AC0010, an irreversible EGFR inhibitor selectively targeting mutated EGFR and overcoming T790M-induced resistance in animal models and lung cancer patients

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Running title
Novel covalent EGFR TKI overcomes T790M-mediated resistance

Keywords
AC0010, EGFR inhibitor, T790M, NSCLC, Resistance

Abbreviations list
EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; TKIs, tyrosine kinase inhibitors; TV, tumor volume; PK, Pharmacokinetics; PD, pharmacodynamics; DLT, dose limited toxicity; AE, adverse event; IGF-1R, insulin-like growth factor 1 receptor.

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Conflict of interest:
Xiao Xu, Long Mao, Wanhong Xu, Wei Tang, Xiaoying Zhang, Biao Xi, Ron Xu, Xin Fang, Jia Liu, Che Fang, Li Zhao, Xiaobo Wang are employees of ACEA Pharmaceutical Research and ACEA Biosciences.

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Abstract

AC0010 is a pyrrolopyrimidine-based irreversible epidermal growth factor receptor (EGFR) inhibitor, structurally distinct from previously reported pyrimidine-based irreversible EGFR inhibitors such as osimertinib and rociletinib. AC0010 selectively inhibits EGFR active and T790M mutations with up to 298-fold increase in potency compared to wild-type EGFR. In a xenograft model, oral administration of AC0010 at daily dose of 500 mg/kg resulted in complete remission of tumors with EGFR active and T790M mutations for over 143 days with no weight loss. Three major metabolites of AC0010 were tested and showed no wild-type EGFR inhibition and off-target effects such as inhibition of IGF-1R. AC0010 is safe in non-small cell lung cancer (NSCLC) patients at the dose range between 50 mg and 550 mg once per day and no hyperglycemia and other severe adverse effects were detected such as grade 3 QT prolongation. The objective responses were observed in NSCLC patients with EGFR T790M mutation.
Introduction

NSCLC accounts for approximately 85% of all lung cancers and is often insidious, and around 80% of patients have more advanced disease including 25% of patients with regional metastasis, and 55% of patients with distant spread of disease (1, 2). In the previous decade, a more comprehensive molecular understanding of the pathology of NSCLC has led to the development of small molecules that target genetic mutations known to play critical roles in the progression to the metastatic disease in NSCLC patients, such as mutations in EGFR and ALK (3-5). EGFR is expressed on the cell surface of a substantial percentage of NSCLCs and 15%-40% of total NSCLC patients harbor mutations in EGFR. Studies with the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib demonstrated biologic and clinical activity in a subset of lung cancers harboring EGFR active mutations such as small deletions (deLE747–750) and point mutation at codon 858 (L858R) (6-9). Despite the impressive results of genotype-directed therapy, EGFR active mutation-positive patients eventually develop resistance (10, 11). There are at least two mechanisms that contribute to the acquired resistance: (i) the emergence of the gatekeeper T790M EGFR mutation, which prevents binding of the gefitinib and erlotinib to EGFR, and (ii) the up-regulation of a bypass track which activate downstream signals (12-15). As reported, over 50% of patients who developed resistance have the T790M mutation (16). An experimental compound, WZ4002 and two clinical compounds, osimertinib and rociletinib, reported as third-generation EGFR TKIs, demonstrated the inhibition of EGFR T790M resistant mutation in animal models and in patients (17-19).

AC0010 was designed specifically to inhibit EGFR active mutations and the T790M acquired resistant mutation, while sparing wild type EGFR. In the preclinical studies, AC0010 showed potent inhibition of NSCLC that harbors both active mutation(s) and T790M mutation in three xenograft mouse models. In addition, AC0010 and its metabolites result in no observable off-target side effect such as hyperglycemia and grade 3 QT prolongation and appear to contain a safe profile in animal models and in patients. Importantly, AC0010 overcame T790M-induced resistance to
first-generation EGFR drugs in NSCLC patients. It is therefore warranted to further develop AC0010 as an alternative therapeutic agent for NSCLC patients who develop acquired resistance to first-generation EGFR TKIs.
Materials and Methods

Structural Chemistry

Experimental drugs or chemicals, Gefitinib, Afatinib and AZ5104 were purchased from Jinan XuanHong Pharmaceutical Technology Co., Ltd., China Langchem Inc. and Selleckchem, respectively. AC0010 (freebase), AC0010MA (maleate salt), AC0010 metabolites MII-2, MII-6, and M7, Rociletinib and Osimertinib were synthesized by Hangzhou ACEA Pharmaceutical Research Co., Ltd.

Molecular modeling/docking structures were conducted using Schrodinger's LigPrep, Glide and Maestro programs. The crystal structure of T790M-mutant EGFR in complex with WZ4002, HKI-272 and AEE788 were retrieved from the Protein Data Bank (PDB entry 3IKA, 2JIV and 2Jiu, respectively). Water cluster analysis was performed by running scripts from Schrodinger software. Docking was performed with Glide program (Schrodinger). The Protein Preparation Wizard module of Maestro was used to prepare the protein. During protein preparation, all water molecules were deleted, except the 4 conserved water molecules within 10 Å distance of WZ4002. For docking, the scoring grids were centered on the crystal structure of WZ4002 using the default bounding box sizes, with an inner box of 10 Å on each side and an outer box of 24 Å on each side. Flexible docking with default parameters was used. Glide XP (extra precision) was employed for all docking calculations. The best docked poses were selected as the ones with the lowest GlideScore.

