $2^{-\Delta\Delta Ct}$ values were >2. Relative Src levels in tumors and normal lung tissues were estimated with the ΔCt method and normalized by subtracting all ΔCt values by the highest and converted in a linear scale. To test differential Src expression in the overall population of tumor versus normal lung and to test significant associations with clinicopathologic variables, the Mann-Whitney *U* and Kruskal-Wallis tests were used. Univariate analysis of survival was done with the Kaplan-Meier method dividing patients according to different cutoffs of Src levels, and the significance was estimated with the log-rank test. The correlation between the level of Src upregulation and transcript expression in tumor samples was tested by Spearman's correlation test. Statistical significance was set at $P = 0.05$.

Results

Dasatinib Treatment Impairs Src Activity with Modest Effects on the Growth of NSCLC Cell Lines

The total amount of Src kinase (c-Src) protein in the cell lines was quantified by Western blot analysis. As shown in Fig. 1A, basal Src protein levels were slightly different: H522, Calu-1, and H1395 had the lowest and H520 and SK-MES-1 had the highest expression levels, respectively. In quantitative real-time PCR experiments, the amount of Src transcript was not significantly associated with protein levels, although a trend toward significance was observed ($P = 0.08$; data not shown).

Src kinase was found phosphorylated (at Y416) in all cell lines (Fig. 1B). The effect of dasatinib in the inhibition of Src activity and the consequent cell growth block was tested. The exposure to 100 nmol/L dasatinib for 48 h produced a decrease of its phosphorylation level >80% in the majority of the cells (H1395, SK-MES-1, Calu-1, and H520), of 75% in H596 and 52% and 44% in H1299 and H522, respectively (all densitometric data). After treatment, the growth of 4 of 7 cell lines was significantly inhibited (Fig. 1C). The extent of growth inhibition was not significantly associated with Src basal expression (at both mRNA and protein levels) nor to its phosphorylation level. In addition, 24 and 48 h treatment with dasatinib did not modify Src transcript and protein levels (data not shown).

Dasatinib Enhances Cisplatin-Induced Cytotoxicity

The effect of the concomitant administration of dasatinib and cisplatin in the cell lines was evaluated by means of cell viability and apoptosis assays after 48 h of treatment. The results of MTT assays showed that the majority of cell lines (5 of 7) had an enhanced sensitivity to cisplatin in the presence of dasatinib at a dose of 100 nmol/L, whereas little and no differences were observed in H596 and H522 cell lines, respectively (Fig. 2). The observed effects were not related to basal cell sensitivity to cisplatin or dasatinib, and no

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changes were observed when different schedules of drug combinations (simultaneous or subsequent) were tested (data not shown).

Furthermore, although dasatinib showed modest proapoptotic effects when administered as a single agent (the highest effect was observed in H596 cells), a higher rate of apoptotic cells was found with the exposition to the dasatinib/cisplatin combination compared with that observed with cisplatin alone (Fig. 3). The two squamous carcinoma lines Calu-1 and H520 (Figs. 3 and 4A, respectively) showed significantly stronger responses when the drugs were delivered in combination. Similar to cytotoxicity assays, this effect was not detected in the H522. Figure 4A shows light microscopy pictures of the H520 cells in different conditions: both dasatinib and cisplatin, administered at 100 nmol/L and 10 μmol/L doses, respectively, were unable to affect either survival or alter cell morphology, but a strong cytotoxic effect was observed when the two drugs were concomitantly added: the cells rounded-up, losing their original shapes, and as a consequence, their adhesion (either cell-cell or cell-plate) properties were impaired. The cisplatin-induced cell death at 10 μmol/L dose in the presence of dasatinib was found comparable with that induced by cisplatin alone at 20 μmol/L dose (data not shown). The status of Src phosphorylation was evaluated under these experimental conditions. Figure 4B shows that H520 cells undergo an increase in Src phosphorylation levels after cisplatin exposure at 10 μmol/L. Such increase was quantified with integrated densitometry (as the ratio of phospho^{Y416}-Src/pan-Src; Fig. 4B, *bottom*) as 42% compared with untreated cells. A 70% increase in Src phosphorylation was observed after the treatment with 20 μmol/L, but this concentration was already highly cytotoxic to observe differences with combined treatments. Src phosphorylation, both endogenous and induced by cisplatin, was found impaired by dasatinib treatment (Fig. 4B).

