Discovery of Phosphatidylinositol 3-Kinase Inhibitory Compounds from the Screening Committee of Anticancer Drugs (SCADS) Library

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Identification of new uses for existing drugs is known to be an efficient approach in drug discovery. The identification of a novel phosphatidylinositol 3-kinase (PI3K) inhibitor is important in terms of cancer chemotherapy because PI3K is implicated in many types of cancer. In an effort to discover new PI3K inhibitory compounds, we recently carried out a screening of Screening Committee of Anticancer Drugs (SCADS) library, a compound library mainly composed of antitumor drugs and kinase inhibitors. As a result, six new PI3K inhibitory compounds were identified each of which displayed over 60% inhibition of PI3Kα at 10 μM. Baicalein, the most potent of these inhibitors, exhibited 73% inhibition at 1 μM. Further characterization of Baicalein and Akt inhibitor VIII showed that both compounds displayed comparable inhibition against PI3Kβ and δ, but relatively weak activity against PI3Kγ. Growth inhibition effects of Akt inhibitor VIII and Baicalein on human cancer cell line panel JFCR39 were also investigated, and the mean logarithm of the concentration required for 50% growth inhibition of cells (Log GI50) was determined to be −5.59 and −4.70, respectively. In addition, COMPARE analysis of the two compounds together with known PI3K inhibitors was carried out by using PI3K inhibitor ZSTK474 as a seed. Our results show that Akt inhibitor VIII displays a similar fingerprint to that of ZSTK474 (r=0.633), while Baicalein does not (r=0.126). These findings suggest the inhibition profile of Baicalein in cells is different from that of a typical PI3K inhibitor.

Key words phosphatidylinositol 3-kinase inhibitor; compound library; Baicalein; drug discovery; COMPARE analysis

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3-hydroxyl group of the inositol ring of phosphoinositides.1) They are divided into three classes based on their structural features and substrate specificity. Class I PI3K mainly phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits and phosphorylates Akt and therefore plays an important role in various cellular responses such as proliferation.2) This class of PI3K is generally referred to as PI3K because much less is known about the function of other two classes. There are four isoforms of class I PI3K, α, β, δ, and γ. PI3Kα is known to play an important role in tumorigenesis since a high frequency of mutations in the PI3Kα gene, which encodes p110α, has been found in human cancers.3) Besides the involvement in thrombotic diseases,4) PI3Kβ was recently reported to be essential in tumorigenesis of phosphatase and tension homolog deleted on chromosome ten, the catalytic antagonist of PI3K (PTEN) negative cancers.5) Both PI3Kδ and PI3Kγ are known to be involved in various inflammatory responses and the immune system.5)

Because PI3K has been recognized to play important roles in tumorigenesis, development of PI3K inhibitors for cancer therapy has attracted a great deal of attention from both academic and industrial researchers. While the classical PI3K inhibitors Wortmannin and LY294002 failed to enter clinical trials due to their poor stability6) or solubility,7) along with their undesirable toxicities,6,8) over a dozen novel PI3K inhibitors including NVP-BEZ235 and GDC-0941, have demonstrated promising antitumor efficacy on various tumor types9,10) and are now being evaluated in phase I clinical trials.11) We previously identified ZSTK474 as a novel specific PI3K inhibitor, and reported its favorable antitumor efficacy in vitro and in vivo as well as the biochemical inhibition profiles.12—16) ZSTK474 has been approved to start phase I clinical trials.

