**YH25448, an Irreversible EGFR-TKI with Potent Intracranial Activity in EGFR Mutant Non–Small Cell Lung Cancer**

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**Abstract**

**Purpose:** Given that osimertinib is the only approved third-generation EGFR-TKI against EGFR activating and resistant T790M mutated non–small cell lung cancer (NSCLC), additional mutant-selective inhibitors with a higher efficacy, especially for brain metastases, with favorable toxicity profile are still needed. In this study, we investigated the antitumor efficacy of YH25448, an oral, mutant-selective, irreversible third-generation EGFR-TKI in preclinical models.

**Experimental Design:** Antitumor activity of YH25448 was investigated in vitro using mutant EGFR-expressing Ba/F3 cells and various lung cancer cell lines. In vivo antitumor efficacy, ability to penetrate the blood–brain barrier (BBB), and skin toxicity of YH25448 were examined and compared with those of osimertinib using cell lines and PDX model.

**Results:** Compared with osimertinib, YH25448 showed a higher selectivity and potency in kinase assay and mutant EGFR-expressing Ba/F3 cells. In various cell line models harboring EGFR activating and T790M mutation, YH25448 effectively inhibited EGFR downstream signaling pathways, leading to cellular apoptosis. When compared in vivo at equimolar concentrations, YH25448 produced significantly better tumor regression than osimertinib. Importantly, YH25448 induced profound tumor regression in brain metastasis model with excellent brain/plasma and tumor/blood ratio under the concentration–time curve value. YH25448 rarely suppressed the levels of p-EGFR in hair follicles, leading to less keratosis than osimertinib in animal model. The potent systemic and intracranial activity of YH25448 has been shown in an ongoing phase II/III clinical trial for advanced EGFR T790M mutated NSCLC (NCT03046992).

**Conclusions:** Our findings suggest that YH25448 is a promising third-generation EGFR inhibitor, which may be more effective and better tolerated than the currently approved osimertinib.

**Introduction**

EGFR-TKI is an established first-line therapy for non–small-cell lung cancer (NSCLC) with activating EGFR mutations, including an exon 19 deletion (Del19) and the L858R mutation (1). Treatment of first-generation (e.g., gefitinib or erlotinib) and second-generation (e.g., afatinib or dacomitinib) EGFR-TKIs have remarkably improved survival in advanced EGFR mutant NSCLC patients (2–5).

Despite dramatic initial tumor response, the majority of patients inevitably experience disease progression after 9 to 13 months of treatment. Molecular mechanisms of acquired resistance to EGFR-TKIs involve drug target alterations (e.g., EGFR T790M mutation) or bypass signaling activations (e.g., MET amplification, ERBB2 amplification). The gatekeeper T790M mutation in EGFR kinase is the most common (approximately 60%) resistance mechanism following first- and second-generation EGFR-TKI treatment (6, 7). Therefore, efforts have been made to target the EGFR T790M mutation, resulting in the development of third-generation EGFR-TKIs, including rociletinib (CO-1686), olmutinib (HM61713), and osimertinib (AZD9291; refs. 8–10). Unlike the first-/second-generation EGFR-TKIs, the third-generation EGFR-TKIs irreversibly inhibit activating EGFR mutations and T790M mutation, while sparing wild-type (WT) EGFR. Unfortunately, clinical developments of rociletinib and olmutinib have been terminated due to their disappointing efficacy and/or safety profile (11). Nevertheless, osimertinib has been successfully developed for NSCLC with T790M mutation and activating EGFR mutations. In AURA3 trial (second line phase III trial), osimertinib demonstrated significant progression-free survival (PFS).
benefit, compared with chemotherapy, in EGFR T790M-mutant NSCLC (12). Furthermore, in FLAURA trial (first line phase III trial; ref. 13), osimertinib significantly improved PFS compared with first-generation EGFR-TKIs. Based on these two pivotal trials, osimertinib has become a standard of care for T790M-mutant NSCLC or a preferred option for treatment-naive EGFR-mutant NSCLC (14).

