Letter

# Selective Covalent Targeting of Mutated EGFR(T790M) with Chlorofluoroacetamide-Pyrimidines

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# Selective Covalent Targeting of Mutated EGFR(T790M) with Chlorofluoroacetamide-Pyrimidines

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## Abstract

Covalent modification of disease-associated proteins with small molecules is a powerful approach for achieving increased and sustained pharmacological effect. To reduce potential risk of nonselective covalent modification, molecular design of covalent inhibitors is critically important. We report herein the development of targeted covalent inhibitor for mutated epidermal growth factor receptor (EGFR) (L858R/T790M) using α-chlorofluoroacetamide (CFA) as reactive group. The chemically tuned weak reactivity of CFA was suitable for the design of third-generation EGFR inhibitors that possess the pyrimidine scaffold. The structure-activity relationship study revealed that CFA inhibitor **18** (NSP-037) possessed higher inhibition selectivity to the mutated EGFR over wild-type

EGFR when compared to clinically approved osimertinib. Mass-based chemical proteomics analyses further revealed that **18** displayed high covalent modification selectivity for the mutated EGFR in living cells. These findings highlight the utility of CFA as a warhead of targeted covalent inhibitors and the potential application of the CFA-pyrimidines for treatment of non-small-cell lung cancer.

**KEYWORDS:** mutated EGFR, covalent inhibitors, α-chlorofluoroacetamide, chemical proteomics, non-small-cell lung cancer

Irreversible inhibition of protein function by covalent drugs have several possible advantages over conventional reversible inhibitors, including enhanced and prolonged pharmacological effects,[1-4] and high protein isoform selectivity.[5-7] Despite the potential of adverse effects due to unintended reactions with off-target proteins,[8] several targeted covalent inhibitors (TCIs) for protein kinases have been successfully developed and used clinically for cancer treatment.[9-12] A representative example is the TCI that targets the epidermal growth factor receptor (EGFR) for the treatment of non-small-cell lung cancer (NSCLC). Irreversible second-generation EGFR inhibitors such as afatinib were developed for treatment of NSCLC harboring EGFR-T790M mutation.[13-16] These quinazoline-type TCIs have a Michael acceptor as the reactive warhead[17-19] and are designed to form a covalent bond with Cys797 in the ATP binding pocket of EGFR. However, these agents are dose

limited by their non-selective inhibition against wild-type EGFR, which is thought to be responsible for the side-effects such as skin rash and diarrhea.[20] Recent efforts to overcome this limitation led to the development of mutant selective irreversible third-generation EGFR inhibitors.[21-28] Currently, the third-generation inhibitor, osimertinib (**1**), is clinically used for the treatment of NSCLC (Figure 1).[29,30] Osimertinib possesses a 2-phenylaminopyrimidine scaffold appended with acrylamide as the reactive warhead for Cys797.

Recently, we have introduced α-chlorofluoroacetamide (CFA) as a new class of TCI warhead.[31] Despite the weak intrinsic reactivity of CFA, CFA-appended quinazoline serves as a potent and selective covalent inhibitor for EGFR by targeting Cys797 in its ATP binding pocket. To further reveal the proteome-wide reactivity profile of the CFA-based covalent inhibitor and validate its utility in TCI design, we report herein the development of the third-generation EGFR(T790M) covalent inhibitor bearing CFA as a reactive warhead. Structural modification of the pyrimidine scaffold of osimertinib resulted in a CFA-appended inhibitor **18** (NSP-037), which showed a potent antiproliferative activity toward H1975 cells harboring EGFR L858R/T790M double mutation. Notably, **18** exhibited higher selectivity for the mutated EGFR over wild-type EGFR when compared to osimertinib. Mass-based chemical proteomic analyses also revealed that **18** displayed high covalent modification selectivity for the mutated EGFR in H1975 cells. These findings highlight the utility of CFA in TCI design and provide a promising strategy for the development of covalent inhibitor for the treatment of NSCLC.