Cell culture

The NCI-H1975, HCC827, A431, A549, NCI-H460, HT1080, HeLa, BEAS-2B and NIH/3T3 cells were obtained from ATCC on years of 2011-2013 and maintained at 37°C with 5% CO₂, in the media supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/mL) and streptomycin (100 µg/mL). All cell lines were authenticated at ATCC using short tandem repeat profiling and cultured for fewer than 3 months after resuscitation. Cells were routinely screened for mycoplasma and periodically authenticated by morphologic inspections.

Engineered cell lines and resistant cell lines

NIH/3T3 cells harboring EGFR L858R/T790M double mutations were engineered by exposing NIH/3T3 cells to the lentivirus that expressed human EGFR (L858R/T790M), and then followed by clone selection using puromycin. The resistant colonies were selected and expanded for further sequencing analysis to confirm the
integration of EGFR L858R/T790M mutations. A confirmed clone was subsequently established to be a cell line designated as NIH/3T3_TC32T8.

Resistant cell line NCI-H1975-P1 was derived from relapsed tumor tissues of NCI-H1975 xenograft mouse that developed resistance to AC0010. AC0010-resistant NCI-H1975-AVR1 cells and osimertinib-resistant NCI-H1975-OSR1 cells were generated in vitro by exposing NCI-1975 cells to escalating doses of AC0010 and osimertinib respectively for a prolonged period.

Cell proliferation assays

Cell proliferation was assayed by a cell viability reagent, WST-1, per instruction from the manufacturer (Roche). Cells were seeded at optimal density onto 96-well plates and incubated for 24 hours, followed by compound treatment for 72 hours. Cell viability was then assayed by incubating cells with WST-1 reagent for 2~3 hrs.

Immunoblotting analysis

NCI-H1975, HCC827 and A431 cells were seeded onto 6-well plates at the density of $1 \times 10^6$ cells per well. After 24 hours of culture, cells were then incubated in serum-free media for 1 hour, and treated with test compound for 2 hours. A431 cells were stimulated with 30 ng/mL EGF during the last 15 minutes of compound treatment. Immunoblotting analysis was performed using the whole-cell extracts and the blots were probed with phospho-specific EGFR (p-Y1068), total EGFR, phospho-Akt (Ser-473), total Akt, phospho-ERK1/2 (p-T202/p-Y204) and total ERK1/2 antibodies (Cell Signaling Technology).

Immunoblotting analysis was performed with the tumor tissues isolated from mouse xenograft models to detect EGFR phosphorylation in vivo. The tumor tissues were homogenized in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors, and the homogenized solutions were incubated on ice for 30 minutes, followed by two rounds of centrifugation at 14000 rpm for 30 minutes at $4^\circ$ C. The supernatants were collected for immunoblotting analysis to probe p-EGFR, total EGFR, and β-tubulin as the protein loading control. The densities of blotting bands were acquired and analyzed by Image J Software.

ELISA assay

Cells were seeded onto a 96-well plate, grown for 24 hours, and then treated with test compound in serum-free medium for 2 hours. NIH/3T3_TC32T8 and A431 cells
were stimulated with 30 ng/mL EGF during the last 15 minutes of compound treatment. Cells were washed with ice-cold PBS before extraction with 100 μL cell lysis buffer. Phosphorylation of EGFR was measured using a sandwich ELISA assay with the pair of phospho-specific EGFR (pY1068) and total EGFR antibodies.

**Xenograft models**

All studies involving animals handling, care, and treatment were conducted in Hangzhou ACEA Pharmaceutical Research Co., Ltd and performed according to the guidelines and SOPs approved by Department of Science and Technology of Zhejiang Province, China.

The Nu/Nu nude mice were purchased from Beijing Vital River Laboratories of China. 6-8 week old female mice were inoculated subcutaneously at the right flank with approximately 3-5×10^6 cells in 0.2 ml of medium for tumor development. The treatments were started when the tumor size reached approximately 200 mm^3. For NCI-H1975 and A431 models, mice were divided into five groups (n=8 per group) including a vehicle group (0.5% methylcellulose, MC), three AC0010-testing groups treated with AC0010MA at the doses of 12.5 mg/kg, 50 mg/kg and 500 mg/kg, and a control group treated with Gefitinib at 100 mg/kg. All mice were orally administrated once daily for consecutive 14 days.

For the long term treatment study, NCI-H1975 tumor bearing mice with tumor volume (TV) of 170 mm^3 were orally treated with a vehicle control (0.5% MC), AC0010 at dose levels of 12.5 mg/kg and 50 mg/kg for 17 days when TV value in vehicle control group reached approximately 2000 mm^3. After 17-day dosing, animals in the vehicle control group were sacrificed, whereas animals in AC0010 groups were continually daily administrated with increased dose at 500 mg/kg till the test mice cannot tolerate the treatment. Mouse body weight and TV were measured twice per week. TV was then used for the calculation of tumor inhibitory rate and tumor regression rate.

