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Refinement of covalent EGFR inhibitor AZD9291 to eliminate off-target activity

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ABSTRACT

Non-small-cell lung cancer (NSCLC) is a major disease that accounts for 85% of all lung cancer cases which claimed around 1.8 billion lives worldwide in 2020. Tyrosine kinase inhibitors (TKIs) that target EGFR have been used for the treatment of NSCLC, but often develop drug resistance, and the covalent inhibitor AZD9291 has been developed to tackle the problem of drug resistance mediated by the T790M EGFR mutation; however, there is a side effect of hyperglycemia that may be due to off-target activity. This study examines analogs of AZD9291 by chemical proteomics, identifying analogs that maintain T790M-EGFR engagement while showing reduced cross-reactivity with off-targets.

Introduction

The tyrosine kinase domain of EGFR is a well-known target for the treatment of non-small-cell lung cancer (NSCLC) and several generations of inhibitors have been developed. However, resistance against these therapies has emerged, especially due to the T790M mutation. To overcome this problem, new irreversible inhibitors have been developed to target an active-site cysteine residue, and the compound AZD9291 developed by AstraZeneca belongs to this class of inhibitors [1] (Fig. 1). This orally active, potent T790M-EGFR inhibitor has been approved by the FDA as a second line treatment for patients with T790M EGFR NSCLC. However, in the phase I/II clinical trials, this compound has shown an unusual side effect of hyperglycemia [2], suggesting that AZD9291 has off-target inhibition activity.

Since cysteines perform many functions in diverse protein classes, irreversible EGFR inhibitors may affect the activity of proteins outside of the kinome, potentially leading to target-independent side effects. Indeed, the Cravatt laboratory has demonstrated that some irreversible EGFR inhibitors, including AZD9291, have off-target reactivity across the broader proteome [3,4]. AZD9291, in particular, accumulates in the lysosome, which may account for off-target activity with the cysteine-dependent cathepsin proteases [4].

In an attempt to improve the selectivity of AZD9291, we modified key groups in the structure of the compound and evaluated the proteomic reactivity of the analogs in NSCLC cells expressing T790M-EGFR. Our goal was to identify hot spots for further refinement of the compound. While other groups have studied the impact of changing the reactive group on the AZD9291 scaffold [5], we chose to retain the same Michael acceptor and instead modify other sites. Based on AstraZeneca's studies on T790M-EGFR inhibitors that led to the discovery of AZD9291 [6], as well as the propensity of compounds with basic amines to accumulate in the lysosome (the organelle that harbors a major set of cathepsin off-targets of AZD9291), we decided to explore the consequences of modifying the diamino group, as well as other groups on the compound (the methoxy and methylindole moieties) (Fig. 1). The pyrimidine group is essential for binding to T790M-EGFR so we chose to keep this region intact, along with the acrylamide responsible for covalent interaction with C797 in the EGFR active site.

Results and discussion

Analogs with 1-methylindole or 1-*H* indole as R^1 were obtained by reaction of either a 1-methylindole or 1-*H* indole with 2,4-dichloropyrimidine. The former was introduced by a Friedel-Craft reaction using aluminum trichloride whereas the latter was introduced by an S_NAr reaction of the indole, deprotonated with methyl

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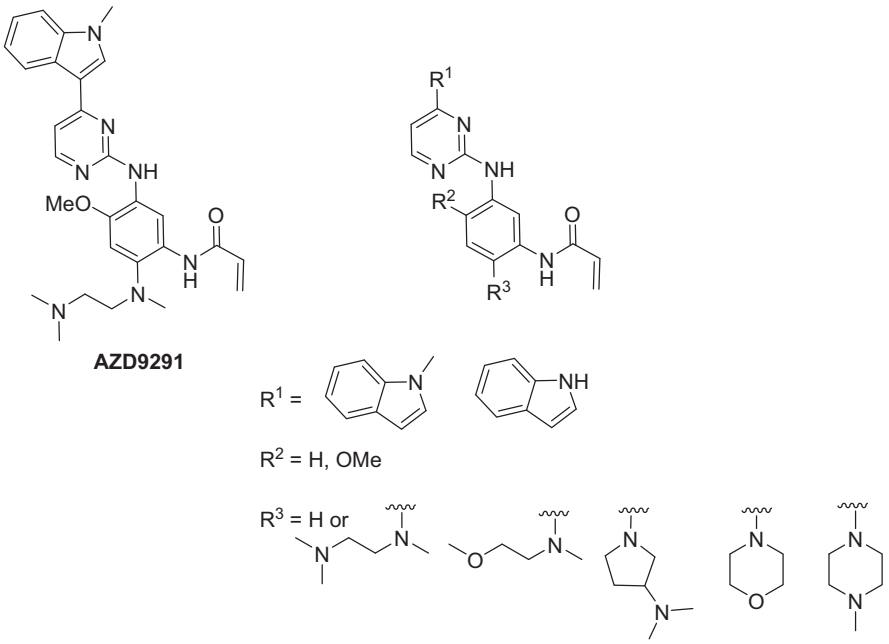

