TAS6417, A Novel EGFR Inhibitor Targeting Exon 20 Insertion Mutations

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Abstract

Activating mutations in the EGFR gene are important targets in cancer therapy because they are key drivers of non–small cell lung cancer (NSCLC). Although almost all common EGFR mutations, such as exon 19 deletions and the L858R point mutation in exon 21, are sensitive to EGFR-tyrosine kinase inhibitor (TKI) therapies, NSCLC driven by EGFR exon 20 insertion mutations is associated with poor clinical outcomes due to dose-limiting toxicity, demonstrating the need for a novel therapy. TAS6417 is a novel EGFR inhibitor that targets EGFR exon 20 insertion mutations while sparing wild-type (WT) EGFR. In cell viability assays using Bx/F3 cells engineered to express human EGFR, TAS6417 inhibited EGFR with various exon 20 insertion mutations more potently than it inhibited the WT. Western blot analysis revealed that TAS6417 inhibited EGFR phosphorylation and downstream molecules in NSCLC cell lines expressing EGFR exon 20 insertions, resulting in caspase activation. These characteristics led to marked tumor regression in a patient-derived xenograft model. Furthermore, TAS6417 provided a survival benefit with good tolerability in a lung orthotopic implantation mouse model. These findings support the clinical evaluation of TAS6417 as an efficacious drug candidate for patients with NSCLC harboring EGFR exon 20 insertion mutations.

Introduction

Somatic mutation of the EGFR is a major oncogenic driver (1) and is present in approximately 30% to 50% and 10% to 20% of non–small cell lung cancer (NSCLC) in Asians and in Americans and Western Europeans, respectively (2–5). Activating mutations in the EGFR kinase domain induce ligand-independent constitutive activation and subsequent downstream molecule phosphorylation, leading to cancer cell growth and survival (6, 7). Because of its important role in cancer, mutant EGFR is an important therapeutic target for lung cancer. Among a wide variety of somatic mutations in EGFR, exon 19 deletion mutations and L858R substitution mutation in exon 21 are most common, accounting for over 80% of mutations (8, 9). These common mutations are sensitive to ATP-mimetic EGFR tyrosine kinase inhibitors (TKI) in preclinical models (10–12), and patients with NSCLC harboring these drug-sensitive mutations show significant responses to EGFR-TKI therapies, including gefitinib, erlotinib, and afatinib, compared with standard chemotherapies (13–18).

The next largest proportion of EGFR mutations is a family of exon 20 insertions, which accounts for roughly 4% to 13% of all EGFR mutations in patients with NSCLC (9, 19–22); these mutations consist of in-frame insertions of 3 to 21 base pairs predominately within the range of codons 762 to 774. These insertions, as well as exon 19 deletions and L858R mutation, have characteristics of oncogenic driver mutations, which induce constitutive activation and cell transformation in preclinical studies. However, in contrast to exon 19 deletions and L858R mutation, exon 20 insertions do not sensitize the kinase domain to EGFR-TKIs, behaving as intrinsic resistant mutations (6, 23–25). Furthermore, patients with NSCLC harboring these mutations exhibit poor clinical responses to monotherapy with afatinib (26), gefitinib (25, 27), and erlotinib (21, 25, 28) because plasma concentrations of these drugs in clinical settings are kept low by dose-limiting toxicity caused by wild-type (WT) EGFR inhibition (22, 29, 30), indicating the importance of development of an EGFR inhibitor targeting exon 20 insertions.

Therefore, clinical trials for EGFR-TKIs designed to overcome exon 20 insertion–mediated resistance in patients are now in progress. AP32788, a TKI designed to inhibit exon 20 insertions in the EGFR or HER2 genes, is currently in a phase I/I clinical trial (NCT02716116) to determine a recommended phase II dose in the dose escalation cohort and the overall response rate in the expansion cohorts. In addition, interventional studies against EGFR exon 20 insertions have been initiated for poziotinib (NCT03066206 and NCT03318939), which is a pan-ErbB inhibitor, and are planned for osimertinib (NCT03191149), which has received approval for patients with NSCLC harboring T790M acquired resistance mutations in the exon 20 region.

