Combined Treatment with Silibinin and Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors Overcomes Drug Resistance Caused by T790M Mutation

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Abstract

Although epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) produce an initially dramatic response in lung cancer patients harboring a mutation in the EGFR gene, development of acquired resistance is almost inevitable. A secondary mutation of threonine 790 (T790M) is associated with approximately half of the cases of acquired resistance. This study investigated whether the addition of silibinin to therapy with gefitinib or erlotinib could overcome T790M-mediated drug resistance considering that silibinin has various antitumor effects, including EGFR modulation. Silibinin selectively reduced the activity of the EGFR family (EGFR, ErbB2, and ErbB3) through the inhibition of receptor dimerization in lung cancer cells with EGFR mutations, but not in those harboring the wild type. In primary and acquired resistant cells with T790M, addition of silibinin enhanced the ability of EGFR-TKIs to downregulate EGFR signals and to inhibit cell growth. Similarly, the combination of silibinin and erlotinib effectively suppressed tumor growth in erlotinib resistance-bearing PC-9 xenografts. The results indicate that the addition of silibinin to EGFR-TKIs is a promising strategy to overcome T790M-mediated drug resistance.

Introduction

The efficacy of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) has been demonstrated in treatment of non-small cell lung cancer (NSCLC). Although sometimes they could induce remarkable or durable response, all patients will ultimately develop progressive tumor growth while receiving gefitinib or erlotinib treatment limiting the median progression-free survival to 9 to 13 months (1). Two different mechanisms of acquired resistance to the EGFR-TKIs in EGFR-mutant NSCLC patients have been identified. These factors are secondary EGFR mutation in threonine 790 (T790M) and MET proto-oncogene (MET) amplification, and are found in approximately 70% of those with acquired resistance (1). Of these 2 mechanisms, T790M mutation is evident in up to 50% of cases and has also been found coincidently with MET amplification in a smaller number of cases (2). Furthermore, the T790M mutation has been reported in a small fraction of tumor cells before drug treatment (3, 4) suggesting that the mutation could be associated with acquired and primary resistance to EGFR-TKIs.

Threonine 790 is a gatekeeper for the ATP-binding pocket in EGFR. A previous report showed that an amino acid substitution to methionine leads to drug resistance by increasing the affinity for ATP (3). Second generation, irreversible EGFR-TKIs may be able to overcome T790M-mediated resistance by virtue of their irreversible mode of binding (5, 6). However, until now, the efficacy of such inhibitors has not been verified in clinical studies, and it remains unclear whether such inhibitors can be active to overcome T790M-mediated resistance at a pharmacological dose (7). Thus, there is a need to explore alternative therapeutic methods to overcome these resistance mechanisms.

Silibinin is a flavonolignan and the major active constituent of silymarin, which is a complex mixture of flavonolignans and polyphenols extracted from milk thistle seeds. Silymarin possesses hepatoprotective properties and has been widely used for decades to treat patients with liver disease (8). Recent studies have shown that silibinin and silymarin inhibits growth of many cancers including breast, lung, colon, pancreas, and prostate cancer cells (9–12). The anticancer efficacy of silibinin is associated with cell cycle arrest, apoptosis, and antiangiogenesis (13). It also inhibits a variety of growth signal

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receptors including EGFR (14), insulin-like growth factor receptor 1 (IGFR-1; 15) and vascular endothelial growth factor receptor 1 (16). Thus, it is reasonable to think that silibinin would enhance the activity of EGFR-TKIs leading to apoptosis and growth inhibition of resistant cells in which EGFR-TKIs have failed to completely abolish EGFR activity. This study investigated the combined effect of silibinin and EGFR-TKIs to overcome the acquired resistance to EGFR-TKIs caused by T790M mutation.