To confirm the specificity of Src inhibition in the observed effects, H520 cells were treated in parallel with imatinib mesylate. Imatinib is a drug that, similar to dasatinib, targets Bcr/Abl, c-kit, and platelet-derived growth factor receptor but has a lower inhibiting activity on Src family kinases. Indeed, scant effects on Src inhibition, cell growth, and cisplatin-induced death were observed at concentration up to 5 μmol/L after 24 to 48 h of treatment (Supplementary Fig. S1).

Finally, a sequence analysis of the dasatinib-binding sites in Src gene done in H520 and H522 cells revealed no differences in terms of nucleotide sequence (data not shown).

Dasatinib Inhibits Cisplatin-Induced mRNA Transcription of DNA Repair and Synthesis Genes

The effect of dasatinib alone or in combination with cisplatin on the expression of gene potentially involved in the resistance to cisplatin was investigated by means of quantitative real-time PCR in all cell lines. A panel of seven genes belonging to DNA repair and synthesis pathways was selected, including ERCC1, TS, Pol η, BRCA1, TopoII-α, RRM1, and E2F1. The results of the experiments indicated that the transcript levels of many of these genes were upmodulated by the cancer cells after cisplatin treatment for 24 h (Fig. 5). By contrast, the presence of dasatinib blocked the cisplatin-induced mRNAs transcription. Again, this effect was evident at different levels of intensity in the different cells tested, being more intense in H520, whereas only minimal changes were observed in H522.

Src mRNA Is Frequently Upregulated in NSCLC Specimens but Lacks Prognostic Effect

Src transcript expression was tested in NSCLC samples and the corresponding normal lung tissues. The results showed a significant upregulation of Src levels, being 2.55 (from 1.4 to 5.9) the median level in nonneoplastic lungs