Note

Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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In this study, we report the preclinical characterization of TAS6417, a novel EGFR-TKI with a unique scaffold fitting into the ATP-binding site of the EGFR hinge region. The structure differs from that of all previously known small-molecule EGFR-TKIs, and our results demonstrate the selective potent inhibitory effect of TAS6417 on EGFR exon 20 insertions compared with WT EGFR, leading to an efficacious anticancer effect in several in vivo models.

### Materials and Methods

#### Materials

TAS6417 was synthesized at Taiho Pharmaceutical Co., Ltd. The structure was confirmed by ESI-MS and NMR, and its purity exceeded 99% as measured by HPLC. Afinatin was purchased from Selleck Chemicals, and erlotinib from LC Laboratories.

#### Mass spectrometry analysis

The human recombinant EGFR D770_N771insNPG was purchased from Carna Biosciences. The recombinant enzyme at 1 μmol/L was incubated with 50 μmol/L TAS6417 at 37°C for 2 hours. After reduction and alkylation with DTT (Thermo Fisher Scientific) and iodoacetamide (Thermo Fisher Scientific), the protein was digested with trypsin/Lys-C (Promega) followed by Asp-N (Thermo Fisher Scientific). The peptide–compound complex was detected with an LC/MS system consisting of an Acquity UPLC I-Class and Xevo G2-S QT of mass spectrometer equipped with an ESI ion source (Waters Corporation) using an Aeris WIDEPORE 3.6 μm C18 150 × 2.1 mm column (Phenomenex) at a flow rate of 0.3 mL/minute and a column temperature of 50°C. The gradient of mobile phase A [water, 0.1% formic acid (v/v)] and B [acetonitrile, 0.1% formic acid (v/v)] was performed as follows: 2% B for 3 minutes, 2% to 50% B for 37 minutes, 50% to 90% B for 10 minutes. The data were acquired in MSE mode with collision energy of 4 eV for low-energy scans and ramped from 15 to 40 eV for high-energy scans, and further data analysis was performed using BioPharMaLynxt 1.3.3 software (Waters Corporation).

#### Cell culture

The NCI-H1975, HCC827, NCI-H23, NCI-H460, and NIH/3T3 cell lines were obtained from ATCC, and the Ba/F3 and PC-9 cell lines were from RIKEN BioResource Center. The LXF 2478L cell line was provided by Charles River Discovery Research Services, GmbH, who generated the original cell line from its proprietary patient-derived xenograft (PDX) model. The NIH/HEK-Neo cell line was obtained from Lonza. Ba/F3 cells were cultured in RPMI1640 supplemented with 10% FBS (MP Biomedicals, LLC) and 1 ng/mL IL-3 (Promega). NIH/3T3 cells were cultured in D-MEM containing 10% NBCS (Thermo Fisher Scientific), and the NIH/HEK-Neo cells were cultured in Keratinocyte Basal Medium (KBM) containing KGM-Gold SingleQuots (Lonza). The NCI-H1975, HCC827, NCI-H23, NCI-H460, and NIH/3T3 cell lines were obtained from Lonza. Ba/F3 cells were cultured in RPMI1640 containing 10% FBS (MP Biomedicals, LLC), all cells were cultured at 37°C with 5% CO₂. All cell lines were authenticated by short tandem repeat profiling before purchase or use in this study.

#### Genetically engineered cell lines

cDNA constructs of WT and mutant EGFR were transfected into Ba/F3 and NIH/3T3 cell lines using Nucleofector (Lonza), followed by clone selection using puromycin. The mutant EGFRs tested in this study were A763_Y764insQEA, V769_D770insASV, D770_N771insSV, D770_N771insGC, H773_V774insNPH, D773_V774insPH, delE746_A750 (exon 19 deletion), and delE746_A750 + T790M (exon 19 deletion + T790M). For NCI-H1975 EGFR D770_N771insSVD cells, NCI-H1975 cells expressing D770_N771insSVD mutant EGFR were established as described above, and then endogenous L858R/T790M mutant EGFR was knocked out by XTN TALEN-mediated mutagenesis (Transposagens Biopharmaceuticals). NCI-H1975 EGFR D770_N771insSVD cells were established by transfection of luciferase expression vector into NCI-H1975 EGFR D770_N771insSVD cells, followed by clone selection using hygromycin B. After establishment, all cell lines were authenticated by short tandem repeat profiling, and sequencing analysis was performed to confirm the integration of WT and mutant EGFR. As for NCI-H1975 EGFR D770_N771insSVD cells, the presence of knockout mutation by mutagenesis was also validated with both sequencing and immunoblotting analysis as described in Supplementary Methods.