Although osimertinib is the only currently approved EGFR-TKI indicated for T790M-mutant NSCLC, it is far from being perfect in terms of toxicity. In AURA3 trial (12), osimertinib treatment produced diarrhea and skin rash, albeit grade 1 or 2, in 41% and 34% of patients, respectively. The incidence of these adverse events seemed numerically higher in the FLAURA trial (13). Furthermore, QT prolongation possibly mediated by HER2 inhibitory effects of osimertinib occurred in 4% of patients in the AURA3 and FLAURA trials (12, 13). Importantly, osimertinib in combination with PD-L1 immune checkpoint inhibitor produced high incidence of interstitial pneumonitis, which may be associated with high concentration (~10% of parent) of a metabolite AZS104 (15).

In addition to its toxicity, osimertinib also has parts to be improved due to intracranial efficacy. Because brain is shielded by the blood–brain barrier (BBB), brain is considered a major pharmacologic sanctuary, likely explaining why the brain is a frequent site of failure after clinical benefit with first-/second-generation EGFR-TKIs (8). Indeed, more than 30% of patients with EGFR-mutant NSCLC experience disease progression during EGFR-TKI treatment due to brain metastases (BM; refs. 16, 17). Moreover, isolated BM often occurs in the presence of continued systemic control. Brain-to-plasma concentration ratio of first-/second-generation EGFR-TKIs is very low, and this suggests that frequent BM during EGFR-TKI treatment is likely due to poor BBB penetration and brain exposure with EGFR-TKIs (18, 19).

Although preclinical data demonstrated greater BBB penetration and brain exposure with osimertinib than with gefitinib, rociletinib, or afatinib (20), no prospective clinical validation has been performed. Furthermore, osimertinib has been known to be a substrate for both MDR1 (P-gp) and BCRP, which are efflux transporters of drugs at BBB (20). Therefore, given the high incidence of BM and poor prognosis in EGFR-mutant NSCLC (21), there is a clinical need for novel EGFR-TKIs with improved efficacy against brain lesions.

Here, we demonstrated superior antitumor activity with potent BBB penetration and better skin toxicity of YH25448, a novel mono-anilino–pyrimidine irreversible mutant-selective EGFR-TKI, compared with osimertinib, using various in vitro cell lines and in vivo mouse models. We also presented a case with T790M-mutant NSCLC who exhibited a dramatic systemic and intracranial response to YH25448 in ongoing phase I/II clinical trial (NCT03046992; ref. 26).

Materials and Methods

Cell cultures and reagents

H2291 and H1975 cells were purchased from the ATCC. The Ba/F3 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures. PC9 and PC9GR cells were provided by J.C. Lee (Korea Institute of Radiological and Medical Science, Seoul, Korea). All cell lines were maintained in RPMI1640 medium supplemented with 10% FBS and 1% solution of antibiotics in a humidified incubator with 5% CO2. All reference compounds were purchased from Selleckchem, except YH25448, which was provided by Yuhan Corporation.

Patient-derived cells

YU-1098, YU-1153, YU-1150, YU-1092, YU-1099, and YU-1097 cell lines were derived from malignant effusions of patients with NSCLC. Patient-derived cells (PDC) were initially cultured on collagen-coated plates in ACL4 medium supplemented with 5% FBS. YUX-1024 cell line was derived from its PDX model. The cells maintained the driver oncogenes that were observed in the patients. When cells were enriched in an epithelial cell adhesion molecule (EpCAM)-positive cell population with a purity of over 95%, cells were subjected to further assays. All patient samples were obtained after written informed consent from the patients using protocols approved by the institutional review board.

Kinase assay

Cell-free kinase assays were conducted using Lance Ultra time-resolved fluorescence energy transfer (TR-FRET) technology from Perkin-Elmer. Briefly, each EGFR enzyme WT, single mutant (Del19, L858R, and T790M), double mutant (L858R/T790M), Del19/T790M), Her2 or Her4, serial diluted EGFR inhibitors, substrate of ULight-poly-GT peptide and variable amounts of ATP (8.5–1,088 μmol/L) were mixed in kinase assay buffer (50 mmol/L, HEPES pH 7.5, 10 mmol/L MgCl2, 1 mmol/L EGTA, 2 mmol/L DTT, and 0.01% Tween-20) and were added to a 96-well plate. Kinase reactions were incubated at room temperature for 1 hour and then stopped by the addition of 5 μL of 10 mmol/L EDTA. The specific Europium-labeled-anti-phosphopeptide antibody (Perkin-Elmer) diluted in LANCE detection buffer was then added to a final concentration of 2 mmol/L. After 30-minute incubation, the LANCE signal was measured on an EnVision Multilabel Reader (Perkin-Elmer). Excitation wavelength was set at 320 nm and emission monitored at 615 nm (donor) and 665 nm (acceptor). The IC50 values were determined using GraphPad Prism (Ver. 5, GraphPad Software Inc.).