**Pharmacokinetics (PK), pharmacodynamics (PD), Metabolites and safety in animal models**

*PK/PD*. NCI-H1975 tumor bearing mice with TV of approximately 200-500 mm^3 were used in the PK/PD study. The dose levels of AC0010 for oral administration were 12.5, 50 mg/kg and 200 mg/kg with either single administration or 8 consecutive
day treatments, while intravenous dose was singly administered at 10 mg/kg. Vehicle control (0.5% MC) and gefitinib at 100 mg/kg were also orally administered for 1 day or 8 consecutive days. Blood samples were collected at pre-dose and at 0.0833, 0.25, 0.50, 1, 2, 4, 6, 8, and 24 hours post-dose and tumor tissues were collected at pre-dose and at 1, 4, 8 and 24 hours post-dose. Tumor tissues for 200 mg/kg AC0010 with 8-dose groups were not collected due to very small size of tumors resulted from the treatments. The tumor tissues were cut into two parts for assaying L858R/T790M EGFR phosphorylation by immunoblotting analysis and for measuring AC0010 concentrations in the tumor tissues. The densities of blotting bands analyzed by Image J software were used for deriving pEGFR/EGFR values. The AC0010 concentrations in plasma and tissues were analyzed by the LC-MS/MS methods.

Metabolite analysis. Metabolite profiling was conducted for AC0010 in vitro in mouse, rat, dog, monkey, and human liver microsomes as well as in vivo in rat plasma, feces, and urine samples. All samples were analyzed by a LC-MS/MS system. The structures of major metabolites were elucidated based on EPI spectra and further confirmed by using synthesized authentic standards. Selected metabolites were quantified in Multiple Reaction Monitoring mode in rat and monkey GLP toxicokinetic study samples as well as in human clinical samples. The biological activities of AC0010 and its metabolites against wild type and mutant EGFR were assessed using WST cell proliferation assay and p-EGFR ELISA assay. The general toxicity was assayed against a panel of A549, HT1080, HeLa, NCI-H460 and Beas-2B cells.

Clinical Studies

Study design. This was a single-center, open-label, two-stage, single/multiple doses and dose-escalation phase I study in histologically confirmed metastatic, or unresectable locally advanced NSCLC patients. The main inclusion/exclusion criteria were described in the protocol (NCT 02274337). The criteria of acquired resistance to EGFR TKIs, e.g. gefitinib and erlotinib, were used based on the clinical definition by Jackman et al. (10).

Based on the modified Fibonacci methods, 5 dose cohorts were designed including 50 mg/d, 100 mg/d, 200 mg/d, 350 mg/d and 550 mg/d. Dose limited toxicity (DLT) was defined as an adverse event (AE) occurring during cycle one following the Common Terminology Criteria for Adverse Events Version 4.0 (CTCAE v4.0)

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criteria. All patients were treated with AC0010 on trial NCT 02274337, with written informed consent from patients and approval by Sun Yat-Sen University Cancer Center Ethics Committee. The permission for publishing patients’ image scan results is included in the informed consent.

Patients’ treatment and pharmacokinetic analysis. The clinical pharmacokinetic study of AC0010 was performed for both single dose and multiple doses. Blood samples were collected from each subject at pre-specified times after the single dose (pre-dose, 1, 2, 3, 4, 5, 6, 8, 12, 24 and 48 hours post dose) and during the multiple dosing cycles (Cycle 1-at pre-dose on Days 1, 8, 15 and 22, as well as at pre-dose, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hours post dose on Day 28).
Results