Materials and Methods

Cell culture and reagents

The human NSCLC lines (A549, H460, H1299, H1975, and HCC827) and 293T cells were purchased from the American Type Culture Collection and characterized by isozyme analysis or short-tandem repeat DNA profiling. LK2 cells were obtained from the Japanese Collection Research Resources Bank. Per the cell bank, LK2 cells were verified through short-tandem repeat DNA profiling. The PC-9 cell line was a kind gift from Dr. Kazuto Nishio (National Cancer Center Hospital, Tokyo, Japan) and has been previously characterized (17, 18). All cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) at 37°C in an atmosphere of 5% CO₂. Gefitinib and erlotinib (Fig. 1A) were kindly provided by AstraZeneca Korea and Roche Korea, respectively. The membrane was treated with antibody against p-EGFR, EGFR, ErbB2, ErbB3, p-Akt, Akt, and Erk (all from Santa Cruz Biotechnology), and p-ErbB2, p-ErbB3, p-Erk, and β-actin (all from Cell Signaling Technology) as the first antibody, and then the membrane was treated with horseradish peroxidase-conjugated secondary antibody. The membrane developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Western blot analysis

The lysates from cultured cells and xenograft tumors were prepared in non-denaturing lysis buffer (10 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.3 mmol/L phenylmethylsulfonyl fluoride; 0.2 mmol/L sodium orthovanadate; 0.5% NP40; and 5 U/mL aprotinin). Western blotting was done as described previously (20). The membrane was probed with antibody against p-EGFR, EGFR, ErbB2, ErbB3, p-Akt, Akt, and Erk (all from Santa Cruz Biotechnology), and p-ErbB2, p-ErbB3, p-Erk, and β-actin (all from Cell Signaling Technology) as the first antibody, and then the membrane was treated with horseradish peroxidase-conjugated secondary antibody. The membrane developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Assessment of EGFR dimerization

After treatment with 50 ng/mL EGF or 100 μmol/L silibinin, cells were washed twice with ice-cold phosphate buffered saline (PBS) and incubated on ice for 30 minutes with the 1 mg/mL BS3. The cross-linking reaction was terminated by adding 250 mmol/L glycine. The cells were lysed and equivalent amounts of protein were resolved by 6% SDS-PAGE. Dimerization of the EGFR was detected by Western blotting using an anti-EGFR antibody. For visualizing the dimerization of EGFR family, the proximity ligation assay was done according to the manufacturer’s protocol (Olink Bioscience). In brief, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and paraffin-embedded sections were prepared according the same protocol used in the immunohistochemistry. The primary antibodies were diluted as follows: EGFR mouse monoclonal antibody 1:200, EGFR rabbit polyclonal antibody 1:400, ErbB2 mouse monoclonal antibody 1:200, and ErbB3 rabbit polyclonal antibody 1:400. Dimerization of EGFR family was stained by in situ proximity ligation assay, and the number of in situ proximity ligation assay...
signals per cell was counted by semiautomated image analysis using the freeware software BlobFinder12 as previously described (21, 22). Images were taken using a LSM710 confocal laser scanning microscope (Carl Zeiss).

Three-dimensional growth assays

Cells were seeded on Growth Factor Reduced Matrigel (BD Biosciences) in wells of 24-well plates following a previously described protocol (23). After incubation for 24 hours, drugs were added into the medium. Photographs were taken after culturing the cells for 10 to 15 days.

Xenograft in vivo study

Female severe combined immunodeficiency (SCID) mice (17–20 g, 6-week old) were purchased from the Animal Resources Centre. Tumors were grown by implanting 5 × 10⁵ cells in Matrigel (BD Biosciences) into the mouse flanks. Treatment of 5 mice per group was started when the tumors had reached a volume of 50 to 100 mm³ with vehicle control, erlotinib (100 mg/kg, 5 days a week), silibinin (200 mg/kg, 5 days a week), or erlotinib plus silibinin. Both drugs were orally administered. Treatment was stopped after 24 days and mice were followed up for tumor recurrence. For tumor size measurements, the length (L) and width (W) of the tumor were measured with calipers, and tumor volume (TV) was calculated as TV = (L × W²)/2. Positron emission tomography (PET) scans were done before and after administration of drugs (0 and 7 days).

PET image acquisition

¹⁸F-FDG PET imaging was done with a dedicated small animal PET scanner (MicroPET R4 scanner; Concorde Microsystems) using LSO crystals, 350 to 750 keV energy window, and timing windows of 6 ns. PET imaging was started 60 minutes after the administration of 7.4 MBq/0.1 mL of ¹⁸F-FDG via tail vein injection. Mice were anesthetized with 2% isoflurane in 100% oxygen (Forane solution; ChoongWae Pharma) for uptake and scanning. After position scanning, each mouse was scanned for 20 minutes to obtain a static image. The acquired 3-dimensional (3-D) emission list-mode data were reconstructed temporally framed sinograms using Fourier 3-rebinning (FORE) and ordered subsets expectation maximization (OSEM2D) reconstruction algorithm with 4 iterations. PET values were first converted to standardized uptake values using the MicroPET ASIPro software (Concorde Microsystems). Image visualization and analysis were done using the nonproprietary Amide’s a Medical Image Data Examiner (AMIDE) software.