Antiproliferation assay

Human NSCLC cell lines or PDCs with an EGFR mutation were incubated with test compounds. Cells were seeded onto 96-well
plates in 100 μL. After treatment with compounds for 72 hours, cell viability was measured by quantifying the total amount of ATP using the CellTiter-Glo 2.0 Assay Kit (Promega) per the manufacturer’s instruction. Dose–response curves were fitted to the data using the GraphPad Prism (Ver. 5, GraphPad Software Inc.).

Antibodies
Primary antibodies specific for p-EGFR (Y1068; 3777), EGFR (4267), p-ERK (T202/Y204; 4370), ERK (9107), p-AKT (S473; 9271), and AKT (9272) were purchased from Cell Signaling Inc. (Danvers, MA).

H1975 subcutaneous implantation and brain metastasis (BM) model
H1975-luc human NSCLC cells were implanted into the right flank and/or brain of female BALB/c nude mice. The tumor burden of intracranial lesions and the tumor size in the right flank were measured using a Bli technique with a real time in vivo imaging system (IVIS Spectrum; Caliper Life Sciences) and a digital calipers (Mitutoyo Corp., respectively. Tumor-bearing mice were treated with a once-daily oral dose of YH25448 or osimertinib from 13 days postimplantation.

In vivo pharmacodynamic study
In the H1975 human NSCLC subcutaneous xenograft model, tumor tissues treated with vehicle, YH25448, or osimertinib (3 or 10 mg/kg) were collected at 2 and 24 hours after 14 days of treatment, and EGFR downstream signals were evaluated by immunoblotting.

Pharmacokinetics
Blood, tumor, and brain samples were collected from the animals at designated time points, and the concentration of YH25448 was determined using a validated LC/MS-MS method.

Patients
The clinical study (NCT03046992) was approved by appropriate institutional review boards at each participating sites and conducted in accordance with good clinical practice guidelines and the ethical principles of the Declaration of Helsinki. All patients provided written informed consent.

Statistical analysis
All data are presented as the mean ± SEM. Data were analyzed by one-way ANOVA, followed by the Dunnett test or Student t test. Survival analysis was performed using a Kaplan–Meier survival curve and a log-rank test comparing each group.

Results
YH25448 is a novel irreversible inhibitor selectively targeting mutant EGFRs
We screened a novel series of highly potent small-molecule inhibitors, which selectively target EGFR tyrosine kinases with sensitizing and T790M mutations over WT EGFR. The compounds targeted the Cys797 residue in the ATP-binding site of the EGFR kinase domain and were irreversibly bound to it through a covalent bond (Fig. 1A). In particular, the aminopyrimidine moiety of YH25448 bound to the hinge residue Met793 through hydrogen bonding. The phenyl substituent in the pyrazole ring pointed to the gatekeeper residue Met790. The morpholine ring faced the solvent exposure region, and the meta-acrylamide formed a covalent bond with Cys797.

To investigate YH25448 in vitro profiles for various targets, we performed kinase profiling of a single-dose inhibition assay against 304 kinases, including WT and mutant EGFRs. The levels of inhibition of kinase activities by 1 μmol/L YH25448, tested in duplicate, were compared with those of the DMSO control (0% inhibition). The average values were plotted, with a 65% cutoff value, in a representation of the human kinase phylogenetic tree using TREESpot from DiscovErx (Fig. 1B). Although osimertinib displayed a broad inhibition profile against 17 kinases, YH25448 had a relatively higher kinase selectivity profile, than osimertinib, based on the finding that it inhibited 11 of the 304 kinases tested (Fig. 1C). In particular, YH25448 at a concentration of 1 μmol/L potently inhibited kinase activities of EGFR T790M, L858R/T790M, and L858R, and mitogen-activated protein kinase kinase MAP3K9 (MLK1), with the respective percentages of inhibition exceeding 90% and an S-score of 0.03. EGFR T790M was the only protein kinase inhibited at a very high level (above 98%). EGFR L858R/T790M and L858R were also found among the hits, with 98% and 91% inhibition, respectively. Although YH25448 showed a high selectivity and a strong activity against the various mutant EGFRs, it exhibited less activity against WT EGFR, compared with that of osimertinib (Fig. 1C). This finding suggested that YH25448 might decrease off-target side effects compared with that of osimertinib. To validate the results of kinase profiling, we carried out cell-free in vitro kinase inhibition assays for mutant EGFRs and the ErbB family with serially diluted YH25448 and osimertinib (Table 1A). Compared with those of osimertinib, YH25448 showed similar or greater potencies in the inhibition of kinase activities of mutant EGFRs, including Del19, L858R, and T790M mutants, with the half-maximal inhibitory concentration (IC50) values ranging from 1.7 to 20.6 nmol/L. Individual EGFR kinase inhibition assay for rare mutant EGFRs that are shown in NSCLC demonstrated that the active metabolite of YH25448 (YH26334) effectively inhibited the rare EGFR mutants at low concentration. On the contrary, we found that the activity of YH25448 (YH26334) for WT EGFR was lower compared with those of osimertinib (AZ5104), indicating that higher selectivity of the active metabolite of YH25448 for mutant EGFR might reduce the possibility of adverse effect of the drug (Table 1B).