**AC0010 as a novel covalent EGFR TKI demonstrates an inhibitory activity selectively against the mutant EGFR in vitro**

Using structure-based drug design and focused-compound library screening approach, a novel series of irreversible, small molecule inhibitors were selected. Many of them showed specific inhibition with different extent of potency to EGFR active mutations, such as L858R, delE746–750 and T790M. Furthermore, these series of compounds displayed enhanced selectivity for the EGFR active mutations over the wild-type EGFR (data not shown). Medicinal chemistry efforts, structure-activity relationship study and structural optimization led to the discovery of the candidate compound, AC0010 (Fig. 1A), which showed the highest inhibitory potency against EGFR T790M mutation and the best selectivity over wild-type EGFR in the series (data not shown). AC0010 covalently modified recombinant EGFR T790M mutation at the target cysteine 797 amino acid (Supplementary Fig. S1). In comparison with the structures of other reported third-generation tyrosine kinase EGFR inhibitors (WZ4002, rociletinib and osimertinib), the designed drug molecule AC0010 has a distinct chemical structure containing pyrrolopyrimidine ring system as its core (Fig. 1A), while all other third-generation EGFR inhibitors such as WZ4002, rociletinib and osimertinib have the pyrimidine core structure (17, 20-21). X-ray structure of EGFR T790M covalently bound to WZ4002 (left, PDB entry 3IKA), and docked structure of WZ4002 (middle) and AC0010 (right) are shown in Figure 1B, using Schrödinger’s Maestro programs. The acrylamide groups on both WZ4002 and AC0010 are at the position that molecular modeling predicts to react with Cys797 (Fig. 1B, middle and right panel), and form H-bonds to Met793 backbone amide & carbonyl. In addition, the hydrogen from NH group of the pyrrole ring from AC0010 forms additional H-bond with Glu 791 backbone carbonyl oxygen. AC0010 ranks significantly higher than WZ4002 in docking analysis (docking scores of -9.640 and -6.812 for AC0010 and WZ4002, respectively), which may be related to the higher inhibitory effect of AC0010 on EGFR T790M mutations than other pyrimidine-based compounds such as WZ4002, rociletinib and osimertinib (Fig. 2A). In our assay system the IC50 value of osimertinib on EGFR phosphorylation in NCI-H1975 was 214 ± 40 nM. However, in the literature, the *in vitro* IC50 value of osimertinib was reported to be at 15 nM (21). Here, the parallel experimental results were used.
In the kinase enzymatic assay, AC0010 exhibited potent inhibitory activity with IC$_{50}$ value of 0.18 nM against EGFR L858R/T790M double mutations, nearly 43-fold greater potency over wild-type EGFR (IC$_{50}$ value, 7.68 nM) (Supplementary Table S1). The inhibitory activity and selectivity of AC0010 were further evaluated in the NCI-H1975 and NIH/3T3_TC32T8 lines harboring EGFR L858R/T790M double mutations, the HCC827 line harboring delE746-A750 mutation, and the A431 cell line which expresses wild-type endogenous EGFR receptor. AC0010 selectively inhibited mutant EGFR phosphorylation with IC$_{50}$ values of 7.3 nM and 2.8 nM in NCI-H1975 and NIH/3T3_TC32T8 cells, about 115- and 298-fold more sensitive than that of the inhibition of wild type EGFR in A431 (Fig. 2A). Consistent with the inhibitory effects of AC0010 on EGFR phosphorylation by ELISA assays, immunoblotting analysis confirmed that AC0010 potently inhibited EGFR-Tyr1068 phosphorylation in NCI-H1975 cells, and the selectivity ratio is at 65-fold for NCI-H1975 cells versus A431 cells (Fig. 2B). As expected gefitinib potently inhibited EGFR phosphorylation in the wild-type EGFR cells but not in the EGFR T790M mutation harboring cells (Fig. 2C). In addition to inhibition of EGFR-Tyr1068 phosphorylation, AC0010 inhibited phosphorylation of the downstream targets Akt and ERK1/2, two important kinases involved in cancer cell proliferation and survival, in NCI-H1975 and HCC827 cells (Fig. 2B). Consistent with the EGFR phosphorylation data, AC0010 was much less potent at inhibiting Akt and ERK1/2 phosphorylation in wild-type EGFR cells. This is well correlated with the weak potency of AC0010 against wild type EGFR (Y1068) phosphorylation with the IC$_{50}$ value of 288 nM in A431 cells. Collectively, the results from EGFR phosphorylation assays demonstrate that AC0010 is a novel third-generation EGFR TKI that inhibits EGFR T790M and other sensitive mutations but spares wild type EGFR.

The selectivity of AC0010 was also assessed by testing its activity against a panel of 349 kinases. At a concentration of 1 µM, AC0010 exhibited greater than 80% inhibition in 33 out of 349 unique kinase assays (9.5%) (Supplementary Fig. S2). Kinase targets with greater than 80% inhibition include JAK3, BTK and 5 TEC family members (Supplementary Fig. S2). However, at the cellular level, the kinase inhibitory potency is much less than with the enzymatic assay. For example, much weaker inhibition was seen in BTK and JAK3 cellular assays with IC$_{50}$ values of 59 nM and 360 nM (Supplementary Table S1), demonstrating a favorable selectivity profile of AC0010.
To further evaluate the potential off-target liability of AC0010, it was tested against a selected panel of 55 key molecular targets including receptors, ion channels and transporters (Eurofins ExpresSProfile, Supplementary Table S2). Out of 55 targets tested in the binding assay, 5 showed over 50% inhibition of radioligand binding in the presence of 1 µM AC0010 including Adenosine A3, L-type calcium (Cav1.2) channel, dopamine transporter, 5-HT2A and 5-HT2B (Supplementary Table S3). Cell-based functional assays were carried out to further characterize AC0010 interaction with the above 5 targets, and no inhibition was detected at the cellular level, implying that at pharmacologically relevant concentrations the risk of off-target binding of AC0010 is minimal (Supplementary Table S3).

**AC0010 inhibits EGFR mutant tumor growth but not wild type EGFR tumor growth in xenograft models over extended duration**

Four types of xenograft mouse models including NCI-H1975, HCC827, NIH/3T3_TC32T8 and A431 were used. Repeat daily oral administration of AC0010 at a dose range between 12.5 mg/kg and 200 mg/kg resulted in a dose-dependent growth inhibition of all three tumors with EGFR active mutations and T790M mutation but not with the wild-type EGFR (A431 model) (Fig. 3A and B, Supplementary Fig. S3). At the dose level of 200 mg/kg, tumor regression was observed with the regression rates of 78%, 98%, 25% in NCI-H1975, HCC827 and NIH/3T3_TC32T8 models, respectively. In contrast, gefitinib, at the dose of 100 mg/kg, showed no tumor growth inhibition in the NCI-H1975 and NIH/3T3_TC32T8 models harboring the T790M. AC0010 at the dose of 200 mg/kg did not exhibit any anti-tumor activity in the A431 xenograft model. As predicted, gefitinib showed strong inhibition in A431 xenograft model at the dose of 100 mg/kg (Fig. 3B).