## Results and Discussion

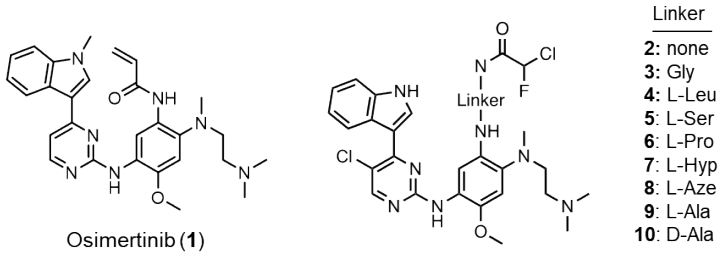



**Figure 1.** Structures of osimertinib (**1**) and CFA-pyrimidine derivatives.

For the development of a CFA-based selective covalent inhibitor for EGFR(T790M), we employed the pyrimidine scaffold of osimertinib (Tagrisso). Preliminary computational docking studies between EGFR(T790M) and CFA-substituted pyrimidine derivatives suggested that the introduction of a linker unit between the pyrimidine core and the CFA unit could accommodate the CFA warhead in an appropriate position close to the targeted Cys797 of EGFR. Based on this molecular design, we initially synthesized a series of CFA-pyrimidine derivatives, **2**–**10,** bearing an amino acid linker and evaluated their antiproliferative activities against H1975 cells harboring EGFR L858R/T790M double mutation. The results are summarized in Table 1. We found that the activity of CFA-pyrimidine derivatives significantly depended on the structure of the linker unit. Compounds **3**, **5**, **7**, and **8**, bearing either a glycine, L-serine, L-3-hydroxyproline (L-Hyp), or L-azetidine-2-carboxylic acid (L-Aze) linker, showed weak antiproliferative activities ($IC_{50}$ > 0.1 μM), while compounds **4**, **6**, and **9** bearing an L-leucine, L-proline, or L-alanine linker, effectively

inhibited H1975 cell proliferation ($IC_{50}$ < 0.1 μM). Among them, compound **9** with an L-alanine linker was the most potent inhibitor with an $IC_{50}$ value of 0.031 μM, which was slightly higher than that of osimertinib (0.016 μM) but lower than that of the non-linker type CFA-pyrimidine **2** (0.050 μM). Interestingly, the inhibitory activity of **10** with a D-alanine linker ($IC_{50}$ = 0.44 μM) was much weaker than that of **9**. The predicted binding model suggests that the methyl group of the L-alanine linker of **9** forms a C-H/π interaction with its indole ring, which may fix the configuration of the CFA unit to be suitable for reaction with Cys797 (Figure S1). In contrast, this stacking interaction was not present in the case of **10** with a D-alanine linker.

**Table 1.** Anti-proliferative activity against EGFR-dependent H1975 cells ($IC_{50}$, μM)[a].

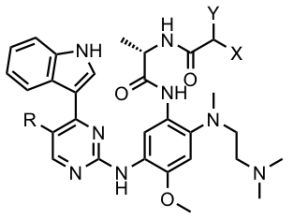
| | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **1** |
|---|---|---|---|---|---|---|---|---|---|---|
| linker | none | Gly | L-Leu | L-Ser | L-Pro | L-Hyp | L-Aze | L-Ala | D-Ala | (osimertinib) |
| H1975 | 0.050 ± 0.005 | 0.13 ± 0.036 | 0.052 ± 0.0014 | 0.32 ± 0.007 | 0.056 ± 0.01 | 0.15 ± 0.03 | 0.38 ± 0.10 | 0.031 ± 0.003 | 0.44 ± 0.13 | 0.016 ± 0.003 |

[a] Data represent mean ± standard error of triplicate experiments.