Consistent with the data from the cell-free in vitro kinase assay, YH25448 significantly and dose dependently inhibited the EGFR phosphorylation in Ba/F3 cells harboring the Del19, L858R, Del19/T790M, and L858R/T790M mutations (Fig. 1D). The viability of the Ba/F3 cells with the mutant EGFRs was remarkably reduced by YH25448, with the mean IC50 values ranging from 3.3 to 5.71 nmol/L (Fig. 1E), which were similar to those obtained for osimertinib-treated cells (Table 2). As previously reported (22, 23), the first-generation TKI gefitinib was overall ineffective at inhibiting the growth of Ba/F3 cells with T790M mutant EGFRs.

Collectively, these findings suggest that YH25448 is a novel mutant-selective EGFR inhibitor with selectivity and potency at least comparable to osimertinib, which is a currently approved third-generation EGFR-TKI.

YH25448 significantly suppresses EGFR-mutant lung cancer in vitro and in vivo
To investigate whether YH25448 could effectively block EGFR and its downstream signaling pathways, we treated EGFR-mutant
Figure 1.
Characterization of the EGFR mutant-selective irreversible inhibitor YH25448. A, Crystal structure of YH25448 in a complex with the EGFR T790M mutant (top). Chemical structure of YH25448 (bottom). B, Kinome tree for YH25448 generated using DiscoveRx TREEspot version 4. Sizes of the red circles are proportional to the percentage inhibition at the test concentration (1 μmol/L): largest circle, 99% inhibition; medium circle, 90% to 99% inhibition; smallest circles, 65% to 90% inhibition. C, Comparison of YH25448 and osimertinib selectivity profiles against approximately 320 kinases. The kinases listed were subject to over 65% inhibition by each compound, compared with DMSO. D, Ba/F3 cells overexpressing the indicated EGFR mutant were treated with YH25448 or osimertinib for 6 hours at the indicated concentrations. p-EGFR levels were detected by Western blot analysis. E, Viability of Ba/F3 cells was determined via Cell Titer Glo. YH25448 was treated for 72 hours.
lung cancer cells with YH25448 at different concentrations for 6 hours (Fig. 2A). YH25448 significantly suppressed EGFR downstream signaling, including phospho-EGFR (p-EGFR), phospho-AKT (p-AKT), and phospho-ERK (p-ERK). The inhibitory effects of YH25448 were stronger than or comparable to those of osimertinib under the same conditions. In contrast, YH25448 did not inhibit EGFR signaling in a WT EGFR cell line (H2291). We further explored whether the blockade of EGFR signaling by YH25448 in lung cancer cell lines correlated with its inhibitory effects on cell proliferation in various NSCLC cell lines including six PDCs harboring with sensitizing EGFR mutations [Del19 (YU-1098), L858R (YUX-1024)], resistant mutations [Del19/T790M (YU-1153), L858R/T790M (YU-1150), Del19/T790M/C797S (YU-1097)], and uncommon mutations [L861Q (YU-1092), G719C/S768I (YU-1099); Fig. 2B; Sanger sequencing results shown in Supplementary Fig. S1A]. The cell viability data showed that YH25448 effectively and significantly inhibited the proliferation of Del19- and/or T790M-mutant NSCLC cells at lower concentrations than those needed to inhibit cells with other EGFR-TKIs (IC50 values ranging from 1.9 to 12.4 nmol/L against PC9, YU-1098, PC9GR, YU-1153, and YU-1150), while showing much less activity against cells with WT EGFR (H2291; Fig. 2B). YH25448 exhibited modest antiproliferative effects in PDCs (YU-1092 and YU-1099) harboring uncommon EGFR mutations with IC50 values of 42.5 and 1224.7 nmol/L, respectively. As previously reported [24], L861Q-mutant lung cancer cells (YU-1092) showed significant inhibition to afatinib. YU-1097 derived from a patient with osimertinib-resistant NSCLC showed resistance to YH25448 and other third-generation EGFR-TKIs. Treatment with YH25448 induced the apoptosis in EGFR-mutant cell lines, as demonstrated by an increase in the cleaved Bim-EL protein or activated caspase-3/7, in H1975 and PC9 cells (Supplementary Fig. S1B and S1C). To determine whether YH25448 is effective in vivo, H1975 tumor-bearing mice were randomly grouped and dosed orally, once daily, with the vehicle and 1, 3, and 10 mg/kg YH25448 or osimertinib for a period of 13 days. YH25448 significantly shrunk the tumor volume within a very short period after the first administration at doses as low as 3 and 10 mg/kg/day (Fig. 2C). After 13-day treatment at a dose of 3 mg/kg treatment, YH25448 suppressed 86.85% of the tumor growth compared with only 7.24% tumor regression in the osimertinib group. Surprisingly, near complete tumor regression (90%) was achieved at a higher dose, 10 mg/kg, without any body weight changes (Supplementary Fig. S1D). A similar degree of tumor shrinkage was observed after administration of 3 mg/kg YH25448 to a PC9 tumor-bearing xenografts after 25 days (Supplementary Fig. S1E and S1F). As shown in Fig. 2D and E, the complete inhibition of p-EGFR was observed as early as 2 hours post-dose in tumor samples from the xenografts, which were treated with YH25448 (3 or 10 mg/kg) for 3 days. The complete inhibition of p-EGFR activity was maintained until 24 hours post-dose. Although YH25448 treatment at 3 mg/kg resulted in partial inhibitions of