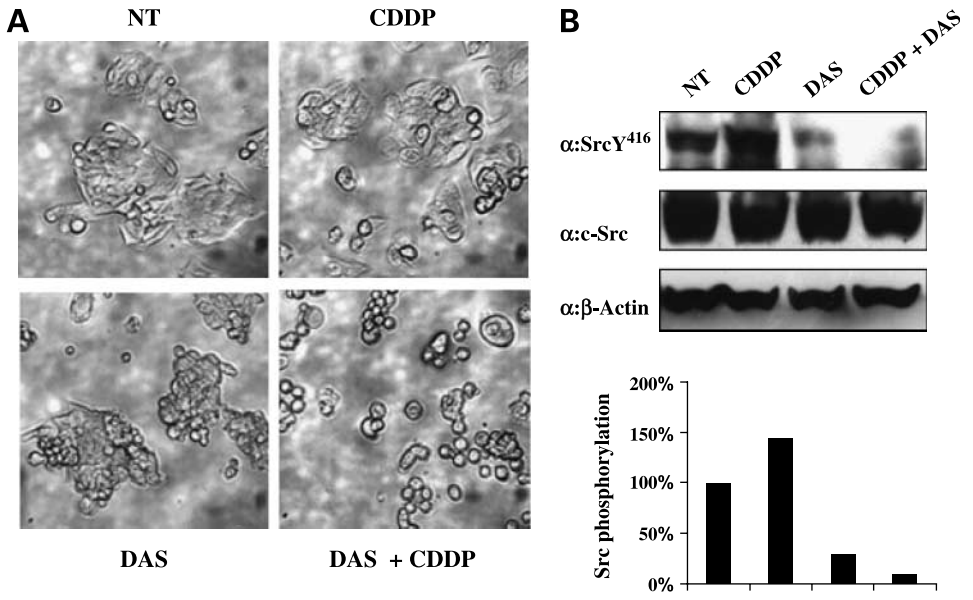

Figure 4. **A,** effect of combined dasatinib and cisplatin treatment in lung squamous carcinoma cell H520. Cells were treated for 48 h at the indicated concentration. Light microscopy pictures were taken at ×100 magnification. **B,** whole-cell lysates of H520 treated for 24 h with cisplatin, dasatinib, and cisplatin/dasatinib combinations at the indicated doses were immunoblotted with anti-phospho-Src, pan-Src, and β-actin (loading control). *Bottom,* histograms show integrated densitometry data representing phosphorylation levels (phospho^{Y416}-Src/pan-Src ratio).

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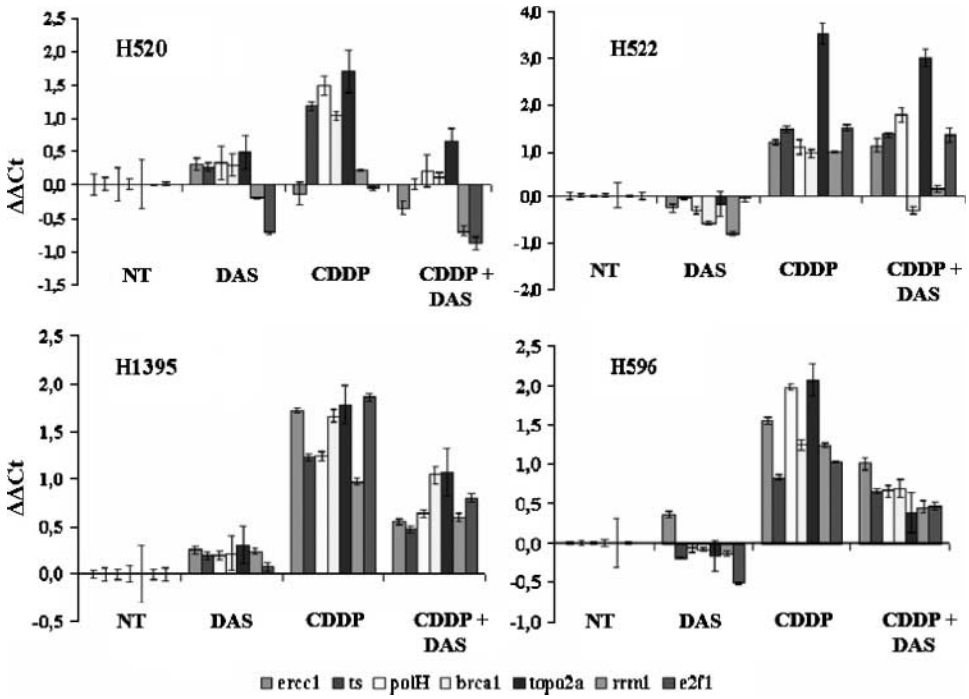

Figure 5. Effects of dasatinib on cisplatin-induced mRNA transcription of seven genes involved in DNA repair and synthesis. Histograms represent ΔΔCt ± SD values of at least three replicates.

compared with 3.51 (from 1 to 9.2; all unit-less ratios) for the tumor tissues ($P = 0.03$; Fig. 6A). The level of Src upregulation (expressed as tumor/normal ratios) was significantly correlated with the levels of expression in the tumors ($R_S = 0.76$; $P < 0.0001$; Fig. 6B). In addition, by the analysis of individual tumor/normal ratios adopting a cutoff value >2-fold for Src upregulation, 10 of 44 (23%) patients had a significant upregulation of Src, whereas, in 16 (36%) patients, a ratio between 0.5 and 2 was found, and only 2 (4.5%) patients had a ratio <0.5-fold, that is, significantly downregulated (Fig. 6C). No correlation between Src relative levels in tumoral tissues or between Src upregulation ratios and patients' characteristics such as gender, age, histotype, tumor size, lymph node status, clinical stage, and survival was found (Supplementary Table S1).

