A771726, an orally available anti-inflammatory agent, has been approved for the treatment of multiple sclerosis by diminishing entire inflammatory responses through multiple signaling pathways. Recently, a few emerging studies have focused on the potential application of A771726 in cancer therapy, less on the treatment of breast cancer and particularly on overcoming drug resistance in breast cancer. We report here for the first time the cytotoxic activity and drug resistance reversal of A771726 in acquired tamoxifen-resistant breast cancer cell line MCF-7/LCC9. We discovered that A771726 treatment showed antiproliferative activities in MCF-7/LCC9 cells, which were even more sensitive to A771726 than their parental tamoxifen-sensitive cells (MCF-7). A771726 also exerted pro-apoptotic activities and induced cell cycle arrest at the G1 phase. Notably, treatment of A771726 restored the sensitivity of MCF-7/LCC9 cells to tamoxifen. Western blot analysis revealed that A771726 decreased the phosphorylation level of Src, one key driver of tamoxifen resistance. Moreover, in order to comprehensively clarify the mechanisms of A771726 in anti-estrogen-resistant cells, we explored a genome-wide transcriptomic analysis, and showed that A771726 could modulate multiple signaling pathways (e.g. cell cycle, apoptosis, MAPK, metabolism and p53 signaling pathway) and cellular processes (e.g. signal transduction, transcription and cell cycle). Overall, our results indicate that A771726 alone and the combination of A771726 with anti-estrogens may be of therapeutic benefit for ER-positive and endocrine-resistant breast cancer.

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related mortality among women in the US (1). Breast cancer is a type of hormone-dependent malignancy, and sustained exposure to estrogen is known to contribute to the development and progression of this disease (2). The biological effects of estrogen are mediated by the estrogen receptor (ER) through classic genomic and non-genomic actions (3). In the US, ~75% of breast cancers express ER (4), and are classified as ER-positive breast cancer, thus corroborating the use of selective ER modulators (SERMs) as estrogen antagonists (5). Tamoxifen, the most commonly used SERM, has been accepted as the gold standard of endocrine therapy and has been utilized successfully both in neoadjuvant and adjuvant settings (6). However, a substantial proportion of breast cancer patients with localized disease and nearly all with advanced disease, despite an initial positive drug response, develop acquired tamoxifen resistance (7). To date, application of commercially available drugs to circumvent tamoxifen resistance remains sparse, and therefore, searching for novel pharmaceutical agents for re-sensitization to endocrine therapy is urgent.

A771726, the main active metabolite of leflunomide, also named teriflunomide, has been approved by the FDA for the treatment of multiple sclerosis (MS) (8). A771726 exerts anti-inflammatory and immunomodulatory actions by inhibiting the activity of proliferating B- and T-cells through dihydroorotate dehydrogenase (DHODH) inhibition-dependent and -independent patterns (9). Although the precise underlying mechanisms remain elusive, A771726 has high plasma concentrations (10),
a long elimination half-time (11), high treatment compliance (12) and multiple molecular targets (13), implying more possibilities for broader clinical applications, even beyond the treatment of MS.

Actually, A771726 has been reported to exert a potent anti-cancer effect in treating multiple human neoplasms (14-17). Notably, A771726 has been used as an agent with direct cytotoxicity to chemotherapy resistant chronic lymphocytic leukemia (CLL) (18), and has also been utilized to enhance chemosensitization (19,20) and assist targeting therapy (21,22) in the treatment of other types of tumors. Further investigation on the application of A771726 in endocrine therapy for breast cancer has not yet been documented. Primary mechanisms of acquired resistance to tamoxifen in ER-positive breast cancer include deregulation of cell cycle and apoptotic machinery accompanied by altered modulation of various regulators, such as Bcl-2, p21, activation of receptor tyrosine kinase signaling leading to the increased activity of Src and MAPK pathways, and alteration of ER and ER co-regulators (23). In the present study, using an acquired endocrine-resistant cell model, MCF-7/LCC9, we investigated, for the first time, to our knowledge, the anticancer activity and tamoxifen-resistance reversal of A771726. Furthermore, we performed a comprehensive profiling analysis of A771726-regulated molecules in MCF-7/LCC9 cells and validated that A771726 may modulate extensive cellular processes and multiple signaling pathways that are critical for endocrine resistance. Here, our study provides new clues for the reversal of resistance to endocrine therapy and the potential clinical applications of A771726 in the management of tamoxifen resistance in breast cancer patients.