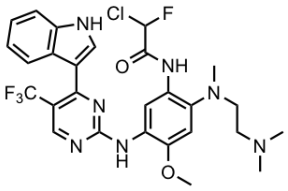
We next optimized the substituent at the 5-position of the pyrimidine ring. It has been reported that this substituent group directs to the gate-keeper residue Met790,[22] and their interaction influences the inhibitory activity of the pyrimidine derivative. Among the series of 5-substituted derivatives **9**, **11**–**18** (Table S1) bearing an L-alanine linker, we found that compound **18** with a trifluoromethyl substituent showed the most potent activity ($IC_{50}$ = 0.015 μM), the value of which was comparable to that of osimertinib ($IC_{50}$ = 0.016 μM)

(Table 2 and Figure 2). We next examined the antiproliferative activity of the 5-substituted derivatives against H292 cells expressing wild-type EGFR and assessed their inhibition selectivity for H1975 cells harboring the mutated EGFR(L858R/T790M). We found that all the tested CFA-pyrimidines showed weak antiproliferative activities against H292 cells ($IC_{50}$ > 0.57 μM). Among them, **18** exhibited the highest cell selectivity index (H292 / H1975 = 91.3), which was higher than that of osimertinib (H292 / H1975 = 13.8) (Table 2). The high selectivity profile of **18** for the mutated EGFR over wild-type EGFR was confirmed by an *in vitro* kinase activity inhibitory assay. As shown in Table S2, the ratio of kinase inhibitory activity (wild-type EGFR / mutated EGFR) of **18** was 44.7, which was higher than that of **1** (wild-type EGFR / mutated EGFR = 4.1). As compared to **18**, the non-linker type compound **19** with a 5-$CF_3$ substituent showed a weaker antiproliferative activity against H1975 ($IC_{50}$ = 0.033 μM) and a lower cell selectivity index (H292 / H1975 = 17.3) (Table 2), indicating that the inserted alanine linker of **18** contributes to its preferable inhibitory activity for the mutated EGFR. The substitution of CFA warhead of **18** with the non-reactive acetyl group as in **20** dramatically decreased the activity ($IC_{50}$ = 0.46 μM), suggesting that the potency of **18** is attributable to covalent bond formation between CFA warhead and the mutated EGFR. LC/MS/MS analysis revealed that **18** covalently bound to Cys797 of recombinant EGFR (L858R/T790M) kinase domain (Figure S2).

**Table 2.** Anti-proliferative activity against EGFR-dependent cell lines ($IC_{50}$, μM)[a] and cell selectivity index.



**9**: R = Cl, X = Cl, Y = F
**18**: R = $CF_3$, X = Cl, Y = F
**20**: R = $CF_3$, X = H, Y = H



**19**

| | **9** | **18** | **19** | **20** | **1** |
|---|---|---|---|---|---|
| R | Cl | $CF_3$ | $CF_3$ | $CF_3$ | (osimertinib) |
| H1975 | 0.031 ± 0.003 | 0.015 ± 0.002 | 0.033 ± 0.007 | 0.46 ± 0.044 | 0.016 ± 0.003 |
| H292 | 0.71 ± 0.15 | 1.37 ± 0.018 | 0.57 ± 0.048 | 3.59 ± 0.10 | 0.22 ± 0.015 |
| selectivity index | 22.9 | 91.3 | 17.3 | 7.8 | 13.8 |

[a] Data represent mean ± standard error of triplicate experiments.

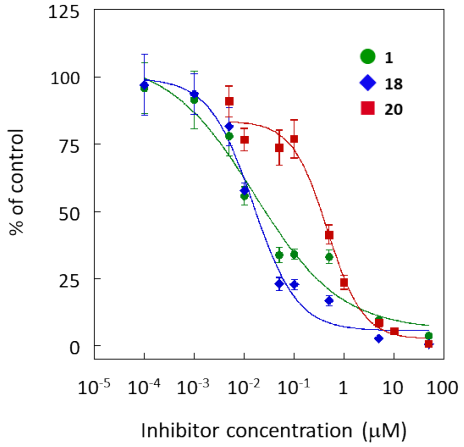



**Figure 2.** Anti-proliferative activities of pyrimidine derivatives **1**, **18**, and **20** against H1975 cells. H1975 cells were grown in the presence of the inhibitor for 72 h. Each plot represents the average ± standard deviation of triplicate experiments.