Fig. 1. Structure of AZD9291 and library compounds.

magnesium bromide, on the 4-position of the 2,4-dichloropyrimidine. The second chlorine was then substituted by various nitroanilines bearing either a hydrogen or a methoxy group as R^2 and either a hydrogen or a fluorine in the 4 position. In the latter case, the fluorine was substituted by different amines as R^3 groups. The nitro group was reduced into an amine, using hydrogen and palladium on carbon or iron powder and ammonium chloride, in order to introduce the Michael acceptor moiety (Fig. 2).

The introduction of the acrylamide group in the final step proved to be more tedious than expected for some of the analogs. With only one equivalent of acryloyl chloride, the reaction was incomplete and the starting material and the product could not be separated (Fig. 3a). The use of a slight excess of acryloyl chloride lead to a mixture of product and a by-product bearing 2 acrylamide groups, also non-separable (Fig. 3b). The use of acrylic acid and a coupling agent, T3P, gave the product with non-separable impurities. The same issue occurred when using acrylic acid and triphenylphosphite (Fig. 3c). To avoid the formation of the bis-acrylamide compound, the synthesis route was modified to protect the secondary amine with a Boc group. However, during the deprotection of the Boc group, the acrylamide was partially cleaved leading to a mixture of products that were not separable (Fig. 3d). We also used *tert*-butyl acrylate and ethyl acrylate in an attempt to achieve selective introduction on the primary amine, but no reaction was observed (Fig. 3e). We then decided to use 2 equivalents of acryloyl chloride to introduce the acrylamide and hydrolyze the acrylamide on the guanidine-like nitrogen. Several acidic conditions were tested and a solution of HCl 3 M in AcOEt was determined to be optimal, despite the formation of another by-product resulting from the addition of a chlorine on the Michael acceptor. After elimination of the chlorine with triethylamine in acetonitrile, the final compounds were obtained (Fig. 3f).

Using this synthetic route, the following compounds were obtained (Fig. 4).

The *in situ* activity of the various analogs was evaluated using the NSCLC lung cancer line H1975, which harbors a T790M-EGFR variant, by competitive activity-based protein profiling (ABPP) [3]. Cells were first treated with an inhibitor analog or DMSO vehicle (1 and 10 μM). After 2 h, the cells were treated with the alkyne-bearing AZD9291 probe (Fig. 5). After 4 h, cells were lysed, and membrane and soluble proteomes isolated and subject to treatment with rhodamine-azide by copper(I)-catalyzed azide-alkyne cycloaddition [7], as described previously [4]. Probe-labeled proteins were then separated by SDS-PAGE and visualized by in-gel fluorescence scanning (Fig. 6). In this competitive ABPP experiment, a compound is considered inhibitory against T790M-EGFR or off-targets if it blocks the AZD9291 probe labeling of these proteins at 1 or 10 μM test concentrations (reflected in a loss of in-gel fluorescence signal for these proteins).

These studies demonstrated that replacing the diamino group with other basic amines had variable impact on EGFR and cathepsin reactivity, but did not furnish analogs with superior selectivity. In contrast, an analog where the diamino group was removed along with removal of the *N*-methyl of the indole unit (EB037) maintained T790M-EGFR engagement at 1 and 10 μM while showing reduced reactivity with cathepsins. In contrast, analog EB017, which also lacked the diamino group, but maintained the *N*-methyl indole lost interactions with both EGFR and cathepsins. We should also note that other off-targets of AZD9291 were retained with EB037 (e.g., ~100 kDa soluble protein).