Materials and methods

Cell culture and drug treatment. MCF-7 and tamoxifen-resistant MCF-7/LCC9 cells were kindly provided by Dr Robert Clarke (Georgetown University Medical Centre, Washington, DC, USA). Cells were routinely cultured in complete medium with 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂, 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and A771726 was kindly provided by Cinkate Corporation (Oak Park, IL, USA). For the tamoxifen sensitivity analysis, 2,000 cells/well were plated in phenol red-free IMEM plus 5% charcoal dextran-treated FBS (Tissue Culture Biologicals, Oak Park, IL, USA). For the tamoxifen sensitivity analysis, cells were routinely cultured in complete medium with 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂. 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and A771726 was kindly provided by Cinkate Corporation (Oak Park, IL, USA). For the tamoxifen sensitivity analysis, 2,000 cells/well were plated in phenol red-free IMEM plus 5% charcoal dextran-treated FBS (Tissue Culture Biologicals, Los Alamitos, CA, USA) for 24 h and were then treated with a combination of A771726 and OHT for 7 days.

Sulforhodamine B (SRB) assay. Cells were seeded in 96-well plates (Corning, Acton, MA, USA) and fixed by trichloroacetic acid (Sinopharm Reagent, Shanghai, China). Dried cells were stained by SRB (Sigma-Aldrich), which then was solubilized by Tris-base (Sinopharm Reagent). The absorbance was read on an automated microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA).

Clonogenic assay. Cells were plated at 500 cells/well in 6-well plates (Corning) and incubated with various concentrations of A771726 for 2 weeks. Then, the colonies were stained with 0.5% crystal violet and counted.

Flow cytometric assay. For cell apoptosis analysis, after treatment of A771726 for 48 h, cells were detached and washed with cooled PBS. Cells were then re-suspended and stained by Annexin V and propidium iodide (PI) (BD Pharmingen, San Diego, CA, USA). Analysis was performed on a FACSCalibur analyzer (Becton-Dickinson, San Jose, CA, USA). With respect to cell cycle analysis, cells were harvested and fixed by 70% cooled ethanol, and then stained with PI (Sigma-Aldrich). Analysis was also carried out on a FACSCalibur analyzer.

Western blot assay. All cells were treated with A771726 for 72 h. The protein extracts were separated by SDS-PAGE and transferred to NC membranes (Millipore, Bedford, MA, USA). Src and p-Src proteins were visualized by a chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

Microarray analysis. Briefly, samples were utilized to synthesize double-stranded complementary DNAs (cDNAs), which were labelled and hybridized to dual-channel mRNA microarray version 1.0 (CapitalBio Corp., Beijing, China) following the manufacturer's instructions. Data were selected by using a threshold of 2- and 2-fold change under false discovery rate (FDR) protection (P<0.05).

Statistical analysis. Statistical significance was evaluated by the Student's t-test with GraphPad Prism software 5.0. P-values <0.05 were considered to indicate statistically significant results.

Results

A771726 inhibits the cell proliferation of endocrine-resistant breast cancer cells. To examine the biological effects on cell proliferation of tamoxifen-resistant cells and their sensitive counterparts, we treated the cells with different doses of A771726 and performed an SRB assay (Fig. 1A). Viability of the cells decreased in a dose- and time-dependent manner. Moreover, tamoxifen-resistant MCF-7/LCC9 cells were more sensitive to inhibition by this agent than the tamoxifen-sensitive MCF-7 cells; marked early antiproliferative effects were observed within a 24-h period in the MCF-7/LCC9 cells but not until 48 h in the MCF-7 cells, respectively. Lower IC₅₀ values were observed for A771726 in the MCF-7/LCC9 cells (72 h post-treatment: IC₅₀=64.72 µM in MCF-7/LCC9 and 122.79 µM in MCF-7 cells).