To explore the PK/PD relationship *in vivo*, the plasma and tumor tissues from the AC0010-treated NCI-H1975 xenograft models were examined. For PK analysis, following intravenous administration of 10 mg/kg of AC0010, total body clearance and volume of distribution of AC0010 were estimated to be 5.91 L/h/kg and 14.76 L/kg, respectively. The elimination half-life (t1/2) of AC0010 was about 1.73 hour, indicating AC0010 is rapidly distributed into tissues including tumor tissues. Following oral administration of 12.5 mg/kg, 50 mg/kg and 200 mg/kg of AC0010 for 1 day or 8 consecutive days, AC0010 was absorbed with the T_max of 1 to 2 hours, and bioavailability of 15.9-41.4%. AUC_{0-4} values of AC0010 from plasma were 333, 2623,
and 6774 ng·h/mL on day 1, respectively, and 678, 3476, and 7299 ng·h/mL on day 8, respectively (Fig. 3C and D). To further study the PD of AC0010, the phosphorylation of mutant EGFR in tumor tissues was examined showing complete inhibition by AC0010 after 4 hour treatment at the dose of 12.5 mg/kg, and the inhibition was sustained up to 8 hours (Fig. 3E). At the dose of 50 mg/kg, complete inhibition was achieved after 1 hour treatment and such inhibition was sustained up to 8 hours. At the dose of 200 mg/kg, complete inhibition of phosphorylation was observed in tumor tissues through the measurement period from 1 hour to 24 hours. The dose-dependent inhibition of mutant EGFR phosphorylation in tumor tissues were well correlated with the in vivo efficacy and plasma and tissue concentrations of AC0010 in a parallel study (Fig. 3A, 3C and D). Again, no inhibition of EGFR L858R/T790M phosphorylation was detected in the tumor sample treated with 100 mg/kg gefitinib (Fig. 3E).

Next, the duration of AC0010-induced tumor shrinkage in NCI-H1975 xenograft models was studied (Fig. 3F). In comparison with the vehicle control mice, the tumor growth inhibition rates were 71% and 79% in 12.5 mg/kg and 50 mg/kg of dose groups, respectively after 17 days dosing. In order to achieve the maxim efficacy and evaluate tolerability of AC0010 in long term treatment we select the dose of 500 mg/kg and continued the daily dosing for another consecutive 143 days in the mice previously treated at 12.5 mg/kg and 50 mg/kg. Significant tumor regression was quickly observed in all animals (total 14 mice) after first three doses of AC0010 at 500 mg/kg (Day 21). The tumor volume (TV) became immeasurable from starting TV of 510 mm³, indicating a very strong dose dependent activity against EGFR T790M mutation bearing tumors. During the dosing period of 143 days, 50% of mice showed complete remission of the tumors. Interestingly, tumor relapses were observed in 2 out of 14 mice (14%). The relapses were found on Day 106 and Day 135 post AC0010 dosing and may indicate development of resistance against the AC0010 in NCI-H1975 tumor xenograft model. The mouse with the relapse on Day 106 was sacrificed at Day 115 when the tumor volume reached 660 mm³ from the undetectable tumor. The relapsed tumor tissue was collected and an in vivo resistance cell line, NCI-H1975-P1 was generated. To test the cross resistance of AC0010 with other third-generation EGFR TKIs, NCI-H1975-P1 together with NCI-H1975-AVR1 and NCI-H1975-OSR1, two resistant cell lines induced in vitro by AC0010 and osimertinib, were further investigated. The cross-resistance was seen between
AC0010 and other two third-generation compounds, rociletinib and osimertinib, in these three cell lines (Supplementary Table S4). Interestingly, in NCI-H1975-AVR1 line and NCI-H1975-OSR1 line, AC0010 was more potent than both osimertinib and rociletinib. However such phenomena can be seen in both osimertinib and AC0010 induced resistant cell lines, but not in the resistant cell line generated from relapsed mouse tumor tissues (Supplementary Table S4). Although the duration of tumor growth inhibition by AC0010 was demonstrated, the relationship between duration of EGFR T790M phosphorylation inhibition and tumor growth inhibition/resistance development was not analyzed in this study. A further investigation together with the resistance development and relevant mechanisms studies for exploring functional uniqueness of AC0010 is ongoing and will be reported separately.

**AC0010 and its metabolites show no off-target effects and no skin lesion in animal models**

As indicated previously, AC0010 showed adequate selectivity in kinase panel screening assay and additional 55 targets including receptors, ion-channels and transports (Supplementary Fig S2 and Table S2). In order to further evaluate the safety liability of AC0010 metabolites, we elucidated the structures of six metabolites found in rats, monkey or patients and proposed the AC0010 metabolic pathways (Fig. 4A). Based on preliminary human data, none of these metabolites are observed at exposures greater than 10 percent of total drug-related exposure. We further evaluated three of these Phase I and Phase II metabolites that are observed with the highest exposures in patients, which include: M7 (double bond reduction), MII-2 (cysteine conjugate), and MII-6 (N-acetylation of M4). All three major metabolites exhibited no toxicity against a panel of cell lines (Supplementary Table S5), and showed no activities against either wild-type or mutant EGFR in both cell proliferation and EGFR phosphorylation assays (Fig. 4B). No potent inhibitory activities against insulin-like growth factor 1 receptor (IGF-1R) were detected for M7, MII-2 and MII-6 (Supplementary Table S6). Accordingly, no hyperglycemia was seen in patients receiving AC0010 treatment (Table 1). The bioactive metabolite (M502) of rociletinib showed a strong inhibitory activity against IGF-1R (Supplementary Table S6) and exhibited unexpectedly high exposures in patients (22), which results in hyperglycemia in patients. The major metabolite (AZ5104) of osimertinib showed much more potent activity to both wild type EGFR and mutant EGFR than its parent compound.
osimertinib (21). The lack of activities of the major metabolites of AC0010 against the wild type EGFR and IGF-1R strongly indicates that AC0010 might have a unique safety profile in patients in comparison with osimertinib and rociletinib.