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<th>Table 1. YH25448 selectively inhibits EGFR mutant</th>
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<td><strong>A</strong></td>
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<tr>
<td>EGFR kinase genotype</td>
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<tr>
<td>Wild type</td>
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<tr>
<td>Del19</td>
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<td>L858R</td>
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<td>Del19/T790M</td>
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IC50 values of YH25448 and osimertinib. Each value represents the mean ± SEM calculated from at least two independent experiments.

*The active metabolite of YH25448 (YH26334) is present in humans at levels approximately 3% those of the parent (NCT03046992).

**The active metabolite of osimertinib (AZ5104a) is present in humans at levels approximately 10% those of the parent.

<table>
<thead>
<tr>
<th>Ba/F3 EGFR</th>
<th><strong>IC50, nmol/L</strong></th>
<th><strong>Osimertinib</strong></th>
<th>Gefitinib</th>
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<tr>
<td>Wild type</td>
<td>722.7</td>
<td>519.1</td>
<td>585.3</td>
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<td>Del19</td>
<td>3.5</td>
<td>3.5</td>
<td>10.2</td>
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<td>L858R</td>
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<td>L858R/T790M</td>
<td>5.7</td>
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<td>7628.2</td>
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IC50 values (nmol/L) of YH25448, osimertinib, and gefitinib were calculated.
Figure 2.

A, Immunoblot analysis was performed for EGFR, AKT, and ERK expression after YH25448 or osimertinib treatment for 6 hours at the indicated concentrations in lung cancer cell lines. Antitumor effects of YH25448 in EGFR T790M mutant NSCLC (H1975, L858R/T790M)-bearing mice (n = 7/group). B, IC_{50} values for the indicated EGFR-TKIs in the cells (Treatment of the drugs for 72 h). C, Antitumor effects of YH25448 in H975 (L858R/T790M) tumor-bearing mice (n = 7/group). Mice were treated with YH25448, osimertinib, or vehicle once daily for 2 weeks after the tumor volume reached 100 mm³. Data represent the mean ± SEM (n = 7/group). *, P < 0.001 vs. vehicle control; #, P < 0.0001 vs. osimertinib at the same dose. D and E, Tumor lysates of vehicle-, YH25448-, or osimertinib-treated H975 xenograft mice for 3 days were harvested at the indicated time point after the last treatment. Lysates were subjected to immunoblotting for p-EGFR (Y1068), p-AKT, and p-ERK1/2.
When bioluminescent imaging (BLI) signals reached in vivo photons/second, the mice were assigned to different treatment groups. Comparing the inhibitory efficacy of YH25448 with that of osimertinib at different doses of YH25448 (Supplementary Fig. S2C). Based on positive substrate criteria of efflux ratio (ER > 2) and a previous report (20), whereas osimertinib was a substrate of both BCRP and MDR1 (P-gp), YH25448 was not a substrate of BCRP and only a weak substrate of MDR1. These results suggested that YH25448 may be less affected by efflux transporters.