We assessed the biological activity of **18** in living cells. A western blot analysis revealed that **18** effectively inhibited EGFR (Y1068) autophosphorylation in H1975 cells at 10 nM (Figure 3 and S3). This activity is comparable to that with **1** (Figure S4 and S5). We also confirmed that the inhibitory activity persisted for at least 8 h after washout of **18** from the culture medium, suggesting that **18** irreversibly inhibited EGFR activity via the formation of a covalent bond in living cells. In single oral administration to mice (25 mg/kg), the plasma level of **18** peaked to 0.51 ± 0.17 μM at 2 h and the mean residence time (MRT) was estimated to be 8.9 h ± 0.98 h (Figure S6).

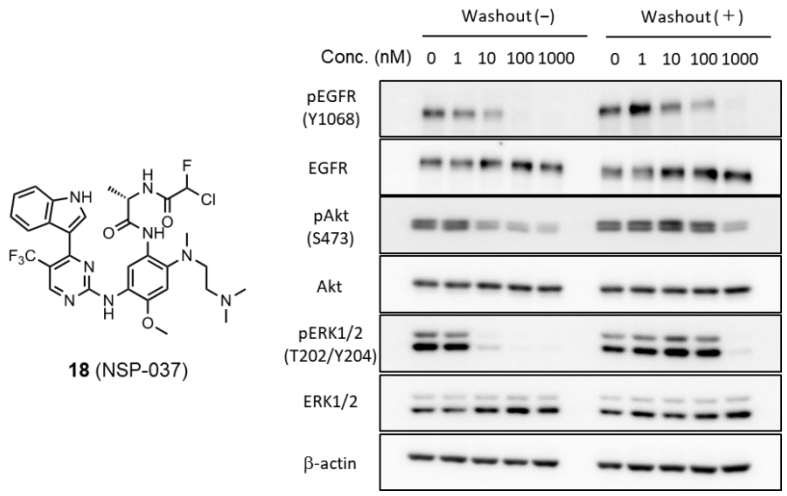



**Figure 3.** Western blot analysis of inhibitory activity of **18** against phosphorylation of EGFR (L858R/T790M) and the related signaling proteins in H1975 cells.

For chemical proteomic analysis, we synthesized probes **21** and **22**[32] as the alkynylated analogs of **18** and **1** (osimertinib), respectively. (Figure 4). Probe **21** and **22** exhibited the strong anti-proliferative activity against H1975 cells ($IC_{50}$ = 0.051 and 0.072 μM,

respectively) (Table S3), suggesting that these probes are good surrogates for interrogating the proteome reactivity of their parent inhibitors. Incubation of the recombinant kinase domain with CFA probe **21** and subsequent copper-catalyzed azide-alkyne cycloaddition (CuAAC) with rhodamine-azide yielded a fluorescent band in the in-gel fluorescence analysis (Figure 4a). The time-trace analysis revealed that adduct formation proceeded rapidly and was completed within 20 min. Notably, this reaction rate was much faster than that of **18** with excess glutathione, wherein the half-reaction time ($t_{1/2}$) was determined to be 49.4 h under neutral aqueous conditions (pH 7.4, 37 °C) (Figure S7). These data suggest that the reactivity of **18** is greatly facilitated in the binding complex with the kinase domain. Rapid covalent modification of the kinase domain was also observed with Michael acceptor probe **22** (Figure S8). In previously published results,[31] we found that CFA–thiol adducts can be gradually hydrolyzed under neutral aqueous conditions. To evaluate the stability of the kinase domain adduct with **21**, the reaction mixture was incubated for an extended period of 72 h. The data revealed that the adduct can stably exist without degradation at least for 24 h (Figure 4b). In contrast, compound **23**, an *N*-acetylcysteine adduct of **18**, was gradually hydrolyzed in a neutral aqueous buffer (pH 7.4, 37 °C, $t_{1/2}$ = 11.2 h) (Figure S9). These observations suggest that the covalent adduct of the CFA-pyrimidine with Cys797 of EGFR was stabilized in the solvent-sequestered ATP binding pocket, as was observed in the CFA-quinazoline derivative[31].