Discussion

The present study shows that (*a*) Src inhibition by dasatinib could be effective in tumor growth control of lung cancer cells; (*b*) dasatinib can enhance cisplatin cytotoxicity, likely impairing the cisplatin-induced modulation of DNA repair and synthesis genes; and (*c*) a subset of NSCLC have an increased Src kinase mRNA expression in tumors.

Src kinase is emerging as a potential critical target to be preclinically investigated in NSCLC (3, 4) and the present study tested the effects of Src inhibition by dasatinib in a panel of NSCLC cell lines. Consistent with data reported previously, treatment with dasatinib at a relatively low concentration impaired Src phosphorylation in the majority of cell lines tested (Fig. 1B), showing a modest effect on cell survival apparently not correlated with both Src basal level and phosphorylation, similar to previous findings in different lung cancer cell lines (17). Recent reports have related the sensitivity to dasatinib with the presence of EGFR-activating mutations (17), but no data on the combined administration of dasatinib and cytotoxic agents were available. A higher cytotoxicity and/or apoptotic rate was observed in all cell lines when cisplatin and dasatinib were simultaneously administered, except for the H522 cells. This unresponsiveness could be due to the residual Src activity observed after dasatinib treatment, and the possible mechanisms of resistance involved are worth of further investigations. A preclinical study has recently indicated a significant correlation between the level of inhibition of Src phosphorylation by dasatinib in peripheral blood mononuclear cells and that observed in tumors in a mouse xenograft model (25), and this could represent a potential strategy for the selection of the patients who may benefit from the treatment. To investigate one of the possible mechanisms of the differential responsiveness, the nucleotide sequences in the ATP-binding pocket of Src of the "responsive" H520 and the "unresponsive" H522 cells were analyzed, but no difference was detected. In the H520 cells, which showed remarkable cytotoxic effects when the two drugs were delivered in combination (compared with controls treated with cisplatin alone) in both viability and apoptosis assays, the effective block of cisplatin-induced Src phosphorylation on catalytic site was shown. This is in line with recent *in vitro* findings reporting that EGFR phosphorylation following chemotherapy could determine the response to combined gefitinib and chemotherapy treatment (26). Altogether, these data contribute to highlight the mechanisms of the acquired resistance to cytotoxic drugs as

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possible targets of the new class of anticancer agents (27) and support future investigations addressed to test the correlation between Src phosphorylation/activation levels and tumor chemosensitivity.

Src kinase inhibition by means of RNA interference or specific drugs has already been shown to enhance *in vitro* toxicity of cancer cells to different agents (28, 29), and although in the present article, for most of the responsive cell lines, the effects of the cisplatin/dasatinib combinations seemed to be additive rather than synergistic (Fig. 2), further studies both *in vitro* and *in vivo* should be addressed to clarify the nature of the interaction between these two drugs in NSCLC, similar to what recently reported in colorectal cancer model (30). Dasatinib targets other proteins with tyrosine kinase activity such as Bcr/Abl, c-kit, and platelet-derived growth factor receptor (12). Thus, to test the specificity of Src inhibition in the phenomena observed in H520, all the experiments were done in parallel with imatinib mesylate, a drug mainly targeting Bcr/Abl that also inhibits c-kit and platelet-derived growth factor receptor but with low efficacy on Src family kinases (31). The results showed that imatinib had minimal effects on Src inhibition and on cisplatin-induced death in H520 even at relatively high concentrations able to inhibit other kinases (16). Therefore, the effects of dasatinib here presented seem specifically attributable to the inhibition of Src family kinases, although the precise family member involved remains to be determined.

c-Src kinase has been implicated in the resistance to various anticancer treatments in different *in vitro* experimental settings (28, 29, 32) and the transfection of cancer cells with v-Src showed to increase the repair of cisplatin-DNA interstrand cross-links (10). Similarly, the results of the present study reported lower expression levels of a panel of genes belonging to DNA repair pathways in dasatinib/cisplatin compared with cells treated with cisplatin alone. Whether this effect was due to the effective block of Src activity or to the higher cytotoxicity of the combined treatment remains unclear, but these findings are intriguing because different retrospective studies have shown the mRNA overexpression of these genes as strongly associated with the resistance of NSCLC patients to platinum-based chemotherapy (22, 33–35).