The results suggested that YH25448 might be more effective than osimertinib in treating metastatic brain tumors from lung cancer.

YH25448 demonstrates potent antitumor activity in an EGFR-mutant patient-derived xenograft model and in an EGFR T790M-mutant NSCLC patient

To further validate antitumor activity of YH25448 in vivo, PDX models established from an EGFR Del19 mutant NSCLC patient were treated orally with YH25448 and osimertinib at 25 mg/kg once daily for 20 days. YH25448 exhibited more potent tumor regression than osimertinib (87.5% and 83.6% inhibition of tumor growth, respectively; P < 0.001; Fig. 4A). Both treatments were well tolerated without a significant body weight loss (Fig. 4B).

In an ongoing phase I/II study of YH25448 in EGFR mutant NSCLC progressing on prior EGFR-TKI, dramatic and durable responses have been observed even at the lowest dose (20 mg, once daily; ref. 26). The first patient was a 62-year-old Korean male with EGFR L858R/T790M mutant lung adenocarcinoma who had progressed following 1-month treatment of gefitinib. The patient was treated with YH25448 20 mg orally once daily, resulting in 51% tumor reduction following 6-week treatment (Fig. 4C). During the treatment, the patient reported no significant adverse events and response lasted for over 8 months. The second patient was a 37-year-old Korean male with EGFR Del19/T790M mutant lung adenocarcinoma who had experienced systemic progression including brain following 8-month treatment of AZD3759 and 2-month treatment of gefitinib. Upon YH25448 40 mg treatment, tumor assessment confirmed a 49.3% intracranial tumor reduction, with a 12.7% extracranial tumor reduction (overall 25.9% tumor reduction) without significant adverse events (Fig. 4D).

YH25448 shows less selectivity for WT EGFR and less skin toxicity than osimertinib does

EGFR is widely expressed in undifferentiated, proliferating keratinocytes in the epidermis and in the outer layer of hair follicles (27). Therefore, cutaneous toxicities such as acneiform rash, itching, and xerosis are most commonly associated with EGFR-TKI treatments including osimertinib (28–31).

Following the observation that both YH25448 and osimertinib did not exhibit severe skin toxicity at 10 mg/kg YH25448 (clinical equivalent to 240 mg once daily) and 25 mg/kg osimertinib (clinical equivalent to 60 mg once daily), we escalated the dose up to 50 and 75 mg/kg to demonstrate a wider therapeutic index of YH25448 over osimertinib.