In order to further assess the potential skin toxicity of AC0010, a rat model was used. Rats were administrated daily with AC0010 at 300 mg/kg for 4 weeks, and in control groups gefitinib at 50mg/kg or vehicle control (0.5% MC) was administrated. Results showed that the skin lesions were observed in gefitinib treated group, whereas, no apparent skin damage was found in AC0010 treated group (Supplementary Fig. S4).

**AC0010 is safe and overcomes T790M-induced resistance in NSCLC patients**

In this study, 25 patients were enrolled in a dose escalation study (Supplementary Table S7). Only 1 DLT was observed at dose level of 550 mg/day, where a female patient had developed a CTC-AE grade 3 rash, due to the allergy to AC0010 (Table 1) but not to the inhibition of wild type EGFR. AEs of any grade and of grade 3 or higher were summarized in Table 1. The most common adverse events considered to be drug related were diarrhea (44% of patients), rash (20% of patients), pruritus (16%). Although diarrhea and rash increased in frequency in a dose-dependent manner, the majority of them were grade 1. There was no drug discontinuation in all treated patients. No pneumonitis, hyperglycemia or grade 3 prolongation of the corrected QT interval event was observed in these 25 patients.

Two relapsed NSCLC patients with positive EGFR T790M mutation detected in the biopsy samples after first-generation EGFR TKI treatment were treated with AC0010 at the dose level of 100 mg and 550 mg and radiographic responses were confirmed with both patients (Fig. 5A and B). The first patient was a 59-year old female with stage IV disease. The patient received 6 cycles of pemetrexed plus cisplatin as first-line chemotherapy, followed by the treatments with 2 cycles of pemetrexed, EGFR TKI (gefitinib) for 27 months, 2 cycles of pemetrexed plus avastin, 6 cycles of pemetrexed plus cisplatin and 1 cycle of docetaxel plus capecitabine. The patient was enrolled to receive AC0010 treatment at 100 mg dose cohort. Tumor shrinkage of 20% was detected after first cycle treatment (4 weeks) and 31% after three cycle treatments (12 weeks) (Fig. 5A). The second NSCLC patient was a 48-year male with stage IV disease. The patient received 3 cycles of pemetrexed plus carboplatin regimen as first-line chemotherapy, followed by gefitinib treatment for 41
months until the tumor relapsed. The patient was enrolled to receive AC0010 treatment at 550 mg dose cohort. Tumor shrinkage of 33% was detected after the first cycle treatment and 36% after three cycle treatments (Fig. 5B). Figure 5C shows the plasma pharmacokinetic profiles of the two patients after dosing at 100 mg and 550 mg once per day for 28 days, respectively.
Discussion

In recent clinical studies, the third-generation EGFR inhibitors such as osimertinib and rociletinib are capable of inhibiting mutant EGFR with T790M and produce responses in over 50% of patients who acquired the resistance against first-generation EGFR TKIs (18, 19). Structurally, osimertinib, rociletinib, and previously reported tool compound WZ4002 are pyrimidine based EGFR TKIs (17, 20-21). The new EGFR TKI, AC0010, reported in this study is a pyrrolopyrimidine based third-generation EGFR TKI, which is structurally distinct from the currently reported 3rd EGFR TKIs. Using the docking model, the pyrrolopyrimidine core of AC0010 showed more stable binding than the pyrimidine core in tool compound WZ4002 in a computer-added model, and such stable binding also correlated well with the increase in the inhibitory potency of the mutant EGFR with T790M and a good selectivity over wild-type EGFR (Fig. 2). In both cell-based assays and animal models, AC0010 revealed the unique features of the third-generation EGFR TKI previously reported in other third-generation EGFR TKIs (17, 20-21), which include (1) irreversibly binding EGFR by forming a covalent bound with Cys 797 in the ATP-binding pocket (Supplementary Fig. S1), (2) sparing wild-type EGFR, and (3) overcoming T790M-induced resistance (Figs. 2 and 3). In in vivo duration study, 14 mice bearing the NCI-H1975 tumors were treated with AC0010 daily for 160 days, and tumors in 12 out of 14 mice were inhibited during the 160-day treatment, suggesting the durable inhibition activity of AC0010. However, tumor relapses were detected in two mice at Day 106 and Day 135 indicating acquisition of resistance against AC0010 may have developed in these two mice. Acquired drug resistance was also seen in the clinical studies of pyrimidine-based irreversible EGFR inhibitors such as osimertinib and rociletinib (23-26). The resistance against the newly developed third-generation EGFR TKIs will significantly limit the long-term clinical success of third-generation EGFR TKIs. Results from non-clinical study models indicated that mechanisms by which the third-generation EGFR TKIs including WZ4002, osimertinib and rociletinib induce the resistance were similar and drugs are cross-resistant (27). Emerging clinical data also revealed that the C797S mutation is detected in ~40% of EGFR mutant NSCLC patients with T790M who developed acquired resistance to osimertinib (23). Interestingly, some EGFR resistant mutations induced by pyrimidine-based irreversible EGFR inhibitors, such as Exon19 Del/C797S, are still
sensitive to quinazoline based EGFR inhibitors gefitinib and afatinib (27, 28). Therefore, irreversible EGFR inhibitors with different chemical core structure may reveal different resistant mechanisms. Using resistant cells derived from AC0010 long-term treated xenograft tumors and drug-induced resistant clones from cell cultures, we found that AC0010 showed cross-resistance with other third-generation compounds such as osimertinib and rociletinib. However, our preliminary data showed that the resistant level is different between AC0010 and osimertinib (Supplementary Table S4). Studies to further understand the underlying mechanisms of acquired resistance against third-generation EGFR TKIs with different chemical structures are warranted to help us design better clinical treatment strategies for patients to gain maximum benefits from EGFR-TKI based target therapy.