A771726 suppresses the colony-forming ability of endocrine-resistant breast cancer cells. To further demonstrate the antiproliferative effects of A771726 on the growth of MCF-7/LCC9 cells, a colony formation assay was carried out. As shown in Fig. 1B, the colony numbers of MCF-7 and MCF-7/LCC9 cells were significantly decreased in dose-dependent manner, and long-term treatment of A771726 at the concentration of 100 µM almost completely diminished the colony formation capability. The results were consistent with that of the SRB assay further indicating that A771726 inhibits the in vitro proliferation of MCF-7/LCC9 cells.

A771726 induces apoptosis in endocrine-resistant breast cancer cells. We aimed to ascertain whether the cell growth
inhibition induced by A771726 was due to the induction of apoptosis in tamoxifen-resistant cells. Therefore, we incubated MCF-7/LCC9 and MCF-7 cells with A771726 for 48 h. Our experiments showed that A771726 induced apoptosis in a dose-dependent manner in the endocrine-resistant and wild-type cells (Fig. 2A). When exposed to 50 and 100 µM A771726, few MCF-7 cells were PI+ or Annexin V+, indicating that low doses of A771726 did not induce significant apoptosis while the highest concentration of 200 µM did. However, MCF-7/LCC9 cells were more sensitive to A771726; the lowest concentration of 50 µM significantly increased the percentage of Annexin V+/PI- cells. Following treatment with higher doses of A771726 at 100 and 200 µM, the percentage of Annexin V+/PI- cells was increased from 4.48 to 18.63 and 27.85%, as well as the percentage of Annexin V+/PI+ cells from 3.06 to 37.05% at 200 µM, indicating A771726 at various concentrations induced apoptosis and necrosis in endocrine-resistant cell.

A771726 induces G1 phase arrest in endocrine-resistant breast cancer cells. In order to determine whether A771726 has a cell cycle arrest effect, MCF-7/LCC9 and MCF-7 cells treated with increasing doses of A771726 for 48 h were subjected to flow cytometric analysis. As shown in Fig. 2B, A771726 caused a dose-dependent accumulation of cells in the G1 phase fraction, while reducing cell accumulation in the S and G2/M phases. The G1 phase fraction increased from 57.12 to 61.95%, 63.59 and 77.74% at 50, 100 and 200 µM A771726 in resistant cells, and from 54.30 to 62.60%, 68.78 and 74.00% in wild-type cells at the same concentrations of A771726, accompanied by maximal decreases of 6.82% in the S phase and 13.79% in the G2/M phase in MCF-7/LCC9 cells and 10.53% in the S phase and 8.59% in the G2/M phase in MCF-7 cells.

A771726 reverses tamoxifen resistance in endocrine-resistant breast cancer cells. To further examine the effects of A771726 on tamoxifen sensitivity, hormone-resistant MCF-7/LCC9 cells were treated with different doses of A771726 and tamoxifen (alone or in combination) for 7 days in estrogen-depleted media containing 5% charcoal-stripped serum (CS). As shown in Fig. 3, cell viability assay recorded over a range of 4-OHT concentrations from 1 to 7 µM confirmed that these cells indeed were resistant to OHT, compared with their sensitive counterparts (data not shown). After receiving A771726 plus OHT, dose-dependent increases in the sensitivity of MCF-7/LCC9 cells to OHT were observed. The IC50 value of OHT in the A771726-treated MCF-7/LCC9 cells was decreased to 7.0 and 5.9 µM at 2.5 and 10 µM, indicating significant recovery of drug sensitivity.

A771726 inhibits Src activation in endocrine-resistant breast cancer cells. To investigate the mechanisms by which A771726 reverses the drug resistance of MCF-7/LCC9 cells,
we analyzed the state of Src phosphorylation, one key driver of tamoxifen resistance (Fig. 4A). Although no change in total Src expression was detected between MCF-7 and MCF-7/LCC9 cells, our results revealed that the phosphorylation of Src (Tyr418) was increased in the resistant cells, both in complete medium with 5% CS, and interestingly at a higher level in FBS. Following A771726 treatment, the Src phosphorylation was significantly suppressed at 100 µM A771726 at 72 h.