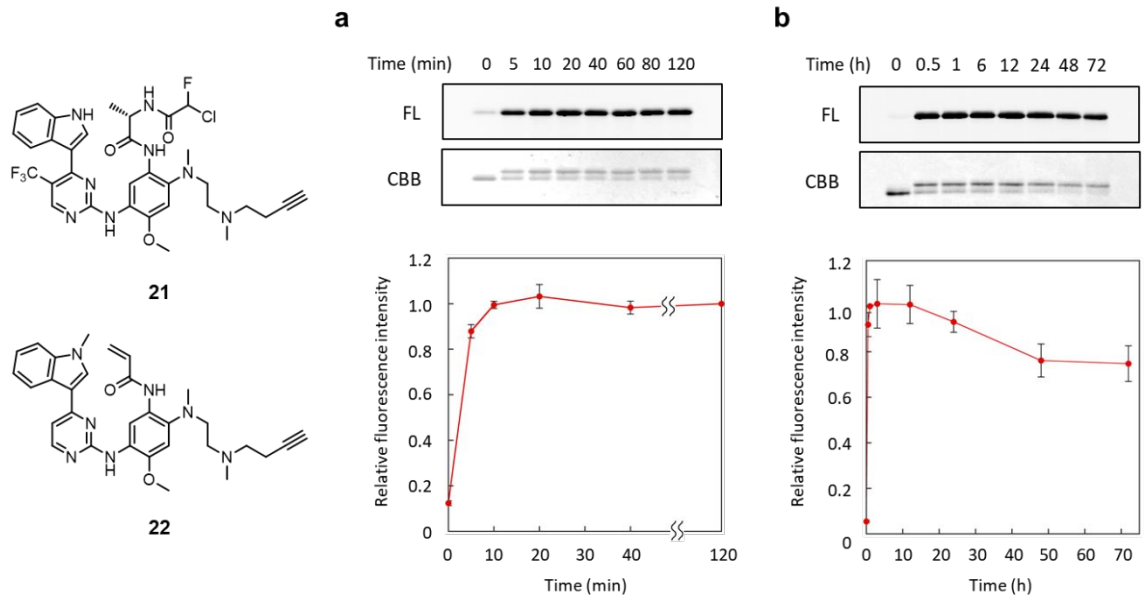



**Figure 4.** Reactivity profiles of probe **21** with kinase domain of mutated EGFR (L858R/T790M). In-gel fluorescence (FL) and Coomassie Brilliant Blue (CBB) analysis of the kinase domain labeled with **21** for short incubation time (0-120 min) (a) and long incubation time (0-72 h) (b). The lower panels show time plot of the relative fluorescence intensity (mean ± s.d. obtained from three independent experiments). The fluorescence intensity at 120 min was set to arbitrary value of 1.0. Conditions: [**21**] = 2 μM, [EGFR kinase domain] = 3 μM, 25 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 10% glycerol, 0.01% Tween-20, 37 °C.