The preclinical PK/PD study demonstrated that the tumor inhibition of AC0010 is well correlated with duration of inhibition of EGFR phosphorylation in NCI-H1975 tumors (Fig. 3). At the dose of 200 mg/kg, the double mutant EGFR phosphorylation can be completely inhibited for 24 hours resulting in 98% tumor growth inhibition, which suggests that the persistent exposure of AC0010 is required to gain the best therapeutic advantage in NSCLC patients. Based on the efficacy data of three EGFR-mutant xenograft mouse models at 50 and 200 mg/kg which resulted in stable and regressive xenografted tumors, respectively, the human efficacious dose projected based on body surface area conversion factor (12.3) can be predicated to be in the range of 244 and 976 mg per day for a 60 kg human. Notably, in the phase I clinical trial (NCT02274337), a patient was responsive to AC0010 at the dose of 100 mg once per day (Fig 5A). Interestingly, for this responsive case, AC0010 blood exposure was high reaching to AUC$_{0-24h}$ value of 10800 ng·h/mL, which is above blood drug exposure at the effective doses in the mouse model, and close to the drug exposure of the patient at the dose of 550 mg per day (ACU$_{0-24h}$ value, 19500 ng·h/mL), who was also responsive to AC0010 (Fig 5B). Detailed clinical PK study will be reported in separate reports.

The selective inhibition of the mutant EGFR by third-generation EGFR inhibitors greatly improves the on-target adverse effects resulted from the equal-inhibition of both wild type and mutant EGFR by first-generation EGFR TKIs and second-generation EGFR TKIs such as afatinib (29-31). In the rat model, AC0010 showed no skin lesion after 28 day-treatment at the dose as high as 300 mg/kg (Supplementary Fig. S4).
clinical study, although the patient number is still small, much less occurrence of rash (24%) was seen in the patients treated with AC0010 and most of them were grade 1. Both in animal safety studies (data not shown) and in clinical trials no severe off-target effect induced by AC0010 parent drug and its metabolites were seen, which indeed is consistent with weak to no binding of 55 safety-related target screening (Supplementary Table S2) and results from the biological analysis on AC0010 metabolite profile (Fig. 4). Off-target-related AEs were reported in patients who received the treatment of rociletinib (19). The predominant adverse event of rociletinib is hyperglycemia, which occurred in 47% of tested patients and 22% of them are grade 3. The hyperglycemia is caused by a rociletinib metabolite that inhibits IGF-1R (19, 22) (Supplementary Table S6). AC0010 parent compound and three major metabolites do not inhibit IGF-1R (Supplementary Table S6). As a consequence, no hyperglycemia was detected in rats and monkeys in the long-term animal safety studies (data not shown) and in patients enrolled in the phase I dose escalation study (Table 1). Furthermore, the three major metabolites of AC0010 revealed no inhibitory activity against either wild type EGFR or mutant EGFR, in contrast to a metabolite of osimertinib (AZ5104). Indeed, in patients, osimertinib showed frequent wild type EGFR inhibition-related AEs such as skin damage (40%), which might also be resulted from the very potent activities of AZ5104 (21). Minimal effects on IGF1-R, on the other hand, were reported for Osimertinib (32). The different safety profile of AC0010 and its major metabolites strongly suggests that AC0010 is distinct from other two third-generation EGFR inhibitors, osimertinib and rociletinib.

AC0010 is a new third-generation EGFR inhibitor and showed potent activity against EGFR T790M mutation. Due to its distinct structure and metabolite profile, AC0010 might demonstrate unique therapeutic property in future clinical trials and provide another option for patients who develop resistance against first-generation EGFR inhibitors or for combination therapy with other anticancer agents.
Acknowledgements

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References

Tables

Table 1. Adverse events of evaluable-for-safety population in AC0010 dose escalation stage (N=25)

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number of patients (percent)

Abbreviations: CTC, Common Terminology Criteria; AE, Adverse Events; WBC, White Blood Cell.
Figure Legends

Figure 1
AC0010 chemical structure and T790M EGFR binding modes. **A.** Chemical Structure of AC010 maleate salt with dihydrate, which contains a pyrrolopyrimidine core. **B.** The binding modes of WZ4002 and AC0010 in T790M EGFR ATP binding site. Left: X-ray crystal structure of WZ4002 covalently bound to T790M EGFR (PDB: 3IKA). Middle: docking structure of WZ4002 to T790M EGFR. Right: docking structure of AC0010 to T790M EGFR. Schrödinger’s Maestro program was used for structure preparation and docking (see Material and Method for details).