We observed that the YH25448-treated (50 mg/kg) H1975-bearing BALB/c nude mice appeared phenotypically normal. On the contrary, one of the five mice treated with osimertinib (50 mg/kg) showed minimal signs of keratosis on the skin (Fig. 5A). We further extended the experiment with the treatment of mice with a
Figure 3.
Intracranial antitumor effects of YH25448 in the H1975-luc BM model. A and B, An intracranial tumor growth model was established with BALB/c nude mice using H1975-luc cells. Two weeks after H1975-luc injection, animals were treated with YH25448 or osimertinib once daily. BLI was used to detect intracranial tumor growth in vivo. Data represent the mean ± SEM (n = 7/group). *, P < 0.001 vs. vehicle control; #, P < 0.01; ###, P < 0.05 vs. osimertinib at the same dose. C, Histopathologic examination of brain sections obtained following H1975-luc intracranial implantation. H&E staining and immune histochemical staining for Ki-67. Scale bars, 25 μm. D, Kaplan-Meier survival curves of H1975-luc cells in mice treated with YH25448 or osimertinib at 10 and 25 mg/kg, once daily, respectively. E, Plasma, intracranial tumor, and brain tissue samples obtained at 4, 8, and 24 hours post dosing of YH25448 (10 mg/kg) on day 21 post-dose were analyzed with a validated LC/MS-MS method.
75 mg/kg dose of each drug. Forty percent of the mice (2/5) in the YH25448 treatment group showed minimal keratosis, whereas severe keratosis on the face, neck, and abdomen was observed in all osimertinib-treated mice (Fig. 5A–C; Supplementary Fig. S3A). To determine whether these phenotypical differences were due to less selectivity of YH25448 for WT EGFR, we performed histologic examination of the skin from the mice in both groups. As shown in Fig. 5D and Supplementary Fig. S3B, at 75 mg/kg dose, YH25448 rarely suppressed the levels of p-EGFR (WT) in hair follicles, whereas osimertinib strongly reduced the
phosphorylation of EGFR (WT). Based on the results of hematoxylin and eosin (H&E) staining, the epidermis of the osimertinib-treated mice was significantly thicker than that of the YH25448-treated mice (Fig. 5E, top and 5F). From the results showing that a higher H&E staining intensity observed in the epidermis of osimertinib-treated mice than that of YH25448-treated mice (Fig. 5G), we predicted strong infiltration of immune cells by osimertinib, compared with YH25448 did. The blockade of EGFR leads to the upregulation of chemokine expression and massive infiltration of T cells and macrophages into the skin, which causes skin inflammation (32). Because BALB/c nude mice were used for these experiments, we were only able to test the levels of macrophages (detected with F4/80 antibody) infiltrated into the dermis in each group. Consistent with the above data,
YH25448 did not affect the macrophage infiltration in the epidermis (Fig. 5E; bottom). However, osimertinib enhanced the macrophage infiltration at the same dose, indicating that the inflammation caused by macrophage occurred in the osimertinib-treated mice, which thickened the epidermis.

Taken together, these data strongly suggest that YH25448 has less selectivity for WT EGFR, resulting in less cutaneous toxicity than osimertinib at high doses.

Discussion

In this study, we comprehensively analyzed the selective antitumor activity of YH25448 against EGFR sensitizing- and T790M-mutant NSCLC using kinase assays, various patient-derived cell line xenografts, and patient cases enrolled in an ongoing phase I/II study. Our findings suggest that YH25448 had a superior preclinical activity and safety to osimertinib, which is a current standard of care for T790M-mutant NSCLC (33, 34).

Given the frequent CNS progression while EGFR-TKI treatment (16, 17), AUC-based brain-to-plasma ratio (0.9) and intracranial tumor-to-plasma ratio (7.0) of YH25448 shown in our study is noteworthy (Fig. 3E). These outstanding in vivo PK parameters were supported by superior CNS activity of YH25448 in H1975-Luc BM mouse models (Fig. 3A and B) and a dramatic CNS response in a clinical case report (Fig. 4D). Furthermore, we demonstrated that YH25448 was not a substrate of BCRP and only weakly a substrate of MDR1, suggesting that it may be less affected by efflux transporters than osimertinib. Additionally, given that YH25448 at 10 mg/kg in murine approximates to the recommended dose of 120 mg in clinical phase I/II study. Our mouse safety data, YH25448 demonstrated a good safety profile across all dose levels (95% CI, 21.1–78.9). Consistent with our mouse safety data, YH25448 demonstrated a good safety profile and tolerability without dose-limiting toxicity at the maximum tolerated dose (80 mg once daily). Taken together, these results suggested that at least equivalent activity of YH25448 for CNS metastasis may be expected to that of osimertinib.