Current drug development remains a costly and time-consuming process, although computer-based drug design has improved the situation to some extent. Recently, identification of new uses for existing drugs has been recognized to be a far more efficient approach for drug discovery, primarily because the safety profiles and pharmacokinetics have already been determined.17) In the case of the chemical tools used in biological research, since target specificity is essential, identification of an additional target of a given chemical tool may update the biological information previously obtained based on the knowledge that it specifically acts on the original target. So far, various libraries containing drugs in clinical use and chemical tools have been made available.17) The Screening Committee of Anticancer Drugs (SCADS) compound library, containing 285 compounds in three 96-well microplates (http://gantoku-shien.jfcr.or.jp/), has been developed by the Screening Committee of Anticancer Drugs which is supported by Grant-in-Aid for Scientific Research on Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The compounds, mainly composed of antitumor drugs and kinase inhibitors, are provided at 10 mM concentrations in dimethyl sulfoxide (DMSO) solution.

JFCR39 anticancer drug screening system is an informatic drug-activity database that we established previously by exploiting a panel of 39 human cancer cell lines.18—21) This system is able to provide information about disease-oriented chemotherapy based on the differential growth inhibition activity of a certain agent against 39 cancer cell lines. Moreover, action mechanism of a particular agent can be predicted

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by comparing its growth inhibition profiles for JFCR39 (fingerprint) with those of the standard anticancer drugs using the COMPARE algorithm, since the inhibition profiles against growth-related targets in cells are reflected by each fingerprint. So far, utilizing this method we have succeeded in predicting the action mechanisms of MS-247 (topoisomerase inhibitor), FJ5002 (telomerase inhibitor), and ZSTK474 (PI3K inhibitor).

Herein, aiming to discover novel PI3K inhibitors, we recently carried out a screening of the SCADS compound library, and then determined the growth inhibition activity against JFCR39 panel of two hit compounds: Akt inhibitor VIII and Baicalein (Fig. 1).

MATERIALS AND METHODS

Materials SCADS library (http://gantoku-shien.jfcr.or.jp/) was kindly provided by Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The PI3K Homogenous Time Resolved Fluorescence (HTRF) Assay Kit and human recombinant PI3Kα, β, δ and γ were purchased from Millipore (Billerica, MA, U.S.A.).

Cell Lines A panel of 39 human cancer cell lines, known as JFCR39, was used as described previously, which consists of the following cell lines: lung cancer, NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273 and DMS114; colorectal cancer, HCC-2998, KM-12, HT-29, HCT-15 and HCT-116; gastric cancer, MKN-1, MKN-7, MKN-28, MKN-45, MKN-74 and St-4; ovarian cancer, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8 and SK-OV-3; breast cancer, BSY-1, HBC-4, HBC-5, MDA-MB-231 and MCF-7; renal cancer, RXF-631L and ACHN; melanoma, LOX-IMVI; glioma, U251, SF-295, SF-539, SF-268, SNB-75 and SNB-78; prostate cancer, DU-145 and PC-3. All the cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in humidified air containing 5% CO₂.

Homogenous Time Resolved Fluorescence (HTRF) Assay for the Determination of PI3K Activity The HTRF assay was carried out as described previously. Briefly, test compounds were incubated with the recombinant PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ in the assay buffer supplemented with 10 μM (final concentration, the same as following) of PIP2 in the wells of a 384-well plate at room temperature. Reaction was initiated by addition of 10 μM ATP and was stopped after 30 min of incubation by adding the stop solution containing ethylenediaminetetraacetic acid (EDTA) and biotin-PIP3. Detection buffer was then added and the resulting mixture was further incubated for 14 h. Signals from the wells were read using the EnVision 2103 Multilabel Reader (PerkinElmer, Wellesley, MA, U.S.A.). The PI3K inhibitory activity of each compound was calculated according to the following formula: PI3K inhibition (%) = (plus-enzyme control − sample)/(plus-enzyme control − minus-enzyme control) × 100. For the plus-enzyme control, the kinase was incubated with PIP2 and ATP in the absence of the test compound, and for the minus-enzyme control, PIP2 was incubated with ATP in the absence of the kinase and test compound. Representative data from at least two independent experiments, each carried out in triplicate, were used for plotting.