#### Cell proliferation assay

Cell proliferation was assessed using the CellTitier-Glo luminescent cell viability assay (Promega). For a panel of Ba/F3 cell lines expressing human EGFRs, the cells were plated at an optimal density onto 96-well plates, followed by 72 hours of exposure to the compound in mIL-3-free conditions. Ba/F3 parent cells and Ba/F3 EGFR WT cells were stimulated with 1 ng/mL mIL-3 and 50 ng/mL EGF, respectively, during compound treatment. For a panel of human lung cancer cell lines and NIH/HEK-Neo cells, the cells were plated as described above and incubated for 24 hours for attachment, followed by compound treatment for 72 hours. After compound exposure, a volume of CellTitier-Glo reagent equal to the volume of medium in each well was added, followed by measurement of luminescence in each well using an EnSpire Multimode Plate Reader (Perkin Elmer). IC₅₀ and GI₅₀ values were determined using the SAS software package in EXSIS (CAC Croit).

#### Caspase induction analysis

Caspase induction was assessed using the Caspase-Glo 3/7 assay (Promega). Cells were plated at optimal density onto 96-well plates and incubated for 24 hours for attachment, followed by compound treatment for 24 hours. After compound exposure, a volume of Caspase-Glo 3/7 reagent equal to the volume of medium in each well was added, followed by measurement of luminescence in each well as described above. The measurement was corrected with respect to cell viability determined as described above, and relative caspase-3/7 activation against DMSO control was determined.

#### Immunoblotting analysis

For cultured cell lines, cells were seeded at optimal density onto culture plates or dishes with medium including 10% serum. Following culture for 24 hours, the cells were exposed to the compound and harvested at the indicated time points. NIH/3T3 EGFR WT was stimulated with 50 ng/mL EGF during compound exposure. Xenograft tumors and mouse skin tissues were excised at each time point after a single dosing of test compound. The collected samples were lysed and homogenized with RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) on ice. Following centrifugation, the
supernatants were collected, and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

For quantification of pEGFR, proteins at optimal concentrations were dispensed into Wes 12 to 230 kDa prefiltered plates (ProteinSimple) with primary antibody, anti-rabbit secondary antibody, and streptavidin horseradish peroxidase (HRP) and its substrate, followed by separation with capillary electrophoresis and chemiluminescence detection using Simple Western (ProteinSimple). Peak areas (in the range of 162–198 kDa) were used to determine IC50 values using the SAS software package in EXSUS (CAC Croit).

For Western blotting analysis, proteins were separated by SDS-PAGE using 4% to 15% Criterion TGX precast gels (Bio-Rad Laboratories) and transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). After probing with the primary antibodies, the membranes were incubated with HRP-conjugated secondary antibody. The antigen–antibody complexes were visualized using ECL prime (GE Healthcare), followed by detection with the Amersham Imager 600 QC (GE Healthcare). Primary and secondary antibodies were obtained from Cell Signaling Technologies and GE Healthcare, respectively.

### In vivo efficacy experiments

All animal protocols were reviewed and approved according to regional Institutional Animal Care and Use Committees. For the LXF 2478 and LU0387 models, animal experiments were performed by Charles River Discovery Research Services, GmbH, and Crown Bioscience Inc., respectively. Male BALB/cAlcl-nu/nu mice from Charles River Laboratories, and BALB/c nude mice from Harlan Laboratories; animals ranged from 4 to 6 weeks of age.

In the subcutaneous implantation model, LXF 2478 and LU0387, human lung cancer PDXs, were transplanted into NMRI nu/nu mice and BALB/c nude mice, respectively. The genetically engineered cell lines were suspended in PBS and inoculated into BALB/cAlcl-nu/nu mice for NCI-H1975 EGFR D770_N771insSVD (0.8 × 10^7 cells/100 μL) and NIH/3T3 EGFR H773_V774insNPH (0.5 × 10^7 cells/100 μL), and into F344/NiCl-rnu/nu rats for NCI-H1975 EGFR D770_N771insSVD (1.6 × 10^7 cells/200 μL). After reaching optimal tumor volume, animals were randomized into groups (six to eight animals per group) and orally administered the compound once daily for 10, 14, or 28 days. In both PDX models, the treatment period was followed by an observation period of 14 days. Antitumor activity was evaluated using tumor volume calculated according to the following formula: [tumor volume] = [major axis] × [minor axis]^2 × 0.5. Statistical analysis was performed using repeated measures ANOVA followed by Dunnett test for tumor volume (P < 0.05).