Immunohistochemistry analysis

Three tumor-bearing mice were used in each group for immunohistochemical studies. Immunohistochemical staining was done using specific primary antibody (Ki-67; DakoCytomation), the EnVision Plus staining kit (DakoCytomation) and APO-Direct terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit (Millipore) according to the supplier’s instructions. Quantitative analysis of section staining was done by counting immunopositive cells in 5 arbitrarily selected fields at ×400 magnification.

Results

Silibinin inhibits EGFR activity in cells with EGFR mutations

To investigate whether silibinin affected EGFR activity in NSCLC cells, cells were treated with 100 µmol/L silibinin in a time-dependent manner. As shown in Figure 2A and B, silibinin inhibited EGFR activity in cells harboring EGFR mutations but not in those harboring wild type, although the effect of silibinin on EGFR was observed in cells with wild-type EGFR at a dose exceeding 300 µmol/L (Supplementary Fig. S1). The effect of silibinin on EGFR activity in cells transfected with wild or mutant EGFR (delE746-A750) was further assessed using LK2 NSCLC cells and 293T human embryonic kidney cells that express very low levels of EGFR to limit endogenous EGFR expression. Consistent with above results, silibinin inhibited EGFR activity only in cells transfected with mutant EGFR (Fig. 2C). Moreover, silibinin affected the activity of other members of the EGFR family, inhibiting ErbB2 and ErbB3 activity in a ligand-dependent manner (Fig. 2D). Taken together, these results indicated that silibinin could effectively inhibit the activities of EGFR and the other EGFR family members in cells with EGFR mutations.

Silibinin inhibits homo- and heterodimerization of EGFR in cells harboring EGFR mutations

It was previously shown that silibinin is able to inhibit EGF binding to EGFR in vitro (14). Silibinin can inhibit EGFR activity through alternative mechanisms in cells with EGFR mutations because kinase domain mutations in the EGFR gene lead to constitutive ligand-independent stimulation of the tyrosine kinase activity (24). To determine how silibinin inhibits the activity of EGFR family in cells with EGFR mutations, the affinity of silibinin to the ATP-binding pocket of the EGFR tyrosine kinase domain was measured by using a direct binding assay (3). However, silibinin did not have any affinity for the ATP-binding pocket of wild- and mutant-type EGFR tyrosine kinase (data not shown). The next experiment examined the effects of silibinin on the dimerization of EGFR by doing cross-linking experiments as previous described (25). As shown in Figure 3A, the formation of EGFR dimers was observed in all cells under conditions with or without serum, and was markedly increased by the addition of EGF. Silibinin treatment inhibited EGFR dimerization under all conditions including complete serum, serum starvation, and EGF stimulation. It was further examined whether silibinin could interfere with the homodimerization of EGFR or the heterodimerization between members of the EGFR family. Proximity ligation confirmed the presence of homo- (EGFR/EGFR)
and heterodimerization (EGFR/ErbB2 and EGFR/ErbB3) of EGFR under serum-free conditions. Consistent with the results of cross-linking experiments, preformed dimers of EGFR were disrupted by silibinin treatment (Fig. 3B). Collectively, these data support the suggestion that silibinin inhibits EGFR activity by interfering with ligand-dependent and -independent dimerization of EGFR.

Figure 2. Silibinin inhibits EGFR activity in cells harboring EGFR mutations. A, cells were treated with silibinin (100 μmol/L) in full serum (10% FBS) for the indicated times, lysed, and EGFR and its downstream molecules were analyzed by Western blotting. B, serum-starved cells were treated with the indicated doses of silibinin for 6 hours prior to treatment with EGF (100 ng/mL) for 10 minutes. EGFR activity was detected by Western blotting. C, LK2 and 293T cells were transfected with vector containing wild-type EGFR or del E746-E750 and treated with the indicated doses of silibinin for 6 hours before exposure to EGF (100 ng/mL) for 10 minutes. D, Western blots were done in panel B. Cells were treated with silibinin (100 μmol/L) for the indicated times. The activity of multiple EGFR family members was analyzed by using phospho-EGFR, ErbB2, and ErbB3 antibodies.
Silibinin enhances the activity of EGFR-TKIs to overcome T790M-mediated drug resistance by downregulating EGFR signaling pathways.