A771726 regulates global gene expression and multiple signaling pathways in endocrine-resistant breast cancer
To systematically gain further insight into the functional effects of A771726 in tamoxifen resistance reversal and other biological functions, we performed large-scale mRNA expression profiling following treatment of 100 µM A771726 for 72 h in MCF-7/LCC9 cells. As shown in Fig. 4B, clustering results of all significantly regulated probes were demonstrated. Respectively, we identified 410 upregulated probes and 285 downregulated probes (Table I).

### Table I. Differentially regulated genes in cell cycle, apoptosis and MAPK signaling pathways following A771726 treatment in MCF-7/LCC9 cells.

<table>
<thead>
<tr>
<th>Signaling pathway (oligo ID)</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold-change (LCC9-A771726 vs. LCC9-DMSO)</th>
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<tr>
<td>Cell cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H200008365</td>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1 (p21)</td>
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<tr>
<td>H300012891</td>
<td>CHEK1</td>
<td>Serine/threonine-protein kinase Chk1</td>
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<tr>
<td>H300020974</td>
<td>CCNE2</td>
<td>G1/S-specific cyclin-E2</td>
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<td>H200015433</td>
<td>E2F2</td>
<td>Transcription factor E2F2</td>
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<tr>
<td>H200006619</td>
<td>MAD2L1</td>
<td>Mitotic spindle assembly checkpoint protein MAD2A</td>
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<tr>
<td>H200007139</td>
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<td>opHSV04000006063</td>
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<td>Calpain-2 catalytic subunit precursor</td>
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<tr>
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<td>BAX</td>
<td>BAX protein</td>
<td>2.00</td>
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<td>H300008499</td>
<td>IRF1</td>
<td>Interferon regulatory factor 1</td>
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<td>HSPA5</td>
<td>78 kDa glucose-regulated protein precursor (GRP 78)</td>
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<td>Baculoviral IAP repeat-containing protein 5 (survivin)</td>
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<td>BCL2</td>
<td>Apoptosis regulator Bcl-2</td>
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<td>MAPK</td>
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<td></td>
<td></td>
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<tr>
<td>H300008649</td>
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<td>Voltage-dependent calcium channel γ-1 subunit</td>
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<td>Dual specificity protein phosphatase 4</td>
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Discussion

In the present study, we provide evidence that A771726 may have direct chemopreventive potential and resistance reversal when concurrently used in endocrine-resistant cells. We found that this anti-inflammatory agent is capable of suppressing the growth of MCF-7/LCC9 cells in vitro by inducing cell apoptosis and G1 phase arrest. Moreover, by analyzing A771726-regulated gene expression profiles in anti-estrogen-resistant cells, we demonstrated the multiple biological functions and signaling pathways that were involved.

Although recently approved by the FDA for use in the relapsing forms of MS in the US, A771726 has also been...
investigated as a potential anticancer agent in a handful of human tumors (14-17,21). However, the lethal effect of A771726 in breast cancer has not been thoroughly investigated. In the present study, we report the potent cytotoxicity and attenuation of colony-forming activity of A771726 in sensitive ER-positive breast cancer cells (Fig. 1A and B). Notably, consistent with the direct anticancer effect in drug-resistance CLL (18), we also showed that A771726 led to cell death in endocrine-resistant cells (Fig. 1A and B). Moreover, we discovered that the tamoxifen-resistant MCF-7/LCC9 cells were more sensitive to A771726-mediated killing than the parental MCF-7 cells (Fig. 1A), indicating that resistance-related signals
may be responsible for the differential response to A771726. Transcriptional repression or somatic deletion of CDKN1A (encoding p21), resulting in the alleviation of the inhibitory effect on CDK activity, contributes to decreased anti-estrogen sensitivity in breast cancer (24,25). Although no change in Myc, a suppressor of p21 (24), was observed (data not shown), significant upregulation of p21 mRNA (Table I) was determined, leading to the inability of growth maintenance and retrieval of tamoxifen sensitivity.