The proteome reactivity of the CFA-pyrimidine was next evaluated by in-gel fluorescence analysis using CFA probe **21** (Figure 5). Treatment of H1975 cells for 1 h at 37 °C and subsequent CuAAC with rhodamine-azide yielded a fluorescent band at 175 kDa. This band disappeared in the presence of excess osimertinib, suggesting that **21** reacted with EGFR. The weak band intensity of EGFR was attributable to its low expression level in H1975 cells[33]. We next compared the off-target activity of **21** with Michael acceptor probe **22**. Concentration-dependent labeling experiments revealed that **21** exhibited higher reaction selectivity for EGFR when compared to **22**, especially at high concentrations of the probes (1–10 μM) (Figure 5b). The rampant off-target reactivity of Michael acceptor probe in the micromolar concentration range was also reported for the EGFR inhibitor with a quinazoline scaffold and the Bruton's tyrosine kinase inhibitor.[31,34] In contrast, the corresponding CFA probes maintained their high target selectivity. The time-dependent labeling experiment in H1975 cells with 1 μM of the probe also revealed the lower off-target reactivity of **21** compared to **22** (Figure S10). Of note, **21** sustained its low off-target activity even after extended 10 h incubation. We confirmed that intracellular level of the probes was almost the same in H1975 cells (Figure S11). Taken together, these results indicate the usefulness of CFA as a target selective warhead of TCI. The low off-target activity of **21** was also confirmed in H292 cells expressing wild-type EGFR and EGFR-independent HEK293 cells (Figure S12 and S13). The distinct band pattern of **21** from **22** indicates their different proteome selectivity in these cell lines.

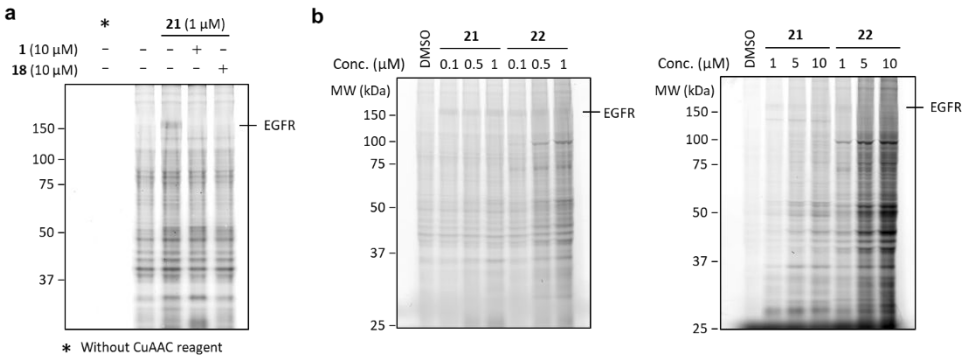



**Figure 5.** Reactivity profiles of CFA probe **21** and Michael acceptor probe **22** in H1975 cells. (a) Competitive protein reaction profiles of **21** in H1975 cells. (b) Concentration-dependent reactivity profiles of **21** and **22** ( [probe] = 0.1–1 (left panel) and 1–10 μM (right panel), 2 h incubation, 37°C).

To quantitatively evaluate off-target reactivity profiles of the probes, we performed stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometry analysis using probe **21** and **22**.[35] The initial experiment compared the isotopically labeled cells treated with **21** or **22** versus dimethyl sulfoxide (DMSO) (Figure 6a, S14 and Dataset S1). The result showed that both probes exhibited the high SILAC ratio values for the targeted EGFR ($\log_2$(probe / DMSO ratio) > 2). We found that **22** exhibited higher off-target reactivity than CFA probe **21**: 33 proteins were significantly enriched by probe **22** ($\log_2$(ratio) > 1), while 6 proteins were enriched by **21** ($\log_2$(ratio) > 1) (see Methods in Supporting Information for the criteria of data filtration). We further performed competitive SILAC experiment