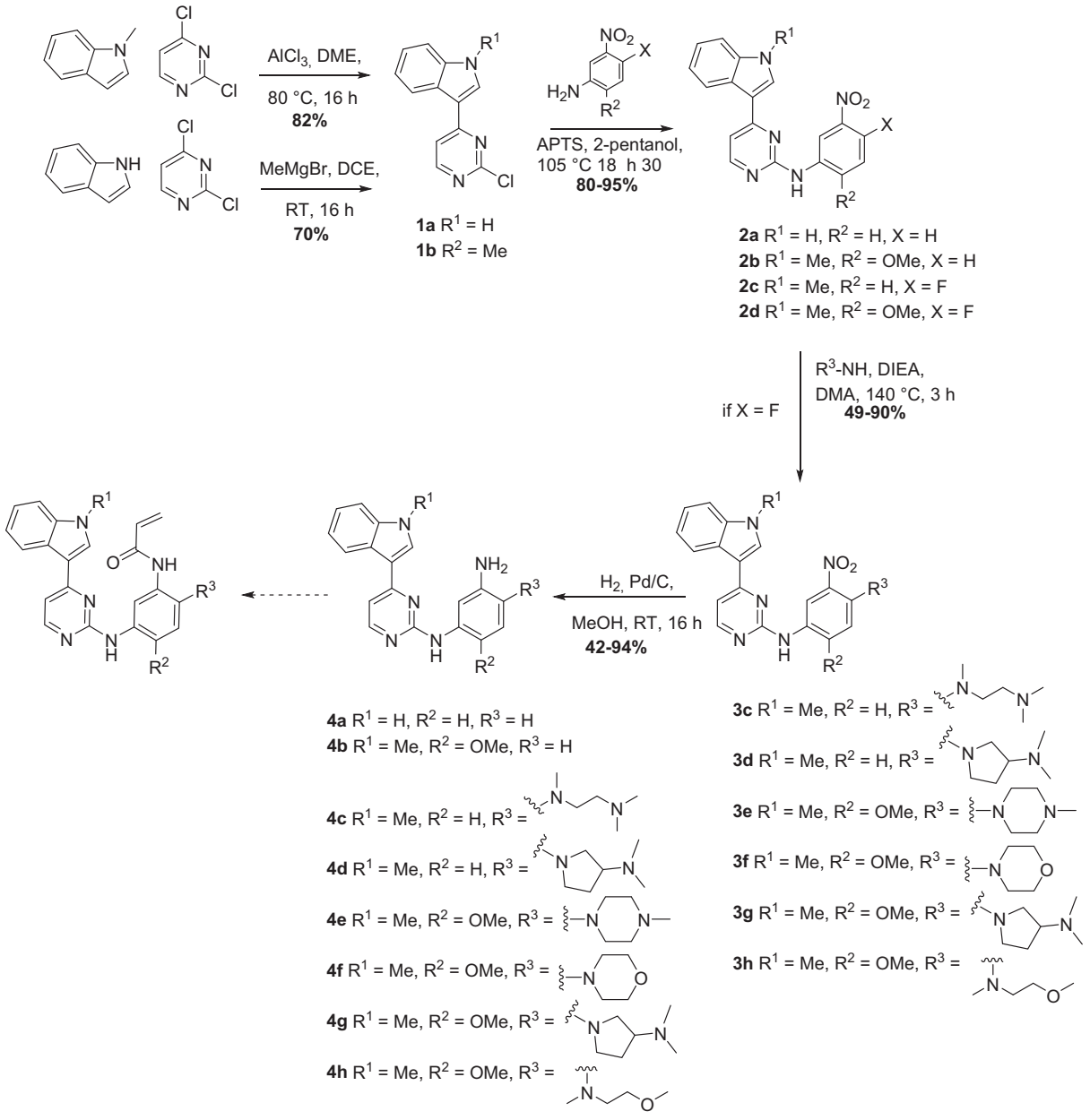

Fig. 2. Synthetic route for the variation of R^1, R^2 and R^3.

Conclusion

In summary, this study revealed that modification of the AZD9291 scaffold can reduce off-targets of the parent compound, while maintaining reactivity with T790M-EGFR. In particular, our findings indicate that the basic amine in AZD9291 is not required for engagement of T790M-EGFR and replacement of this group with suitable neutral analogs may yield compounds with lower propensity to enter the lysosome and cross-react with cathepsins. Further work is required to identify compounds with optimal selectivity for T790M-EGFR over cathepsins, as well as to further improve selectivity against other off-targets in the proteome. Work continues to develop next generation covalent inhibitors against T790M EGFR with reduced off-target reactivity and to explore the lysosomotropic properties of the new compounds using established assays [8].