In addition, YH25448 has potential advantage over osimertinib or other third-generation EGFR-TKIs in terms of toxicity. Because of the important role of EGFR signaling in skin, cutaneous toxicities have been frequently reported with the first- or second-generation EGFR-TKIs. The most frequent cutaneous toxicities are papulopustular rash, itching, and inflammation around the nails, affecting 49% to 89%, 10% to 20%, and 14% to 61%, respectively (2, 3, 5, 35, 36). Although these cutaneous toxicities seemed lower with osimertinib, it also produced papulopustular rash in 34% to 58%, itching in 13% to 18%, and inflammation around the nails in 22% to 36%, respectively (12, 13). Although rarely life-threatening, these cutaneous toxicities cause significant physical and psycho-social discomfort, which may lead to a decreased quality of life and modification or discontinuation of treatment (37). In our study, we observed that YH25448 produced less keratosis on the skin than osimertinib in H1975-bearing BALB/c nude mice (Fig. 5A–C; Supplementary Fig. S3A). By histologic examination, we demonstrated that the minimal skin toxicity in YH25448-treated mice was due to less selectivity of YH25448 for WT EGFR (Fig. 5D; Supplementary Fig. S3B). Indeed, unlike osimertinib, YH25448 rarely suppressed the levels of WT p-EGFR in hair follicles, leading to less macrophage-mediated cutaneous inflammation in the dermis (Fig. 5E, bottom and 5G). Although the dose of YH25448 and osimertinib used in the skin toxicity experiments was higher than the clinical equivalent doses (10 mg/kg YH25448 and 25 mg/kg osimertinib, respectively), these data supports a wider therapeutic index of YH25448, compared with osimertinib, in terms of skin toxicity. Based on our results, we expected less cutaneous toxicities in patients treated with YH25448 than those with osimertinib, which lead to improved patient’s quality of life.

In addition to cutaneous toxicity, YH25448 may be associated with less cardiac dysfunction and hyperglycemia due to lack of activity on HER2 and type 1 insulin-like growth factor receptor (IGF-1R; Fig. 1C). In contrast, changes in QT interval was reported with osimertinib in 10% due to HER-2 inhibition and/h ERG channel inhibition, whereas hyperglycemia was reported with rociletinib in 47% due to IGF-1R inhibition (10, 13). Importantly, our preclinical findings were translated into promising efficacy in the phase I/II study in advanced EGFR-mutant NSCLC with acquired resistance to EGFR-TKIs (26). This clinical trial included dose escalation and dose-expansion cohorts. In the expansion cohort, tumor biopsies prior to treatment were required for central confirmation of EGFR T790M status. Among 38 patients enrolled in the dose-escalation cohorts, no dose-limiting toxic effects occurred at the doses evaluated. An additional 89 patients were treated in five expansion cohorts. Among 99 patients with T790M mutation, objective response rate (ORR) was 66% (95% confidence interval [CI], 56.9–75.2) across all dose levels (20–320 mg once daily). In particular, ORR was 71% at a dose of 240 mg once daily whereas deep responses were seen in low doses (40–120 mg). In patients with BM, the intracranial ORR was 50% across all dose levels (95% CI, 21.1–78.9). Consistent with our mouse safety data, YH25448 demonstrated a good safety profile and tolerability without dose-limiting toxicity at 240 mg. Although it always needs a great caution when comparing results between different clinical trials, the frequency of overall treatment-related adverse events (AE) and treatment-related grade 3 to 5 AEs were numerically lower than osimertinib phase II/II trial (69% vs. 80% and 3% vs. 13%; refs. 9, 26). In addition, the incidences of skin rash and diarrhea which are commonly observed in EGFR-TKIs were lower in YH25448 compared with osimertinib, respectively (26% vs. 40% for any grade skin rash and 13% vs. 47% in any grade diarrhea). Most AEs of patients treated with YH25448 were grade 1 or 2 (9, 26). YH25448, as a mono-anilino–pyrimidine compound structurally similar to osimertinib, irreversibly binds to the EGFR kinase by targeting the cysteine-797 residue in the ATP binding site via covalent bond formation (Fig. 1A). The substitution of cysteine with serine at codon 797 (C797S) is a common resistance mechanism to osimertinib, because C797S mutation prevents formation of a covalent bond between osimertinib and the thiol group (~SH) of cysteine in the EGFR C797 residue (38). Therefore, considering the similar binding mode, C797S mutation is anticipated to confer resistance to YH25448. As expected, osimertinib-resistant, C797S-mutant YU-1097 cells was cross-resistant to YH25448 (Fig. 2B).

In conclusion, we demonstrated that YH25448 is a novel, highly potent, BBB-penetrating, irreversible EGFR-TKI, which selectively blocks EGFR sensitizing and T790M mutations. The comparable potency and CNS activity, with the possibility of a wider therapeutic index, of YH25448 to osimertinib strongly support further clinical development of YH25448 for the treatment of EGFR-mutant NSCLC.

Disclosure of Potential Conflicts of Interest

S. Oh is an employee of Yuhan Corporation. No potential conflicts of interest were disclosed by the other authors.
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