between the two probes in H1975 cells. The scatter plot of the SILAC ratio values (**21** / **22**) obtained from the two individual experiments (**21** in Heavy / **22** in Light, and **21** in Light / **22** in Heavy) revealed the differences in proteome-wide reactivity of the probes (Figure 6b and Dataset S2). In the plot, 19 proteins were predominantly enriched by **22** ($\log_2$(**21**/**22**) ≤ −2), while only 2 proteins were primarily enriched by **21** ($\log_2$(**21**/**22**) ≥ 1). These data suggest the lower off-target activity of **21** compared to **22** (see also Figure S15). To identify high-occupancy targets of inhibitors **18** and **1**, we next performed competitive SILAC experiments where isotopically labeled cells were pre-treated with the inhibitors (**18** and **1**) or DMSO followed by treatment with the corresponding probes (**21** and **22**, respectively) (Figure 6c and Dataset S3). This experiment identified the 4 high-occupancy targets of **18**, defined as proteins with high SILAC ratio values ((DMSO + probe) / (inhibitor + probe) ratio > 4). This experiment also identified the 12 high-occupancy targets of **1**, among which 7 proteins including ERBB2, CTSC, and CTSL were also reported as the targets of **1** in the previous manuscript[32]. SELENOT and RPL12 were found to be unique high-occupancy targets of **18**. SELENOT was also found as the predominantly enriched protein by probe **21** as shown in Figure 6b. To evaluate the selective binding profile of the CFA-pyrimidine toward mutated EGFR(L858R/T790M) over wild-type EGFR in cellular context, we performed competitive SILAC experiments between H1975 and H292 cells using

probes **21** and **22** and compared their SILAC ratio values for EGFR (mutated EGFR(L858R/T790M) in H1975 / wild-type EGFR in H292) (Figure S16, Table S5 and Dataset S4). The $\log_2$(ratio) values of **21** and **22** were determined to be 3.2 and -0.059, respectively, suggesting that **21** has a higher mutated EGFR(L858R/T790M) selectivity over wild-type EGFR when compared to **22**. This result was further confirmed by EGFR pull-down assay in the two cell lines (Figure 6d). Quantitative western blot analysis revealed that pull-down efficiency for mutated EGFR(L858R/T790M) in H1975 cells was almost the same in the both probes, whereas that of **21** for wild-type EGFR in H292 cells is significantly lower than that of **22** (Table S6).