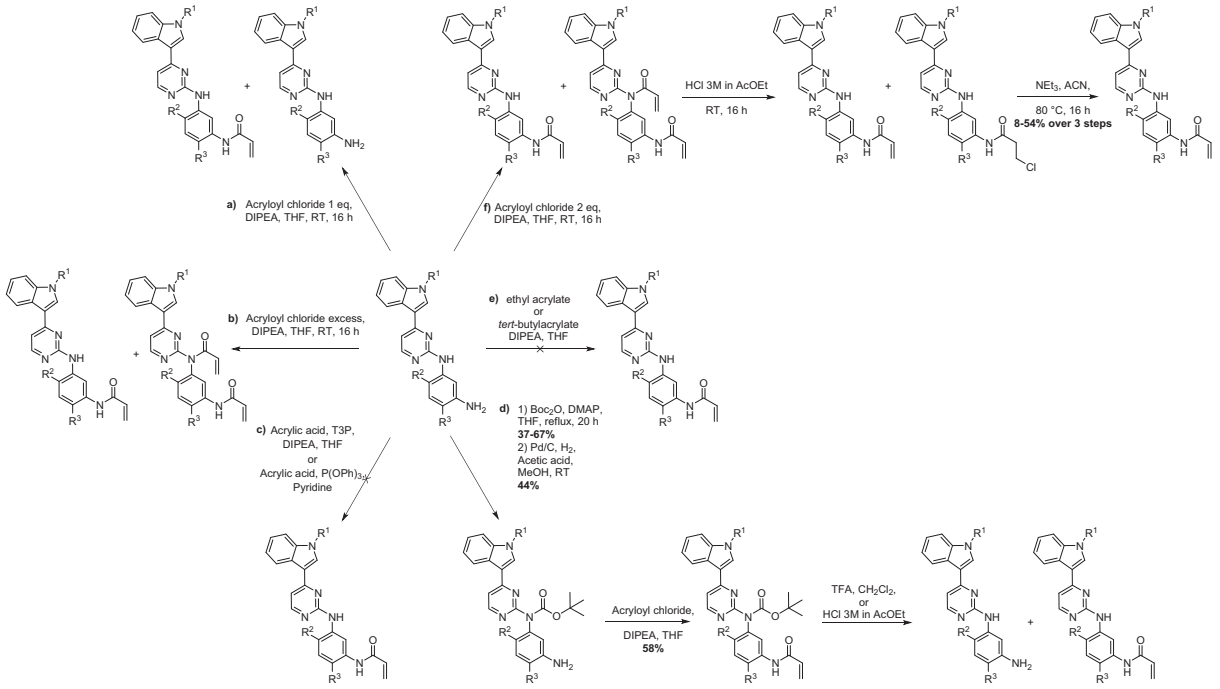

Fig. 3. Different strategies attempted to introduce the acrylamide.

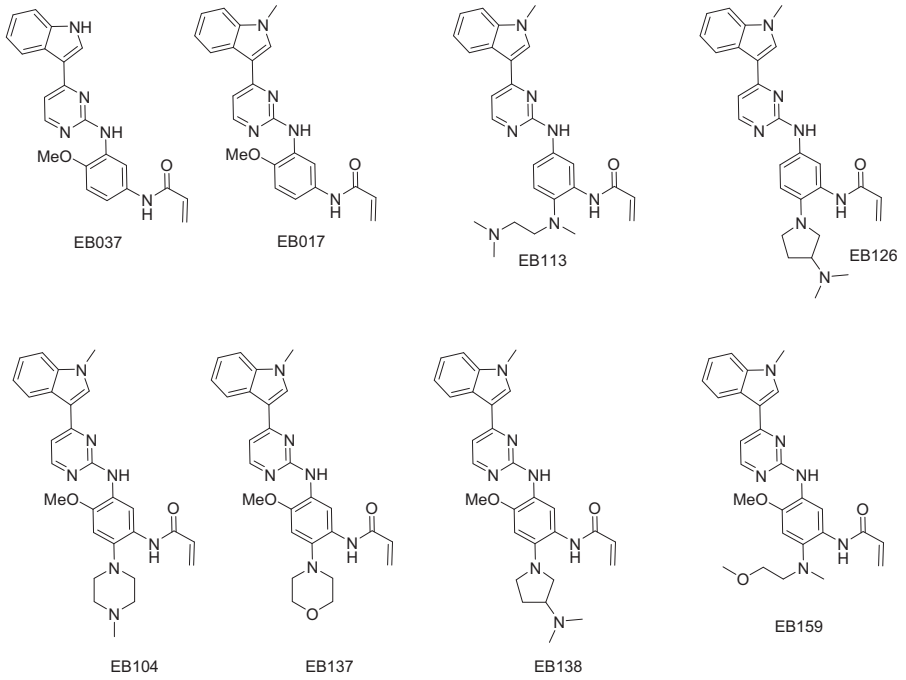

Fig. 4. Library of biologically tested compounds.