In the orthotopic implantation model, NCI-H1975 EGFR D770_N771insSVD_Luc cells were suspended in 50% PBS and 50% Matrigel (BD Biosciences) and directly inoculated into the lungs of BALB/cAlcl-nu/nu nude mice (2.4 × 10^6 cells/20 μL). Six days after inoculation, a luciferin solution was administered via the tail vein, and then photon values were measured by the IVIS Imaging System (Perkin Elmer) to confirm engraftment, followed by grouping using the values. Starting on the following day, animals were orally administered the compound once daily. Survival benefit was evaluated according to median survival time (MST), and increase in life span (ILS) was calculated using the following formula: \[\text{ILS} \% = \left(\frac{\text{MST of the treated group}}{\text{MST of control}} - 1\right) \times 100.\] Statistical analysis was performed using the Wilcoxon test for MST (P < 0.05).

TAS6417 was formulated in 0.1 N HCl with or without 0.5% hypromellose (HPMC) solution. Afatinib was formulated in a 0.5% methylcellulose (MC), 0.4% Tween80 suspension.

### Pharmacokinetic analysis

The plasma concentrations of TAS6417 were determined in BALB/cAlcl-nu/nu mice and F344/NiCl-rnu/nu rats following multiple oral administrations for 14 days at effective doses (three animals per group). The plasma samples were collected before administration on Day 14, as well as at 0.5-, 1-, 2-, 4-, 8-, and 24-hour post-administration for mice and 0.25-, 0.5-, 1-, 2-, 4-, 8-, and 24-hour post-administration for rats. The plasma concentrations were measured with the LC/MS-MS.

### Results

TAS6417 is a novel inhibitor of EGFR exon 20 insertion

TAS6417, with 6-methyl-8,9-dihydropyrimido[5,4-b]indolizine as its core structure, is a novel small-molecule inhibitor designed to be fit to the ATP binding site of the EGFR hinge region and to exhibit inhibitory activity against exon 20 insertion mutations. The chemical structure is shown in Fig. 1A and its synthetic procedure is described in Supplementary Fig. S1. MS analysis demonstrated that TAS6417 covalently modified the cysteine residue at position 797 of recombinant EGFR, harboring an in-frame insertion mutation in the exon 20 region (Fig. 1B; Supplementary Table S1).

TAS6417 inhibited the in vitro phosphorylation activity of EGFR and its mutants including an exon 20 insertion mutation (D770_N771insNPG), with IC50 values ranging from 1.1 ± 0.1 to 8.0 ± 1.1 nmol/L (Supplementary Table S2A). TAS6417 was further evaluated according to its selectivity for EGFR over other kinases as described in Supplementary Methods. Of the 255 kinases tested, TAS6417 inhibited 25 kinases other than EGFR with IC50 values less than 1.000 nmol/L, which was 100 times higher than its IC50 value against WT EGFR. It inhibited only six kinases containing TXK, BMX, HER4, TEC, BTK, and JAK3, with IC50 values less than 10 times that against WT EGFR, as shown in Supplementary Table S2B.

