Preparation of 3-Substituted-1-Isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amines as RET Kinase Inhibitors

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S *Supporting Information*

ABSTRACT: A series of 3-substituted-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amines have been designed, synthesized, and evaluated as RET protein kinase inhibitors. On the basis of docking results, a small library of pyrazolopyrimidine compounds with an extended hydrophobic side arm was synthesized. The most promising of the compounds (**7a**) displayed efficient inhibition in vitro and good selectivity when tested on a panel of kinases. Furthermore, **7a** inhibited GDNF-induced RET phosphorylation of ERK1/2 in MCF-7 breast cancer cells at concentrations as low as 100 nM.

INTRODUCTION

Protein kinases constitute 1.7% of the protein products encoded in the human genome.[1] It encodes 518 protein kinases, of which the majority are serine/threonine kinases and 90 are tyrosine kinases (TKs). The TKs are divided into nonreceptor and receptor tyrosine kinases, of which the receptor tyrosine kinases (RTKs) are a major family of disease-related proteins that are promising therapeutic targets.[2] Under normal conditions, RTK activity is closely regulated, but deregulation of the RTKs can lead to oncogenic conversion,[3] which occurs via three main mechanisms: formation of fusion proteins with altered localization and/or specificity through retroviral transduction or genomic rearrangements, point mutations leading to constitutive activation and/or potentially altered binding of ATP, and overexpression of the normal protein sequence.

RET (REarranged during Transfection) is a transmembrane RTK essential for the development of the sympathetic, parasympathetic, and enteric nervous systems as well as the kidney.[4] Its extracellular part contains four cadherin-like repeats, a calcium-binding site, and a cysteine-rich domain. The intracellular part contains a typical TK domain. The RET protein is activated by ligands of the glial-derived neurotrophic factor (GDNF) family, such as GDNF, neurturin, artemin, persephin, and a coreceptor, GFR-α. The GDNF ligand/receptor complex brings together two RET proteins, thereby triggering autophosphorylation and intracellular signaling.[5]

Deregulation and dysfunction of RET can lead to several different thyroid cancers, including multiple endocrine neoplasia 2 syndromes (MEN2A and MEN2B), familial medullary thyroid carcinoma (FMTC), and papillary thyroid carcinoma (PTC).[4] Point mutations in the RET gene are the cause of the three cancer syndromes MEN2A, MEN2B, and FMTC, while PTC is related to chromosomal inversions or translocations affecting the intracellular kinase domain.

Recent studies have demonstrated that RET is overexpressed in a subset of estrogen-receptor (ER)-positive breast cancers and that crosstalk between ER and RET is important in responses to endocrine therapy.[5,6]

Small-molecule inhibitors have been used to block the auto- and substrate phosphorylation of RET in an ATP competitive way, thereby preventing its oncogenic activity.[7] Several classes of inhibitors have been used as RET inhibitors, including pyrazole-pyrimidines (e.g., **1**), indolocarbazoles, 2-indolinone derivatives, and a 4-anilinoquinazoline (**2**).[4,8−13]

Previously, we have used the pyrazolopyrimidines successfully as a scaffold for the inhibition of the PFPK7 kinase of *Plasmodium falciparum*;[14] therefore, we were also interested in expanding the use of the pyrazolopyrimidine scaffold to other kinases. Furthermore, the pyrazolopyrimidine-based compound **1** has previously been used successfully as an inhibitor of several TKs that are considered important cancer targets.[15,16]

In this work, we present the design, synthesis, and biological evaluation of a small library of 3-substituted pyrazolopyrimidines that inhibit the RET kinase in low nanomolar concentrations (down to 8 nM) in vitro. The most active compounds have been evaluated in RET kinase-expressing MCF-7 breast cancer cells and RET-fusion protein-expressing TPC1 papillary thyroid carcinoma cell lines.