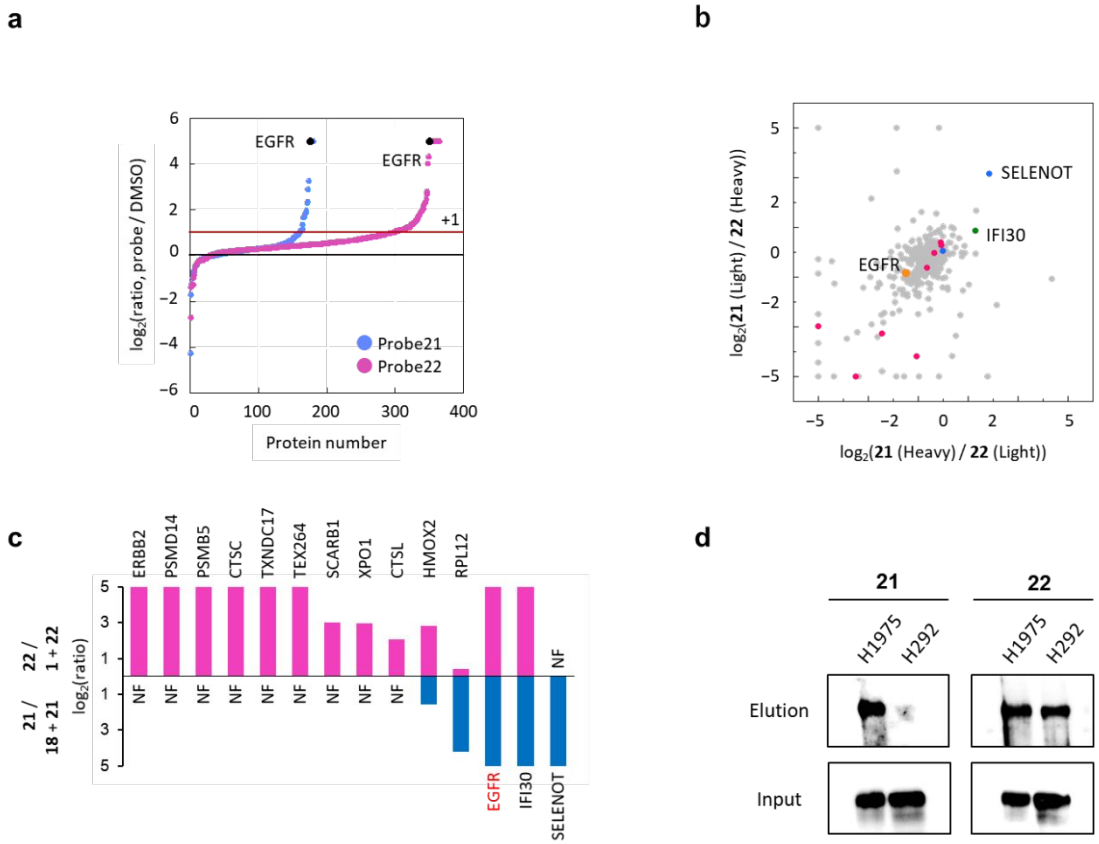



**Figure 6**. Selectivity profiles of probe **21** and **22** in H1975 cells. Plot of SILAC ratio values of proteins in probe/DMSO experiments (a) and probe/probe competitive experiments (b). H1975 cells were treated with **21** or **22** (5 µM, 2 h, 37 °C). Results are plotted as $\log_2$ of the median SILAC ratios obtained from triplicate mass spectrometry (MS) analyses of single streptavidin-enriched sample. EGFR is highlighted in orange. High-occupancy targets of **18** and **1** defined in Figure 6c are highlighted in blue and pink, respectively. IFI30 is highlighted in green. (c) Proteins identified as high occupancy targets of **18** and **1** in the (DMSO + probe) / (inhibitor + probe) competitive SILAC experiments. Data represent the median $\log_2$(ratio) values obtained from triplicate MS analyses of the sample in which Light cells were pre-

treated with the inhibitor. (d) EGFR pull-down experiments in H1975 and H292 cells. Cells were treated with **21** or **22** (1 μM, 1 h, 37 °C). Representative data from two individual experiments are shown.

## Conclusion

We have developed a third-generation covalent inhibitor for EGFR by exploiting CFA as a reactive warhead. We revealed that CFA-pyrimidine **18** (NSP-037) exhibited higher inhibitory selectivity for the mutated EGFR (L858R/T790M) over wild-type EGFR when compared to osimertinib. The chemoproteomics analysis using the alkyne probes suggested the possibility that **18** showed a lower off-target reactivity profile compared to osimertinib. These highly selective profiles, as well as the potent antiproliferative activity against H1975 cells, suggest that **18** is a potentially useful chemical entity for the treatment of NSCLC. The findings presented in this study also provide further evidence for the utility of CFA as a new class of warhead. We envision that CFA will be broadly applicable to covalently target additional proteins associated with abnormal cell properties and disease onset.

## Supporting Information

The Supporting Information is available free of charge via the internet at http://pubs.acs.org.

Additional tables and figures as described in the text, synthetic procedures and spectra data of the target compounds (PDF)

Supplementary datasets (XLSX)

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## Abbreviations

EGFR epidermal growth factor receptor; CFA α-chlorofluoroacetamide; TCI targeted covalent inhibitor; NSCLC non-small-cell lung cancer; Hyp 3-hydroxyproline; Aze azetidine-2-carboxylic acid; MRT mean residence time; CuAAC copper-catalyzed azide-alkyne cycloaddition; CBB Coomasie Brilliant Blue; SILAC stable isotope labeling by amino acids in cell culture; DMSO dimethyl sufoxide; CTSC cathepsin C; CTSL cathepsin L1; SELENOT thioredoxin reductase-like selenoprotein T; RPL12 60S ribosomal protein L12.

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