The cellular potency of TAS6417 against EGFR exon 20 insertion mutations was evaluated using genetically engineered NIH/3T3 cells. After 6 hours of exposure, TAS6417 showed potent inhibition of EGFR phosphorylation in a panel of NIH/3T3 cells stably expressing exon 20 insertion mutant human EGFRs, with an IC50 value of <4.48 nmol/L for A762_Y763insFQEA, 7.82 ± 27.4 nmol/L for V769_D770insASV, 23.4 ± 2.5 nmol/L for D770_N771insSVD, 45.7 ± 19.8 nmol/L for D770_N771insG, 66.7 ± 19.0 nmol/L for H773_V774insNPH, and 117 ± 31 nmol/L for H773_V774insNPH (Table 1A). In contrast, the IC50 value of TAS6417 for WT EGFR was 368 ± 181 nmol/L. Furthermore, TAS6417 inhibited proliferation of Ba/F3 cells driven by various EGFR exon 20 insertion mutations, with IC50 values ranging from 5.05 ± 1.33 nmol/L to 150 ± 53 nmol/L (Fig. 1C). Consistent with an intracellular target inhibition assay in NIH/3T3 cells, TAS6417 showed selectivity against WT EGFR (IC50 = 676 ± 304 nmol/L). In a cell proliferation assay in a Ba/F3 cell panel, the selectivity for exon 20 insertions over the WT, represented as the WT/mut ratio,
was 134-fold for A763_Y764insFQEA, 6.37-fold for V769_D770insASV, 17.4-fold for D770_N771insSVD, 17.2-fold for D770_N771insG, 4.51-fold for H773_V774insNPH, and 4.55-fold for H773_V774insPH (Fig. 1D). In contrast, afatinib and erlotinib showed no selectivity against exon 20 insertions, with less than one-fold WT/mut ratios except for that of A763_Y764insFQEA. Although A763_Y764insFQEA is an insertion mutation at the exon 20 region in the EGFR gene, it has been
reported as an EGFR-TKI-sensitive mutation, and patients with NSCLC harboring this mutation have shown clinical responses to both afatinib and erlotinib (9, 25). These data demonstrated the potency of TAS6417 against cells driven by EGFR exon 20 insertions with mutant selectivity, the spectrum of which was different from that of other EGFR-TKIs.

TAS6417 inhibits in vitro cell growth and EGFR signaling in human cancer cell lines driven by EGFR exon 20 insertions

The effect of TAS6417 on tumor cell proliferation was investigated using a panel of seven human lung cancer cell lines expressing different EGFRs. The panel consisted of LXF 2478L cells established from a PDX expressing an EGFR exon 20 insertion mutation (V769_D770insASV), NCI-H1975 EGFR D770_N771insSVD cells in which the endogenous alleles with L858R and T790M substitutions were knocked out and exogenous EGFR with an exon 20 insertion mutation (D770_N771insSVD) was transfected (Supplementary Fig. S2), HCC827 and PC-9 cells expressing an exon 19 deletion mutation (delE746_A750) of the EGFR gene, NCI-H23 and NCI-H1975 cells expressing different EGFRs. The panel consisted of LXF 2478L cells and H1975 EGFR D770_N771insSVD cells, indicating inhibition of EGFR signal transduction. Accordingly, TAS6417 led to increased levels of Bim, which is a pro-apoptosis protein whose expression is regulated by AKT and ERK signaling (33), and cleaved PARP, which is cleaved by caspases and is a marker of cells undergoing apoptosis (34), in both cell lines after treatment for 24 hours. Consistent with Western blotting analysis, chemiluminescence analysis revealed that TAS6417 caused a concentration-dependent increase in relative caspase-3/7 activity in both cell lines compared with that in the control 24 hours after treatment, implying induction of apoptosis (Fig. 2C and D). In summary, these findings suggest that TAS6417 inhibits EGFR signal transduction, leading to cell growth inhibition and apoptosis induction in NSCLC cells driven by EGFR exon 20 insertion mutations.

TAS6417 causes persistent tumor regression in vivo in EGFR exon 20 insertion-driven tumor models

To investigate in vivo antitumor efficacy of TAS6417 for tumors driven by EGFR exon 20 insertion mutations, we conducted experiments against the three major mutations, namely V769_D770insASV, D770_N771insSVD, and H773_V774insNPH. TAS6417 was administered orally once daily for 14 days to mice implanted with NCI-H1975 EGFR D770_N771insSVD xenografts, resulting in dose-dependent tumor growth inhibition (Fig. 3A). At final evaluation on Day 15, all groups treated with TAS6417 showed statistically significant inhibition of tumor growth compared with that of the control group. Although TAS6417 administered at 50 and 100 mg/kg/day showed marked tumor growth inhibition with treatment/control (T/C) ratios of 51% and 19%, respectively, 200 mg/kg/day TAS6417 achieved tumor regression with a T/C of 5%. The tumor regression occurred quickly after TAS6417

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<table>
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<th>EGFR status</th>
<th>IC50 value, nmol/L</th>
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<tr>
<td>WT (+EGF)</td>
<td>368 ± 181</td>
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<tr>
<td>A763_V764insFQEA</td>
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<td>V769_D770insASV</td>
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Table 1. Cellular potency of TAS6417