RESULTS AND DISCUSSION

The RET kinase has been characterized by X-ray crystallography, with several small molecule inhibitors. Among them, **2** (Figure 1, PDB 2IVU)[10] and **1** (Figure 1, PDB 2IVV)[10] have been shown to inhibit the RET kinase in nanomolar concentrations and **2** is currently undergoing phase II clinical trials. Both inhibitors are ATP competitive and bind into the ATP binding site with small adjustments compared to AMP.[10] In the X-ray structures, **1** forms two hydrogen bonds to the kinase (N to NH of Ala807 and NH_2 to C=O of Glu805), whereas **2** only forms one hydrogen bond (N to NH of Ala807).[10] Furthermore, the tolyl group of **1** and the bromofluorophenyl group of **2** both occupy a hydrophobic pocket in the back of the ATP binding site.[10] We were interested in developing inhibitors, based on the pyrazolopyr-
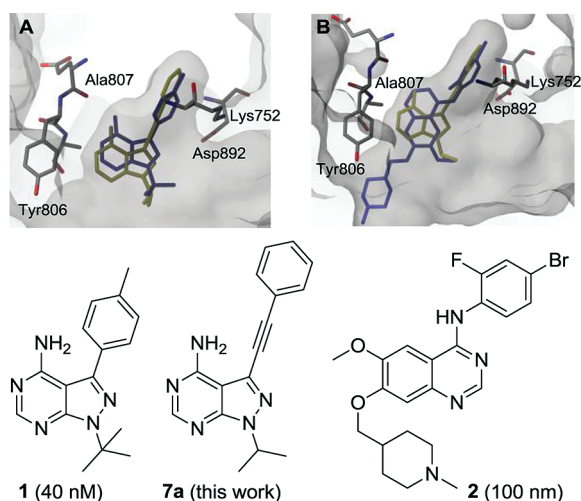
Figure 1. Initial docking of **7a** (yellow, A and B) into the crystal structure of the RET kinase (2IVV and 2IVU) containing the original ligands **1** (blue, A) and **2** (blue, B), respectively. Compound **7a** has the same binding mode as **1** and interacts with the same hydrophobic pocket in the RET kinase that is seen for both **1** (blue, A) and **2** (blue, B).

imidine scaffold, with a hydrophobic arm that utilizes the hydrophobic back pocket. Our design strategy was to extend the pyrazolopyrimidine with an alkynyl linker in the 3-position of the pyrazole ring leading to compound **7a** (Figure 1).

Compound **7a** was docked into the X-ray structures of RET (2IVV and 2IVU) using Schrödinger's Glide. The docking suggests a similar binding mode of **7a** and **1** in both crystal structures, although compound **7a** is slightly shifted out of the pocket (Figure 1A). Moreover, the extended hydrophobic arm of compound **7a** fits deeper into the hydrophobic back pocket than the inhibitors **1** and **2** (Figure 1A,B). This suggests that compound **7a** could potentially inhibit the RET kinase; therefore, we were interested in synthesizing a small library of compounds with an extended linker between the pyrazolopyrimidine scaffold and the carbocyclic part of the inhibitor.

We used a convergent route for preparing 1,3-disubstituted pyrazolopyrimidines (Scheme 1 and Table 1).[17] This route

Scheme 1. Synthesis of 1,3-Disubstituted Pyrazolopyrimidines
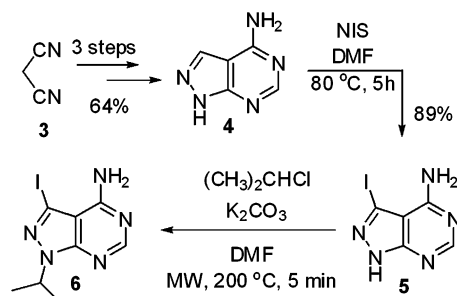
involves the synthesis of a common intermediate, 4-amino-3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidine (**5**) that allowed rapid derivatization of the heterocyclic core scaffold in two steps (Scheme 1).