Additionally, the present study assessed the regulation of Src mRNA transcripts in surgically resected NSCLC and in the corresponding normal lung tissues. In 23% of cases, we detected a significant upregulation of mRNA levels compared with 4.5% of downregulated samples. Although these data are based on the quantification of mRNA, they are consistent with those reported previously about Src protein quantification, which showed higher levels in tumors, as well as a positive correlation with enzymatic activity (5). However, neither the upregulation nor the overexpression of Src in tumors had an effect on patients' survival. These results agree with a study carried out on a large case-series of NSCLC patients ($n = 370$), which evaluated the activity of Src family kinase proteins using a phospho-specific antibody in paraffin-embedded tissues. In 33% of cases, Src activation was detected, but no prognostic association was detected (6). Although there is no effect of Src activity and expression on NSCLC patients' survival, the identification of a subgroup of patients with transcriptional upregulation

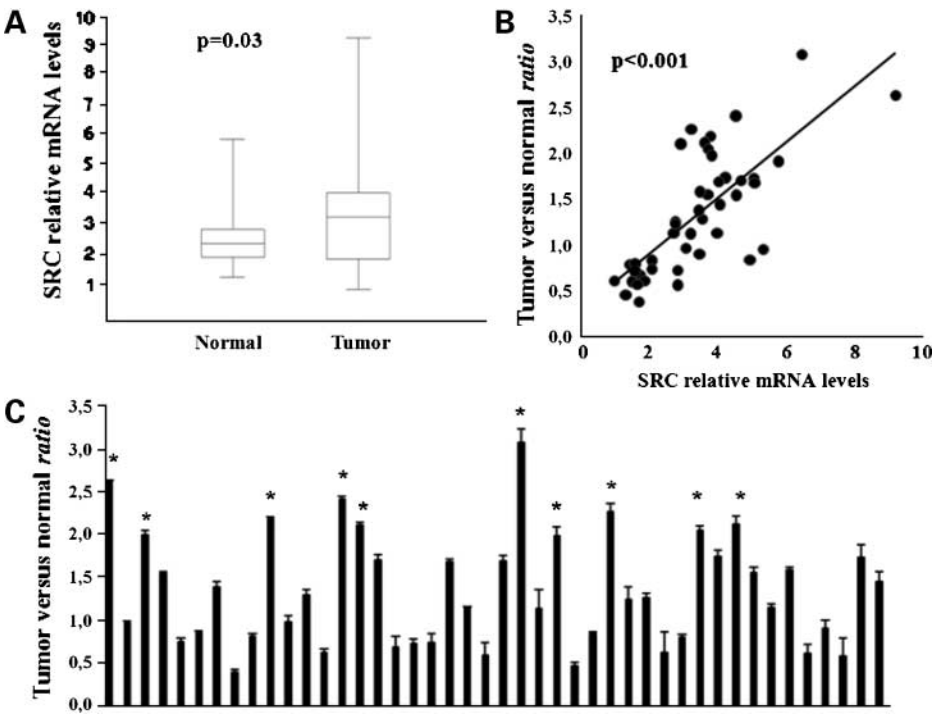

Figure 6. **A,** general upregulation of Src transcript levels in 44 NSCLC fresh-frozen surgical specimens compared with the corresponding normal lung tissues. *P* (Mann-Whitney *U* test). **B,** correlation between the level of Src upregulation expressed as tumor/normal ratios and Src transcript levels in tumor samples. *P* (Spearman's rank correlation test). **C,** individual ratios between Src mRNA levels in tumor compared with normal lung. *Bars,* SD of triplicate measurements. *Asterisk,* cases with at least 2-fold Src transcript upregulation.