RESULTS

Screening of SCADS Compound Library with HTRF PI3K Assay and Recombinant PI3Kα By using human recombinant PI3Kα and the HTRF assay, each compound of the SCADS library was tested at 10 μM for the inhibition against PI3Kα. As a result, eight compounds were found to inhibit PI3Kα by more than 60% at 10 μM (Fig. 2). Among them, Wortmannin and LY294002 are well-known PI3K inhibitors. Other 6 compounds were then tested for the activ-
ity at lower concentrations. Of these, only Baicalein displayed over 50% inhibition against PI3Kα at 1 μM (Fig. 3B), whereas other 5 compounds did not (data not shown). Cyclin dependent kinase 4 (Cdk4) inhibitor, cytosolic phospholipase A2 (cPLA2) inhibitor, replication-defective acutely transforming gene1 (Raf1) inhibitor and SB218078 were reported to show activity against their respective original target at low nanomolar concentrations. Thus, PI3K inhibitory activity might only play a minor role in their anti-tumor effect. Nonetheless, Akt inhibitor VIII is of particular interest because PI3K is located upstream to its original target Akt. Therefore, we continued to investigate the PI3K inhibitory activity and antitumor effect of Akt inhibitor VIII and Baicalein.

**Inhibitory Activities of Akt Inhibitor VIII and Baicalein against 4 PI3K Isoforms** To examine whether Akt inhibitor VIII and Baicalein have selectivity for 4 PI3K isoforms, we determined their inhibition against each PI3K isoform. As shown in Fig. 3, both compounds inhibited PI3Kα, β and δ more than 60% at 10 μM, while showing relatively weak inhibition against PI3Kγ. Baicalein exhibited highest activity against PI3Kα, with a 73% inhibition at 1 μM.

**JFCR39 Fingerprint of Akt Inhibitor VIII and Baicalein, and COMPARE Analysis with PI3K Inhibitor ZSTK474 as Seed** Next, we determined the growth inhibition effect of Akt inhibitor VIII and Baicalein on human cancer cell line panel JFCR39 by SRB assay. Their respective GI50s were calculated against each cell line. The mean logarithm of GI50 for JFCR39 of the two compounds is ~5.59 and ~4.70, respectively (Fig. 4). The JFCR39 fingerprints were then developed to show the differential sensitivity of the 39 cell lines (Fig. 4). Akt inhibitor VIII exhibited the most potent inhibition against breast cancer cell line BSY-1 (Log GI50 = ~6.56), while showing far weaker activity on OVCAR-5 (Log GI50 = ~4.85). In the case of Baicalein, SNB-78 showed highest sensitivity whereas DU-145 exhibited highest resistance.

Our previous reports demonstrated that JFCR39 fingerprints could be used to predict the molecular target of antitumor agents. Thus, compounds with a common molecular target should have similar fingerprints. Both Akt inhibitor VIII and Baicalein directly inhibits PI3K. However, as shown in Fig. 4, Akt inhibitor VIII exhibits a similar fingerprint to that of specific PI3K inhibitor ZSTK474, while Baicalein does not. We further carried out COMPARE analysis of the two compounds together with other PI3K inhibitors in our database by using ZSTK474 as a seed. As indicated in Table 1, GDC-0941, another specific PI3K inhibitor, showed the most similar fingerprint with a correlation coefficient (r value) as high as 0.863. Akt inhibitor VIII, together with PI3K inhibitors including Wortmannin, PX-866, LY294002 and NVP-BEZ235, exhibited an r value of more than 0.5, suggesting similar inhibition profiles. In contrast, Baicalein showed an r value of 0.126, further demonstrating a different inhibition profile from that of PI3K inhibitor ZSTK474.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Correlation coefficient (r)</th>
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<tbody>
<tr>
<td>GDC-0941</td>
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<tr>
<td>Wortmannin</td>
<td>0.753</td>
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<tr>
<td>LY294002</td>
<td>0.749</td>
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<tr>
<td>PX-866</td>
<td>0.708</td>
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<tr>
<td>Akt inhibitor VIII</td>
<td>0.633</td>
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<tr>
<td>NVP-BEZ235</td>
<td>0.565</td>
</tr>
<tr>
<td>Baicalein</td>
<td>0.126</td>
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</tbody>
</table>

Fig. 2. Activities of Compounds Which Inhibit PI3Kα at 10 μM in SCADS Compound Library

Activities of the compounds at 10 μM are shown as the percentage of PI3Kα activity inhibited. Data are mean±S.D. (n=3), representative of 2 independent experiments.