Compound **5** was reacted with isopropyl chloride in the presence of potassium carbonate in DMF and with microwave-

Table 1. Synthesis of Compounds 7a–7p, 8a, 8b, and 9

compd	R^2	yield (%)
7a	phenyl	81
7b	4-fluorophenyl	79
7c	4-pyridinyl	68
7d	3-pyridinyl	63
7e	2-pyridinyl	88
7f	4-aminophenyl	88
7g	3-aminophenyl	90
7h	2-aminophenyl	78
7i	4-trifluoromethyl-phenyl	71
7j	1-methyl-1*H*-imidazol-5-yl	50
7k	1-napthyl	70
7l	6-methoxy-2-napthyl	68
7m	2,5-dimethylphenyl	77
7n	prop-1-en-2-yl	56
7o	*t*-butyl	97
7p	ferrocenyl	51
8a	–(CH$_2$)$_2$-Ph	78
8b	–(CH$_2$)$_2$-4-F-Ph	86
9	phenyl	81

assisted heating at 200 °C for 5 min. The reaction yielded **6** in nearly quantitative yield and high regioselectivity.[18]

Compound **6** was then reacted with several aromatic acetylenes (5 equiv) in the presence of Pd(PPh$_3$)$_4$ (2 mol %), copper iodide (20 mol %), and Amberlite IRA-67 (5 equiv) in THF, using microwave assisted heating (120 °C, 15 min), in order to generate compounds with the general structure **7a–p**. This protocol is a further development of our previous methodology,[18] which now allows for short reaction times (within minutes) and easy workup (use of ion-exchange resin). The reactions proceeded in moderate to excellent yields (51–97%) (Table 1).

To include compounds with an aliphatic linker, the triple bonds of **7a** and **7b** were hydrogenated using Pd/C in ethanol at 40 °C for 48 h to yield compounds **8a** and **8b** in good yields (78% and 86%, respectively). To include an alkenyl moiety as a linker, **6** was also reacted with *trans*-2-phenylvinylboronic acid (1.5 equiv) in the presence of Pd(PPh$_3$)$_4$ (2 mol %) and K$_2$CO$_3$ (3 equiv) in dioxane:water (v/v 3:1) at 150 °C (MW) for 30 min to generate **9** in 81% yield.

The effect of molecules **7a–p**, **8a**, **8b**, and **9** on the activity of RET was tested in a cell-free inhibition assay at 300 nM for compounds **7a–p**, **8a**, **8b**, and **9** (Figure 2A). The assay measured the efficacy of compounds **7a–p**, **8a**, **8b**, and **9** to decrease the substrate phosphorylation. The most efficient inhibitor has a phenyl group as R^2. Introducing larger groups,
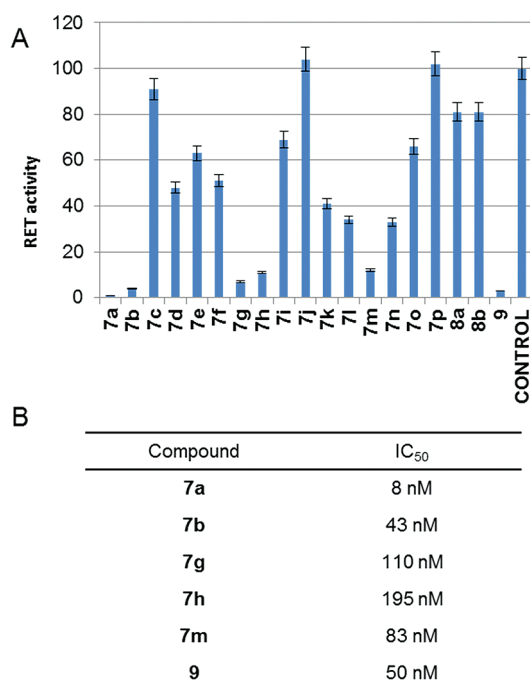
Figure 2. In vitro RET kinase activity assays. (A) Efficacy of **7a**−**p**, **8a**, **8b**, and **9**, using a compound concentration of 300 nM. (B) IC$_{50}$ values for **7a**, **7b**, **7g**, **7h**, **7m**, and **9**. For experimental details, see http://www.millipore.com/techpublications/tech1/pf3036.