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of a kinase involved in many aspects of tumorigenesis and linked to chemoresistance is worth of further consideration. Future studies should be addressed to elucidate the molecular pathways leading to this aberrant transcription/activation in NSCLC or should evaluate the prognostic effect of Src expression levels/activity in patients treated with cytotoxic regimens. Moreover, both the increased activity and the upregulation of its expression in tumors could be used as a biomarker to drive the selection of patients to be treated with Src-targeting agents. The results of a recent study focused on the identification of lesions associated with therapeutically relevant oncogenic pathways have clearly shown that the cells with the amplification of dasatinib target genes were more sensitive to the drug in both *in vitro* and *in vivo* settings (36). Because the wide majority of NSCLC patients are diagnoses in advanced, unresectable stages, and no adjacent normal lung tissues could be used to measure the levels of Src transcription, the high correlation between transcriptional regulation and Src expression in tumors here reported (Fig. 6B) could be useful for future clinical investigations based on the sole assessment of tumor specimens.

In conclusion, the results of this study further support the hypothesis of an integration of cytotoxic chemotherapy with inhibitors of specific molecular pathways in the attempt to ameliorate the outcomes of NSCLC patients. Future studies evaluating the efficacy of Src-inhibiting agents in combination with cisplatin are clearly warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Pfister DG, Johnson DH, Azzoli CG, et al. American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. J Clin Oncol 2004;22:330–53.

2. Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. BMJ 1995;311:899–909.

3. Giaccone G, Zucali PA. Src as a potential therapeutic target in non-small-cell lung cancer. Ann Oncol 2008;19:1219–23.

4. Sarries C, Haura EB, Roig B, et al. Pharmacogenomic strategies for developing customized chemotherapy in non-small cell lung cancer. Pharmacogenomics 2002;3:763–80.

5. Masaki T, Igarashi K, Tokuda M, et al. pp60c-src activation in lung adenocarcinoma. Eur J Cancer 2003;39:1447–55.

6. Zhang J, Kalyankrishna S, Wislez M, et al. SRC-family kinases are activated in non-small cell lung cancer and promote the survival of epidermal growth factor receptor-dependent cell lines. Am J Pathol 2007;170:366–76.

7. Allgayer H, Boyd DD, Heiss MM, Abdalla EK, Curley SA, Gallick GE. Activation of Src kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. Cancer 2002;94:344–51.

8. Wilson GR, Cramer A, Welman A, et al. Activated c-SRC in ductal carcinoma *in situ* correlates with high tumour grade, high proliferation and HER2 positivity. Br J Cancer 2006;95:1410–4.

9. Benhar M, Engelberg D, Levitzki A. Cisplatin-induced activation of the EGF receptor. Oncogene 2002;21:8723–31.

10. Masumoto N, Nakano S, Fujishima H, Kohno K, Niho Y. v-src induces cisplatin resistance by increasing the repair of cisplatin-DNA interstrand cross-links in human gallbladder adenocarcinoma cells. Int J Cancer 1999;80:731–7.

11. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–41.

12. Lombardo LJ, Lee FY, Chen P, et al. Discovery of *N*-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J Med Chem 2004;47:6658–61.

13. Trevino JG, Summy JM, Lesslie DP, et al. Inhibition of SRC expression and activity inhibits tumor progression and metastasis of human pancreatic adenocarcinoma cells in an orthotopic nude mouse model. Am J Pathol 2006;168:962–72.

14. Tsao AS, He D, Saigal B, et al. Inhibition of c-Src expression and activation in malignant pleural mesothelioma tissues leads to apoptosis, cell cycle arrest, and decreased migration and invasion. Mol Cancer Ther 2007;6:1962–72.

15. Shor AC, Keschman EA, Lee FY, et al. Dasatinib inhibits migration and invasion in diverse human sarcoma cell lines and induces apoptosis in bone sarcoma cells dependent on SRC kinase for survival. Cancer Res 2007;67:2800–8.

16. Johnson FM, Saigal B, Talpaz M, Donato NJ. Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. Clin Cancer Res 2005;11:6924–32.

17. Song L, Morris M, Bagui T, Lee FY, Jove R, Haura EB. Dasatinib (BMS-354825) selectively induces apoptosis in lung cancer cells dependent on epidermal growth factor receptor signaling for survival. Cancer Res 2006;66:5542–8.