A. Inhibitory activity of TAS6417 against pEGFR in NIH/3T3 cell lines expressing human EGFR

B. Inhibitory activity of TAS6417 against cell proliferation in lung cancer cell lines and primary keratinocytes
treatment and persisted during the treatment period. Regarding tolerability, mean body weight loss over 5% was not observed in any groups (Supplementary Fig. S3A). In this model, afatinib administered at 20 mg/kg showed minimal, but statistically significant (\(P < 0.049\)), tumor growth inhibition with a T/C of 69%. In rats bearing the same xenograft tumors, similar efficacy was obtained from lower doses compared with those in the mouse model (Fig. 3B). Oral administration of TAS6417 at over 10 mg/kg once daily for 14 days led to statistically significant inhibition of tumor growth (T/C of 20%), and 20 mg/kg/day led to complete inhibition of tumor growth with a T/C of 7%. At 40 mg/kg, the sustained tumor regression (T/C of 3% at Day 15) was observed soon after TAS6417 administration without any effect on body weight gain. Pharmacokinetic analysis revealed that the plasma concentration of TAS6417, administered at 50 and 100 mg/kg to mice, decreased rapidly in the first 4 hours (Fig. 3F). In contrast, although the effective doses in the rat model exhibited lower maximum plasma concentration (\(C_{\text{max}}\)) values than those in the mouse model, the plasma concentrations tended to be more persistent, lasting up to 8 hours.

NIH/3T3, a mouse fibroblast cell line, does not engraft normally in nude mice, but tumorigenicity can be rendered by mutant EGFR expression (6). In vivo antitumor efficacy of TAS6417 against H773_V774insNPH, an exon 20 insertion mutation, was evaluated in nude mice implanted with NIH/3T3 cells genetically engineered to express human EGFR with the mutation. In this model, TAS6417 was administered orally once daily for 10 days. Consistently, with data from the NCI-H1975 EGFR D770_N771insSVD xenograft model, TAS6417 administered at 50 mg/kg/day or more led to statistically significant inhibition of tumor growth in a dose-dependent manner at final evaluation on Day 11 (Fig. 3C). Tumor regression was achieved at 200 mg/kg/day, corresponding to a T/C ratio of 2%; T/C ratios of 25% and 6% were observed at the lower doses of 50 and 100 mg/kg/day, respectively. Furthermore, the in vivo activity of TAS6417 against EGFR H773_V774insNPH was assessed in mice with LU0387 human lung cancer xenografts, a PDX model (Fig. 3D). In this model, 50 mg/kg/day for 28 days induced statistically significant tumor growth inhibition with a T/C of 16%. Striking and persistent tumor regression was observed at 100 mg/kg/day, with a T/C of 2% at Day 28. Although afatinib dosed at 20 mg/kg also
inhibited tumor growth, tumor volume still gradually increased, corresponding to a T/C of 35% at Day 28. After completion of treatment and a 2-week dose-free observation period, the tumors of all tested groups grew over time, resulting in T/C ratios of 31% for 50 mg/kg/day TAS6417, 7% for 100 mg/kg/day TAS6417, and 61% for 20 mg/kg/day afatinib. Although mean body weight loss over 5% was not observed for TAS6417 treatment groups, the body weight of the afatinib treatment group was decreased acutely by over 10% at Day 4, after which it recovered over time during the treatment period (Supplementary Fig. S3D).

In the LXF 2478 human lung cancer PDX model with EGFR V769_D770insASV, one of the most common exon 20 insertion mutations, daily oral administration of TAS6417 resulted in partial to complete remission, and the T/C ratio at Day 28 for the 100 and 200 mg/kg/day groups was 3% and 0.1%, respectively (Fig. 3E). The tumors remained at their reduced sizes
during the 2-week, treatment-free observation period. Although afatinib delayed tumor growth with a T/C ratio of 23% at Day 28, the tumors showed significant regrowth during the observation period. In this model, the maximum mean body weight loss was 3% for the control group, 4% for the TAS6417 100 mg/kg/day group, 11% for the TAS6417 200 mg/kg/day group, and 12% for the afatinib 20 mg/kg/day group (Supplementary Fig. S3E). Solely in the afatinib treatment group, a low grade of dry, flaky, or reddened skin was observed around 3 weeks after initiation of administration. These findings demonstrate that TAS6417 achieved persistent tumor regression in EGFR exon 20 insertion-driven tumors in both genetically engineered xenograft and PDX models, and that TAS6417 was more efficacious and more tolerable than afatinib in an animal model.