PC-9/GR and PC-9/ER cells were generated by stepwise selection using increasing doses of gefitinib or erlotinib over a period of several months. These resistant cells acquired the T790M mutation (20). To evaluate whether addition of silibinin to EGFR-TKIs could overcome resistance caused by the T790M mutation, viability, and 3-D assays were done. Resistant cells were exposed to individual agents or a combination of silibinin with gefitinib or erlotinib. Cotreatment with silibinin enhanced the ability of EGFR-TKIs to induce growth inhibition (Fig. 4A). These combinations showed synergistic growth inhibition (Fig. 4B). Similarly, the combination of silibinin and gefitinib or erlotinib significantly reduced colony size and number in the Matrigel 3-D system (Fig. 4C). To clarify the mechanism by which silibinin restored the antitumor activities of the EGFR-TKIs, the activities of EGFR and Akt were examined. As expected, the inhibitory effect of single treatment with silibinin, gefitinib, or erlotinib on EGFR and Akt activities was modest, whereas the combination of silibinin and gefitinib or erlotinib substantially suppressed EGFR and Akt activities (Fig. 4D).

We further examined whether a lower dose of silibinin could inhibit EGFR activity and overcome resistance to EGFR-TKIs. As shown in Figure 5A, EGFR activity was inhibited in cells treated daily with 10 μmol/L silibinin if the treatment time was extended to 72 hours. In addition, the combination of silibinin and gefitinib or erlotinib showed significant growth inhibition and the suppression of colony formation (Fig. 5B).
Figure 4. Addition of silibinin to EGFR-TKIs overcomes drug resistance caused by the T790M mutation. A and B, cells were treated with various doses of silibinin (50, 100 μmol/L), gefitinib (1 μmol/L), and erlotinib (1 μmol/L) or a combination of gefitinib and silibinin or erlotinib and silibinin for 72 hours. The viability of the cells was determined using the MTT assay. CI plots were computationally generated; CI < 1, 1, and > 1 indicates synergism, additive effect, and antagonism, respectively. C, H1975, PC-9/GR, and PC-9/ER cells were grown in Matrigel with or without gefitinib (1 μmol/L), erlotinib (1 μmol/L), silibinin (100 μmol/L), or combinations of these drugs for 72 hours, and then incubated in drug-free medium for 7 to 10 days. Photographs of the colonies were taken after 10 to 13 days (original magnification, ×4). The lower graph shows cell numbers from Matrigel experiments. Cells were harvested by trypsinization and then counted. Bars represent the mean ± SD of 3 wells. D, cells were treated with drugs as in panel C. After 48 hours, cells were harvested and the modulation of EGFR signaling in the indicated cell lines was detected by Western blotting. C, control; G, gefitinib; E, erlotinib; S, silibinin.
of EGFR and Akt activities compared with single drug treatment (Fig. 5B and C), similar to results obtained with 100 mol/L silibinin. Taken together, these results suggest that silibinin treatment may have advantages in cells with T790M on the basis of the ability to inhibit the activities of EGFR and downstream signaling molecules (Akt and Erk).

**Addition of silibinin to erlotinib inhibits growth of erlotinib-resistant tumor xenografts in vivo**

To further evaluate the antitumor efficacy of the combination with silibinin and EGFR-TKIs, SCID mice bearing established PC-9/ER tumor xenografts were treated orally with silibinin and/or erlotinib. Response to treatment was assessed by \(^{18}\)F-fludeoxyglucose (FDG) uptake changes and tumor growth inhibition. \(^{18}\)F-FDG PET is useful for early prediction of the response to gefitinib in NSCLC (26, 27). Although treatment with erlotinib produced a slight decrease in \(^{18}\)F-FDG uptake, the erlotinib and silibinin combination more drastically reduced the uptake as compared with treatment with either drug alone on day 7 (Fig. 6A). Moreover, the combination of both drugs effectively suppressed tumor growth (Fig. 6B). Interestingly, the inhibition of tumor growth persisted in the combination group even after discontinuation of drugs. Next, EGFR downstream signaling molecules, cell proliferation, and apoptosis in tumors of mice treated with each regimen were assessed. Consistent with the *in vitro* observations, EGFR and Akt activity was reduced only in the combination group, whereas Erk activity did not show any difference (Fig. 6C). Furthermore, the combination of erlotinib and silibinin led to inhibition of tumor proliferation and induction of apoptosis (Fig. 6D). These results support the suggestion that the addition of silibinin to EGFR-TKIs may overcome T790M-mediated resistance through the restoration of the ability of EGFR-TKIs to downregulate EGFR signals, which induces apoptosis and cell growth inhibition.