18. Marchetti A, Martella C, Felicioni L, et al. EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 2005;23:857–65.

19. Volante M, Saviozzi S, Rapa I, et al. Epidermal growth factor ligand/receptor loop and downstream signaling activation pattern in completely resected non small cell lung cancer. Cancer 2007;110:1321–8.

20. Ceppi P, Volante M, Saviozzi S, et al. Squamous cell carcinoma of the lung compared with other histotypes shows higher messenger RNA and protein levels for thymidylate synthase. Cancer 2006;107:1589–96.

21. Ceppi P, Longo M, Volante M, et al. Excision repair cross complementing-1 and topoisomerase IIα gene expression in small-cell lung cancer patients treated with platinum and etoposide: a retrospective study. J Thorac Oncol 2008;3:583–9.

22. Ceppi P, Novello S, Cambieri A, et al. Polymerase η mRNA expression predicts survival of non-small cell lung cancer patients treated with platinum-based chemotherapy. Clin Cancer Res 2009;15:1039–45.

23. Rapa I, Ceppi P, Bollito E, et al. Human ASH1 expression in prostate cancer with neuroendocrine differentiation. Mod Pathol 2008;21:700–7.

24. Scagliotti GV, Fossati R, Torri V, et al. Randomized study of adjuvant chemotherapy for completely resected stage I, II, or IIIA non-small-cell Lung cancer. J Natl Cancer Inst 2003;95:1453–61.

25. Serrels A, Macpherson IR, Evans TR, et al. Identification of potential biomarkers for measuring inhibition of Src kinase activity in colon cancer cells following treatment with dasatinib. Mol Cancer Ther 2006;5:3014–22.

26. Van Schaeybroeck S, Kyula J, Kelly DM, et al. Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. Mol Cancer Ther 2006;5:1154–65.

27. Jackman AL, Kaye S, Workman P. The combination of cytotoxic and molecularly targeted therapies—can it be done? Drug Discov Today 2004;1:445–54.

28. Chen T, Pengetnze Y, Taylor CC. Src inhibition enhances paclitaxel cytotoxicity in ovarian cancer cells by caspase-9-independent activation of caspase-3. Mol Cancer Ther 2005;4:217–24.

29. Ischenko I, Camaj P, Seeliger H, et al. Inhibition of Src tyrosine kinase reverts chemoresistance toward 5-fluorouracil in human pancreatic carcinoma cells: an involvement of epidermal growth factor receptor signaling. Oncogene 2008;27:7212–22.

30. Kopetz S, Lesslie DP, Dallas NA, et al. Synergistic activity of the SRC family kinase inhibitor dasatinib and oxaliplatin in colon carcinoma cells is mediated by oxidative stress. Cancer Res 2009;69:3842–9.

31. Deininger M, Buchdunger E, Druker BJ. The development of imatinib

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as a therapeutic agent for chronic myeloid leukaemia. Blood 2005;105:2640–53.

32. Pengetnze Y, Steed M, Roby KF, Terranova PF, Taylor CC. Src tyrosine kinase promotes survival and resistance to chemotherapeutics in a mouse ovarian cancer cell line. Biochem Biophys Res Commun 2003;309:377–83.

33. Ceppi P, Volante M, Novello S, et al. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. Ann Oncol 2006;17:1818–25.

34. Takizawa M, Kawakami K, Obata T, et al. *In vitro* sensitivity to platinum-derived drugs is associated with expression of thymidylate synthase and dihydropyrimidine dehydrogenase in human lung cancer. Oncol Rep 2006;15:1533–9.

35. Taron M, Rosell R, Felip E, et al. BRCA1 mRNA expression levels as an indicator of chemoresistance in lung cancer. Hum Mol Genet 2004;13:2443–9.

36. Sos ML, Michel K, Zander T, et al. Predicting drug susceptibility of non-small cell lung cancers based on genetic lesions. J Clin Invest 2009;119:1727–40.