Tamoxifen has the ability to elicit cell apoptosis via an ER-dependent and -independent manner, disturbing the balance between pro- and anti-survival functions (26). However, when endocrine resistance develops, cancer cells also lose the response to tamoxifen-induced apoptosis (27). Accumulating evidence suggests that the increased expression of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, and functional defect of pro-apoptotic members, such as BAK and BIK, contribute to the emergence of attenuated responses to tamoxifen (23). In the present study, A771726 treatment in resistant cells induced downregulation of anti-apoptotic Bcl-2 by 40% and upregulation of the pro-apoptotic Bax (Table I), leading to an increase in the BAX:Bcl-2 ratio, shifting the balance towards cell death (28) and restoring the tamoxifen-induced apoptosis in MCF-7/LCC9 cells (29). In addition, compared with the sensitive controls, MCF-7/LCC9 cells expressed lower IRF1 (30), whose enhanced expression by A771726 (by ~60%) may mediate cell death by reversing the balance of Bcl-2 family members and downregulating the inhibitor of apoptosis, survivin (Table I) (31), thus, determining the cell fate decision to again undergo drug apoptosis. Interestingly, we also revealed that autophagy may be involved in A771726-induced sensitivity restoration by inhibiting expression of GRP78 (Table I), which could activate mTOR-regulated dependent pro-survival autophagy with increased LC3-II and decreased p62 in induction of anti-estrogen resistance (32). Overall, our results showed that A771726 could recover the deregulated apoptosis and autophagy in tamoxifen-resistant cells via modulating multiple signals involved in the determination of cell fate.

In addition to the disruption of cell death pathways, cell cycle perturbation by A771726, consistent with data in the treatment of multiple myeloma cells (19), could also be observed in endocrine-resistant cells. Cyclin E2 is induced in the late G1 phase and activates cyclin-dependent kinase CDK2, leading to inactivation of pRB and activation of E2F transcription factors to promote entry into S phase (33). However, in the development of impaired tamoxifen responsiveness, aberrant regulation of several cell cycle regulators, such as cyclin E2 and E2F2, also anti-estrogen targets, was found to be associated with the inability of induction of G1 phase-specific cell cycle arrest and the consequent reduction in cancer growth (34,35). Interestingly, our genome-wide analysis in MCF-7/LCC9 cells showed that A771726 significantly decreased the expression of cyclin E2 and E2F2 (Table I) and CDK2 by 25%, accompanied by the upregulation of the antagonist of CDK inhibitor, p21 (33), contributing to the delay of cell cycle progression from the G1 to the S phase (Fig. 2B) and enhancement of tamoxifen-induced growth inhibition (Fig. 3). Moreover, downregulation of GRP78 (Table I), also a key driver of tamoxifen resistance (32), may be involved in G1 arrest (36).

Prior experiments have demonstrated that the resistant cell line MCF-7/LCC9 has an elevated level of Src phosphorylation (37), which was also observed in the present study, regardless of whether the medium contained estrogen or not (Fig. 4A). Pharmacological inhibition of Src, like that in A771726 treatment in resistant cells, may be effective in preventing the emergence of tamoxifen resistance (38). For the first time, we demonstrated the global pattern of cellular functions and signaling pathways of A771726 targets using GO and pathway analysis. As for pathway mapping, we revealed that multiple oncogenic signaling pathways, cell cycle, apoptosis, MAPK, metabolism and p53 pathway, were regulated by A771726 (Fig. 4D), which have been reported to contribute to acquisition of tamoxifen resistance (23,39). Although precise underlying regulation patterns of the biological processes have not yet been uncovered, A771726 modulated signal transduction, oxidation reduction, transcription, cell cycle and metabolism (Fig. 4E), consistent with that in the pathway analysis. Moreover, the A771726-related gene products were mainly located in the nuclei, indicating its involvement in the regulation of transcription.

In summary, to the best of our knowledge for the first time, we demonstrated the mechanistic evidence for the potent anti-cancer effect and tamoxifen resistance reversal by A771726 in endocrine-resistant MCF-7/LCC9 cells. Our findings demonstrated that A771726 mediated these effects through modulation of extensive related signaling pathways and cellular processes. The data provide support for the potential clinical applications of A771726 for ER-positive breast cancer patients with poor response to endocrine therapy.

Acknowledgements

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