Fig. 3. Inhibitory Activities of Akt Inhibitor VIII (A) and Baicalein (B) against Each PI3K Isoform

Activities of the compounds at 10 μM and 1 μM are shown respectively as the percentage of PI3K activity inhibited. Data are mean±S.D. (n=3), representative of 3 independent experiments.
In this study, we carried out the screening of the SCADS chemical library by use of HTRF PI3K assay. Besides Wortmannin and LY294002, six compounds exhibited over 60% inhibition of PI3Kα at 10 μM. Furthermore, Baicalein inhibited 73% of PI3Kα at 1 μM, while none of the other five compounds showed over 50% of inhibition at the same concentration. Both Baicalein and Akt inhibitor VIII showed relatively weak inhibition against PI3Kγ, compared with that against other 3 PI3K isoforms.

Baicalein is a flavonoid found in Scutellaria baicalensis, a widely used Chinese herbal medicine. Until now, various activities of Baicalein have been reported.30,31 One well known mechanism of antitumor activity is the inhibition of 12-lipoxygenase, thereby inducing the release of cytochrome c, activation of downstream caspases and ultimately inducing apoptosis in tumor cells.30,31 Our biochemical data in this study indicate that Baicalein directly inhibits PI3K at 1 μM.

Baicalein is expected to be developed as anticancer drug candidate in the future.

Akt inhibitor VIII was previously reported to inhibit Akt1 and Akt2 at sub-micromolar concentrations, with more than 10 fold selectivity over Akt3.32 Besides its direct inhibition of Akt, this compound was also found to inhibit the phosphorylation of Akt itself in cellular background like LY294002, while the mechanism was not elucidated.32 Our finding that this compound directly inhibits the upstream PI3K, might at least partially explain why it affects Akt phosphorylation. Akt inhibitor VIII is physically stable. Information about its toxicity has not yet been available until now.

Investigation on the growth inhibition effect on JFCR39 panel indicated that BSY-1 cells showed high sensitivity, while OVCAR5 exhibited resistance, to Akt inhibitor VIII. One reason might be that the former cell line is PTEN negative whereas the latter is Kirsten rat sarcoma oncogene (KRAS) mutant. PTEN status and RAS mutation have been demonstrated to affect the sensitivity to PI3K inhibitors.33 Because Akt inhibitor VIII acts on both PI3K and the downstream Akt, such characteristics of cells might also affect their sensitivity to Akt inhibitor VIII.

We carried out COMPARE analysis of Akt inhibitor VIII, Baicalein and some PI3K inhibitors in our database, by using...
PI3K inhibitor ZSTK474 as a seed. As shown in Table 1, Akt inhibitor VIII exhibited a rather similar fingerprint to that of ZSTK474 (r = 0.633), with comparable correlation coefficient to PI3K inhibitor NVP-BEZ235 (r = 0.565), suggesting a similar mechanism with PI3K inhibitors. These findings indicate that targeting both PI3K and the downstream Akt can result in a similar fingerprint to that obtained by targeting PI3K alone. In contrast, while Baicalein also inhibits PI3K, its fingerprint is rather different from that of ZSTK474, suggesting that the whole inhibition profiles of Baicalein in the cells are different from those of ZSTK474. The precise mechanism by which Baicalein affects the PI3K pathway in a given cellular background remains to be elucidated.

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