Figure 2
Inhibition of cell proliferation and EGFR phosphorylation. **A.** Comparison of the inhibitory activity of AC0010 to gefitinib and other 3G EGFR TKIs on proliferation using WST assay and EGFR phosphorylation in NCI-H1975, HCC827, A431 and NIH/3T3_TC32T8 cells using ELISA. IC$_{50}$ values shown on log10 scale in nM as an average of three independent experiments ± SEM. **B.** AC0010 inhibition of phosphorylation of EGFR-Tyr1068 and its downstream signaling proteins Akt and ERK in NCI-H1975 cells, and wild-type EGFR cell line A431 using Western Blot Analysis. **C.** Gefitinib inhibition of phosphorylation of EGFR-Tyr1068 and its downstream signaling proteins Akt and ERK in NCI-H1975 cells, and wild-type EGFR cell line A431 using Western Blot Analysis. IC$_{50}$ values are derived from the density of blotting band of the whole cell extracts after 2 hours of compound treatments.

Figure 3
In vivo anti-tumor efficacy and PK/PD in xenograft models. **A.** Inhibition of NCI-H1975 tumor growth by AC0010 and gefitinib. Tumor growth curves were plotted as mean ± SEM (N=8) and the inhibition rates in each treated group were indicated. **B.** Inhibition of A431 tumor growth by AC0010 and gefitinib. Tumor growth curves were plotted as mean ± SEM (N=8). **C.** AC0010 concentrations in plasma from AC0010 treated mice on Day 1 and Day 8. The doses were indicated. PO: oral administration. **D.** AC0010 concentrations in tumor tissues from AC0010 treated mice on Day 1 and Day 8. Each dosing group was indicated. 

26
EGFR phosphorylation in tumor tissues by AC0010 and gefitinib. pEGFR/EGFR values were derived from the densities of blotting bands of phosphorylated EGFR and total EGFR in NCI-H1975 tumor tissues from AC0010 treated mice after a single dose (left top panel) or after the last treatment of 8-day consecutive dosing (right top panel). The values (ratios) are indicated in the y-axis, and the lower number of pEGFR/EGFR indicates stronger inhibition.

F. Duration of anti-tumor efficacy and safety of AC0010 in subcutaneous xenograft NCI-H1975 mouse model. NCI-H1975 tumor bearing mice were orally treated with vehicle control (0.5%MC) and AC0010 at dose levels of 12.5 and 50 mg/kg for 17 days when the TV in vehicle control group reached approximately 2000 mm³. After 17 days dosing, the animals in vehicle control group were sacrificed, whereas animals in AC0010 groups were treated with increased dose at 500 mg/kg once per day for another 143 days. The tumor volume changes following total 160-day treatments of AC0010 were plotted as mean ± SEM.

Figure 4
Proposed AC0010 metabolic pathways and activities of its major metabolites. A. Proposed AC0010 metabolic pathways showing the formation of metabolites through N-demethylation (M1), N-oxidation (M2), N-dealkylation (M4) followed by acetylation (MII-6), reduction (M7), and cysteine conjugation (MII-2). B. Inhibitory activities of AC0010 and its metabolites on cell proliferation by WST assay and EGFR phosphorylation in NCI-H1975 and A431 cells by ELSA. The IC₅₀ values are indicated.

Figure 5
Response of NSCLC patients with T790M acquired mutations to AC0010 treatment and their AC0010 plasma concentrations. A. NSCLC patient received AC0010 treatment at the dose of 100 mg once per day for three cycles (28 days/cycle). The lesions indicated by arrows were shown in the CT images of lung (top panel) and liver (bottom panel). B. NSCLC patient received AC0010 treatment at the dose of 550 mg once per day for three cycles. The lesions indicated by arrows were shown in the CT images of lung (top panel) and liver (bottom panel). C. AC0010 from patients treated at the dose of 100 mg per day and 550 mg per day. AUC₀-2₄h values were 10800 ng·h/mL and 19500 ng·h/mL for patient receiving 100 mg per day (A) and for patient receiving 550 mg per day (B), respectively.
Figure 2

A

Inhibition of Cell Proliferation

Inhibition of EGFR Phosphorylation

B

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Figure 4

A

CYP3A4
CYP2C19
CYP2C9
CYP2D6
CYP1A2

M1

M2

CYP3A4
CYP1A2

M4

N-acetyltransferase

CYPs

Intestinal flora

L-cysteine
GSH
N-Acetyl-L-cysteine

MII

MII-2

AC0010

MII-6

M7

B

Inhibition of Cell Proliferation

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Inhibition of EGFR Phosphorylation

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Figure 5

A

B

Pretreated 4W 12W

C

Concentration (ng/mL)

Time (hr)

100 mg QD D28
550 mg QD D28

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Molecular Cancer Therapeutics

AC0010, an irreversible EGFR inhibitor selectively targeting mutated EGFR and overcoming T790M-induced resistance in animal models and lung cancer patients

Xiao Xu, Long Mao, Wanhong Xu, et al.

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