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**Figure 5.** Use of lower dose of silibinin with EGFR-TKIs also overcame drug resistance caused by T790M mutation. A, cells were treated daily with 10 μmol/L silibinin for the indicated times, lysed, and EGFR activity was analyzed by Western blotting. B, three-dimensional assays were done as in Figure 4C. The doses of gefitinib and erlotinib were used as in Figure 4C, and silibinin (10 μmol/L) used a lower dose. Each drug was daily added for 72 hours and then cultured in fresh medium without drug for 10 to 13 days. Lower graph shows cell numbers from Matrigel experiments. Cells were harvested by trypsinization and then counted. Bars represent the mean ± SD of 3 wells. C, control; G, gefitinib; E, erlotinib; S, silibinin. C, cells were treated with drugs as in panel B. After 72 hours, cells were harvested and the modulation of EGFR signaling in the indicated cell lines was detected by Western blotting.
Discussion

In this study, the addition of silibinin to EGFR-TKIs could overcome drug resistance caused by the T790M mutation in primary and acquired resistance cells, which may provide an alternative therapeutic strategy in patients with acquired resistance to EGFR-TKIs through secondary mutation. This alternative is compelling in the light of accumulating data questioning the effects of irreversible EGFR-TKIs that have been reported to be capable of overcoming T790M-mediated resistance. In 1 study, this activity due to HKI-272 was evident only at a suprapharmacologic dose (7). Although BIBW-2992 has shown a remarkable disease control rate in NSCLC patients with EGFR mutations according to preliminary results of ongoing clinical trials (28), its efficacy on
T790M-mediated resistance has been more disappointing (29), indicating that these irreversible inhibitors might be unfeasible for clinical application to manage patients with secondary T790M mutation.

Previous studies showed that silibinin could inhibit EGFR activity through various mechanisms such as suppression of ligand binding (30) or reduction of ligand expression (31). The present observations show that the inhibitory effect of silibinin on EGFR and Akt activity can also be observed in lung cancer cells. All EGFR mutations occur around the ATP-binding domain, leading to structural changes of the ATP-binding cleft. Firstly, the ability of silibinin to bind to the ATP-binding cleft was examined because silibinin is a highly lipophilic compound that can pass across cell membranes (30). Silibinin did not bind directly to the ATP-binding cleft in both wild-type and mutant EGFR. Activating mutations in the EGFR gene confer increased EGFR activity even under serum-free conditions (3, 32). One study showed that this phenomenon results from EGFR-independent dimerization of mutant EGFR (33). Consistent with previous studies, EGFR dimers were observed in cells harboring EGFR mutations under basal conditions, but not in cells with wild-type EGFR (Supplementary Fig. S2). In addition to homodimerization, heterodimerization (EGFR/ErbB2, EGFR/ErbB3) of EGFR also actively occurred in PC-9 cells under serum-free conditions, although it is unclear which type of these dimers was dominant. Interestingly, silibinin treatment inhibited both the EGFR-dependent and -independent dimerization of EGFR. Moreover, it disrupted preformed homo- and heterodimers of EGFR in cells harboring EGFR mutations. Therefore, it can be concluded that the inhibitory effect of silibinin on EGFR activation may be caused by suppression of EGFR dimerization, although further investigations to elucidate its detailed mechanism are required.

Although a higher dose of silibinin inhibited EGFR activation in cells harboring wild-type EGFR, it was not observed in most clinically feasible doses, which could be estimated from a previous phase I study (34, 35). This differential effect between cells with mutant EGFR and wild-type EGFR might be related to the basal status of EGFR dimerization and cellular dependence for survival to EGFR signaling. Cells expressing mutated EGFR showed a higher level of EGFR activity with more dimerized EGFRs, regardless of the presence of ligands when compared with cells harboring wild-type EGFR. Theoretically, preservation of the dimer form is crucial for survival because of the cellular dependence on EGFR signaling. Therefore, suppression of dimerization and disruption of preformed dimers by silibinin leading to downregulation of EGFR signals could cause more lethal damage to cells with mutant EGFR.