TAS6417 inhibits mutant EGFR in tumors but not WT EGFR in skin tissues

To determine whether the in vivo efficacy was associated with effective inhibition of EGFR signaling, phosphorylation of EGFR and its downstream effectors was assessed in tumors by Western blotting after a single dose of TAS6417. In mice bearing xenograft tumors expressing EGFR D770_N771insSVD, a single dose of TAS6417 at 50 mg/kg or more caused intensive reduction of phospho-EGFR (pEGFR) at 1 hour (Fig. 4A). Consistent with this finding, phosphorylation of molecules downstream of EGFR, including phospho-AKT (pAKT) and phospho-ERK (pERK), was also decreased. In contrast, the effect of TAS6417 on pEGFR in mouse skin tissues expressing WT EGFR was partial even at the highest dose of 200 mg/kg, as shown in Fig. 4B. Furthermore, a time-course analysis of EGFR signaling inhibition in NCI-H1975 EGFR D770_N771insSVD tumors was conducted following a single dose of TAS6417 at 100 mg/kg. The inhibition of pEGFR was observed 1 hour after dosing and was maintained partially for 6 hours. In parallel, pAKT and pERK were partially inhibited for 6 hours to levels comparable with the control at 24 hours, as shown in Fig. 4C. Similar results were observed in rats with NCI-H1975 EGFR D770_N771insSVD xenografts, as shown in Fig. 4D. TAS6417 administered at 20 mg/kg, which achieved complete suppression of tumor growth, induced a significant decrease in pEGFR, leading to reduction of pAKT and pERK at 1 hour. The inhibitory effect was still noted at 6 hours, and phosphorylation of EGFR, AKT, and ERK recovered by 24 hours. These pharmacodynamic changes correlated well with plasma concentrations in each model. These results suggest that TAS6417 achieved more potent inhibition of mutant EGFR in tumors than of WT EGFR in skin tissues. The data also indicate that the ability of TAS6417 to sustain the inhibition of EGFR signaling may contribute to its potent antitumor efficacy in the mouse and rat xenograft models.

TAS6417 prolonged survival of animals bearing lung cancer

TAS6417 was further evaluated for its survival benefit in mice with intrapulmonary NCI-H1975 EGFR D770_N771insSVD xenografts. As a result of daily oral administration of TAS6417, dose-dependent prolonged survival was observed (Fig. 5A). TAS6417 administered at 100 and 200 mg/kg/day provided statistically significant prolongation of median survival compared to that of the control group, with ILS of 59% and 102%, respectively. The afatinib group exhibited no statistically significant ILS. Although the body weight of mice in the control group tended to increase by approximately 30 days after implantation, it decreased

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Figure 4.
EGFR inhibition by TAS6417 in the H1975 EGFR D770_N771insSVD xenograft model. A and B, In vivo inhibitory activity of TAS6417 on EGFR in xenograft tumors and skin tissues. Mice bearing NCI-H1975 EGFR D770_N771insSVD xenografts were administered TAS6417 and afatinib. One and 2 hours after administration of TAS6417 and afatinib, respectively, xenograft tumors and skin tissues were collected. Phosphorylation of EGFR-Tyr1068 in xenograft tumors (A) and in skin tissues (B) was detected by immunoblotting. C and D, Duration of inhibitory activity of TAS6417 against EGFR signaling in mice (C) and rats (D). NCI-H1975 EGFR D770_N771insSVD xenografts were collected at each time point. Phosphorylation of EGFR-Tyr1068, AKT-Ser473, and ERK1/2-Thr202/Tyr204 was determined using immunoblotting analysis.
over time during the median survival period until animal death. In contrast, the TAS6417-treated group exhibited an increase in the body weight beyond 30 days. After this period, decreases in body weight associated with animal death were observed, just as in the control group (Fig. 5B). These data demonstrate not only the survival benefit of TAS6417 in mice with intrapulmonary xenograft tumors driven by EGFR exon 20 insertion mutations, but also the good tolerability of long-term daily administration.