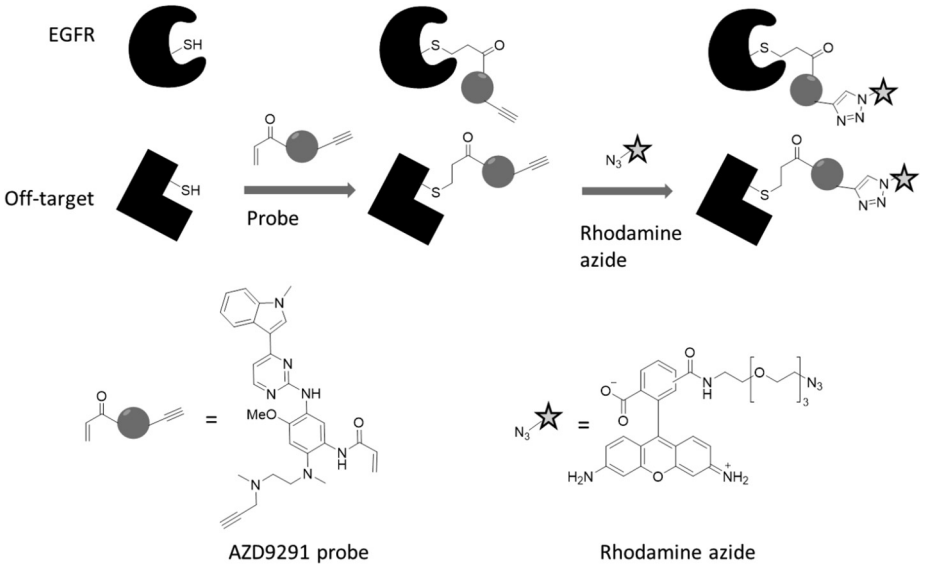

Fig. 5. Structure of AZD9291 probe and Rhodamine azide for competitive activity-based protein profiling of AZD9291 analogs.

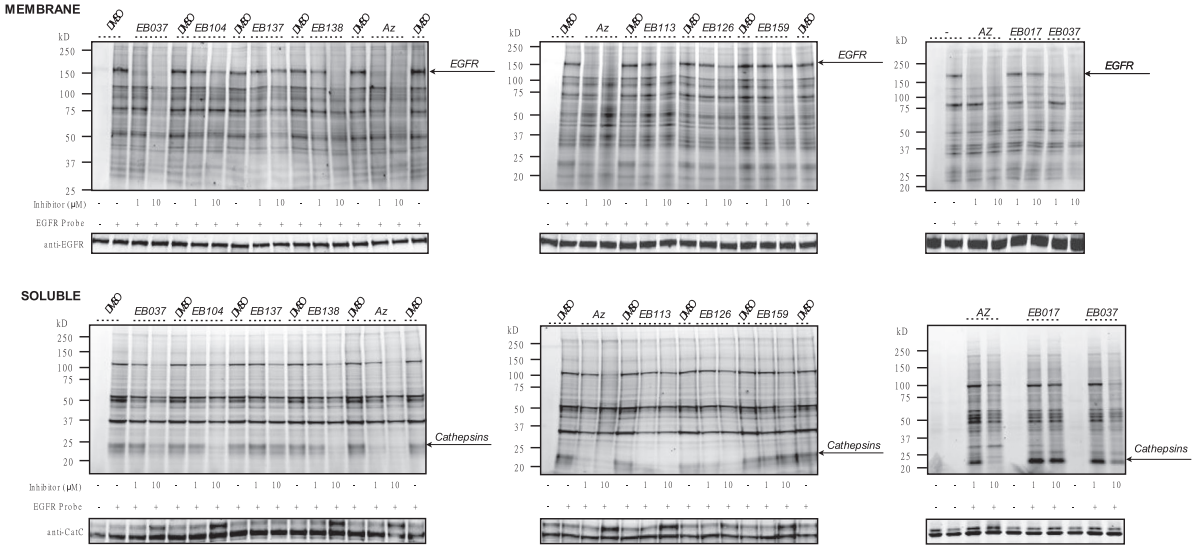

Fig. 6. Gel-based competitive activity-based protein profiling (ABPP) of analogs of AZD9291. H1975 cells were treated at indicated concentrations of inhibitor (AZ = AZD9291) or DMSO vehicle (2 h) prior to incubation with the alkyne AZD9291 probe (1 μM, 4 h). Cells were then lysed and their membrane and soluble proteomes subject to Cu(I)-catalyzed azide-alkyne cycloaddition with rhodamine-azide, SDS-PAGE, and in-gel fluorescence analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2021.153178.

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