Dual targeting of EGFR might overcome T790M-mediated drug resistance (29). These authors reported that the combination of cetuximab and BIBW-2992 induces tumor regressions of mouse lung tumors driven by EGFRΔ858R+T790M. This combination displayed dramatic inhibition of EGFR activity, although the downstream Akt activity was not measured. As a possibility, the authors suggested that cetuximab may induce degradation of total EGFR without affecting its phosphorylation, whereas BIBW-2992 dephosphorylates EGFR without inducing the degradation of EGFR. Therefore, this dual targeting combination allows BIBW-2992 to more efficiently control residual kinase activity. Consistently, our results also suggest that dual targeting may be plausible, in which the reduced dimerization of EGFRs by silibinin enhances the capability of EGFR-TKIs to inhibit kinase activity.

Other mechanisms may also explain the present observations. For example, silibinin increases binding of EGFR-TKIs to the ATP-binding cleft. Because silibinin can interact (or bind) with the membrane-bound EGFR (30), it might lead to conformation changes of EGFR. Other possibility is that silibinin inhibits constitutive activation of EGFR that is related to the acquired resistance to EGFR-TKIs. The occurrence of the T790M mutation induces conformation changes within the receptor that lead to an altered pattern of EGFR dimer such as formation of a homodimer with itself or a heterodimer with other EGFR-related family members. This possibility has been proposed previously (29).

One of the principles in combination chemotherapy is that the side effects of 2 drugs should not overlap. In this aspect, the previously suggested combination of cetuximab with BIBW-2992 (29) raises concerns about toxicity because these drugs share common side effects such as diarrhea and skin eruption. As for BIBW-2992, the adverse events seem to be more common and severe than experienced with erlotinib or gefitinib. Therefore, its clinical feasibility remains unclear in terms of tolerance. In contrast, silibinin has been widely used for a long time with negligible toxicity. Its toxicity observed in very high doses is related with the liver, notably such as an elevation of unconjugated bilirubin or aminotransferase. Rather, silibinin has an anti-inflammatory effect by inhibition of the transcription factor NF-kB (36, 37), which regulates and coordinates various genes expression involved in the inflammatory process. It might lessen the skin problems caused by EGFR-TKIs; this requires further investigation.

Presently, silibinin also inhibited the activity of other members of the EGFR family including ErbB2 and ErbB3. In acquired resistance to EGFR-TKIs, the maintenance of Akt activity in the presence of drugs can be mediated by T790M or the activation of other receptors such as ErbB3 and MET (2, 38). Therefore, silibinin has the potential to overcome the drug resistance caused by Akt activation through the MET-ErbB3 pathway. Moreover, IGFR-1 signaling as a bypass route to activate Akt and loss of IGFBP3 having an apoptosis-inducing effect have been suggested to cause drug resistance, although they were not shown in lung cancer cells (23). Because silibinin also exhibits the capability to modulate IGFR-1
signaling and increase IGFBP3 (39), its possibility to overcome various resistant mechanisms is being investigated in our laboratory.

The peak plasma level of silibinin reached more than 100 μmol/L in phase I and pharmacodynamic studies, although it ranged from 10 to 100 μmol/L in recommended doses showing tolerable toxicity profiles (34, 35). Because the present results were obtained mostly with 100 μmol/L of siliibinin, further studies were done using a lower dose of silibinin to better define its clinical feasibility. Similar effects were observed during daily exposure to 10 μmol/L silibinin indicating its clinical relevance in terms of therapeutic dose. Together, the present results offer a compelling demonstration of silibinin’s efficacy in preclinical settings using resistant cell lines and animals harboring resistant tumors. Based on these data, a clinical trial is planned to prove the efficacy in NSCLC with EGFR mutations.

In summary, silibinin enhances the effect of EGFR-TKIs to overcome T790M-mediated drug resistance in NSCLC by suppression of EGFR dimerization. This combination leads to more complete inhibition of EGFR and Akt signals that may not be achievable by treatment of EGFR-TKI alone in cancer cells harboring T790M mutation. The study results support the suggestion that the combination treatment with silibinin and EGFR-TKI is a promising strategy for preventing selective growth of preexisting T790M mutant cells or treating patients with T790M-mediated acquired resistance to EGFR-TKIs.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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