**Discussion**

In this article, we describe the characteristics of TAS6417, a novel EGFR inhibitor with potential for the treatment of cancer harboring EGFR exon 20 insertion mutations. TAS6417 is a kinase inhibitor that covalently modifies C797 in the ATP-binding site of EGFR, showing inhibitory activity against WT and mutant EGFR including an exon 20 insertion mutation in biochemical assays. In a panel of 255 kinases, TAS6417 at 1,000 nmol/L exhibited >50% inhibition against 25 kinases other than EGFR and only six kinases (TXK, BMX, BTK, HER4, TEC, JAK3) whose ATP-binding pockets harbor a cysteine at a structural position homologous to C797 in EGFR (35), suggesting high selectivity of TAS6417 against EGFR over other kinases. These biochemical data related to kinase selectivity are supported by the results of the cell proliferation assay. Although cells driven by mutant EGFR were sensitive to TAS6417, NCI-H23 cells and NCI-H460 cells, which exhibit EGFR-independent cell growth did not respond to TAS6417. Furthermore, cell-based assays of intracellular EGFR inhibition demonstrated the selectivity of TAS6417 against mutant EGFRs, including exon 20 insertion mutations over WT EGFR. Among a wide variety of exon 20 insertion mutations in EGFR, the most common mutations are V769_D770insASV, D770_N771insSVD, and H773_V774insNPH, which account for approximately 50% of all mutations. TAS6417 inhibited these major mutations and also other insertion mutations, including A763_Y764insFQEA, D770_N771insG, and H773_V774insPH, while sparing WT EGFR, suggesting that TAS6417 has potent and selective inhibitory activity against a diverse range of insertion mutations in the exon 20 region of the EGFR gene over WT EGFR.

Patients with NSCLC harboring common mutations, including exon 19 deletions and L858R, show striking responses to EGFR-TKIs, including gefitinib, erlotinib, and afatinib, leading to prolonged progression-free survival compared with that of standard chemotherapy (13–18). However, the clinical response of NSCLC driven by EGFR exon 20 insertion mutations to these EGFR-TKIs is much lower (25–28). Given that current EGFR-TKIs are not selective against exon 20 insertion mutations and that clinical doses are thus limited by toxicity related to WT EGFR inhibition, poor clinical outcomes may be the result of an insufficient plasma concentration for inhibition (22, 29, 30). Therefore, the development of an EGFR inhibitor selective against mutations in tumors rather than WT EGFR in normal tissues is extremely important for the treatment of NSCLC driven by EGFR exon 20 insertion mutations. This study demonstrates that TAS6417 exhibits selective inhibition of such mutations over WT EGFR in vitro and in vivo. In the cell proliferation assay, TAS6417 exhibited more potent inhibitory activity in human cancer cell lines harboring mutant EGFRs than in primary keratinocytes. Consistent with this, TAS6417 achieved remarkable and durable inhibition of mutant EGFR and its downstream effectors in tumors, while sparing WT EGFR in skin tissues. This mutation-selective characteristic led to significant in vivo antitumor activity in mouse and rat models. Notably, once-daily oral administration of TAS6417 at 100 mg/kg achieved persistent tumor regression with good tolerability in a PDX model of EGFR exon 20 insertions, including V769_D770insASV and H773_V774insNPH. In contrast, afatinib induced
tumor growth inhibition but not tumor regression, and some toxic signatures, including body weight loss over 10% and skin symptoms, were observed. In addition to the tumor growth inhibition experiments, a lung orthotropic implantation model in mice demonstrated the survival benefits of TAS6417, with tolerability in long-term daily administration.

Although EGFR exon 20 insertion mutations are uncommon, occurring in only 2% to 3% of NSCLC cases, there is an unmet medical need for patients. A few clinical trials of EGFR-TKIs for EGFR exon 20 insertion mutation-positive NSCLC are in progress, including trials for AP32788 (NCT02716116), osimertinib (NCT03191149), and poziotinib (NCT03066206 and NCT03318939). As described in this article, TAS6417 has a unique core unit differing from other reported EGFR-TKIs and a unique mutation selectivity profile, achieving remarkable anti-tumor efficacy and prolonged survival in preclinical models. These findings support clinical evaluation of TAS6417 as an efficacious drug candidate for patients with NSCLC driven by EGFR exon 20 insertion mutations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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