such as a naphthyl group in **7k** and **7l**, leads to lower inhibition. The same effect is also seen in compounds with smaller substituents (**7j**, imidazole; **7n**, 3-methylbut-3-en; **7o**, *t*-butyl). On the other hand, substituents in the phenyl ring, such as a fluorine atom in the 4-position (**7b**), an amino group in the 3- or 2-position (**7g** and **7h**), and methyl groups in the 2- and 5-positions (**7m**) also showed efficient inhibition. Replacing the triple bond in the ethynyl linker with a fully saturated linker (**8a** and **8b**) resulted in a less efficient inhibitory effect. However, using a double-bond linker, as seen in **9**, provides good RET kinase inhibition. The result shows that six of the compounds, **7a**, **7b**, **7g**, **7h**, **7m**, and **9**, reduce the phosphorylation considerably (less than 20% remaining activity).

The IC$_{50}$ values for the most promising compounds, **7a**, **7b**, **7g**, **7h**, **7m**, and **9**, were determined using a commercial radiometric RET assay (Figure 2B) by measuring the substrate phosphorylation at different inhibitor concentrations (0.3–3000 nM). All six compounds showed a strong inhibition of the RET activity, with IC$_{50}$ values ranging from 8 to 195 nM (Figure 2). The results show that the lipophilic substituents (phenyl, 4-fluoro-substituted, and 2,5-dimethyl-substituted phenyl) provide the strongest inhibition, which indicates the hydrophobic nature of the back pocket.

To be an efficient inhibitor, in addition to strong and fast inhibition of the kinase, the inhibitor should also bind, selectively, only to the RET kinase. The selectivity of compound **7a** was evaluated toward a panel of 53 serine/threonine/tyrosine kinases (40 Ser/Thr kinases, 12 Tyr kinases, and 1 Ser/Thr/Tyr kinase) that were selected in order to reflect the human kinome (Figure 1S, Supporting Information (SI)). The panel was selected to cover each of the major kinase subfamilies, and it was further based on sequence similarity of the catalytic domain of the kinase as well as experimentally determined inhibition profiles with common kinase inhibitors.

The efficacy was then measured radiometrically, in the presence of **7a** (10 μM); the remaining activity is a measurement of the inhibition for each kinase. The inhibition profile of the panel of 53 kinases is a measurement of the selectivity of the compound. The high inhibitor concentration (10 μM) was chosen in order to ensure a reliable readout of a potential inhibition even though this concentration is much higher than the observed IC$_{50}$ value for **7a** (8 nM). To evaluate the inhibition on the panel of 53 kinases, we classified the inhibition as strong (0–25% remaining activity), intermediate (26%–75% remaining activity), and no inhibition (76%–100% remaining activity). Out of the 53 kinases, **7a** shows "no inhibition" for 29 kinases, "intermediate inhibition" for 15 kinases, and "strong inhibition" for 9 kinases (Figure 2S, SI). A closer analysis of the selectivity screen shows that only a small part of the serine/threonine kinases (7%, 3 out of 41 kinases, including c-Raf, MLK1, SAPK2) was inhibited strongly by **7a**, while a larger part of the tyrosine kinases (>50%, 7 out of 13, including Abl, EphA5, EphB4, Fyn, KDR, Lyn, MLK1) showed strong inhibition. Therefore, we extended the selectivity screen to include a larger number of receptor and nonreceptor TKs. At 10 μM, compound **7a** shows "strong inhibition" for 38 kinases, "intermediate inhibition" for 23 kinases, and "no inhibition" for 16 kinases, including the RET kinase. This means that at high concentrations (10 μM), **7a** inhibits about half of the tested TKs strongly, which has previously been seen for inhibitors such as **1** and 1NM-1.[19] However, the IC$_{50}$ value for **7a** is much lower (8 nM) compared to the concentration (10 μM) that is used in the inhibitory selectivity study. Therefore, we conducted a second selectivity screen at a concentration (0.4 μM) that corresponds to a concentration 50 times higher than the observed IC$_{50}$ value for compound **7a** (see SI) At this concentration (0.4 μM), the selectivity screen shows that only six kinases, including RET (EphA1, FGFR1, Flt4, Lck, RET, and Yes), show "strong inhibition", 26 kinases show "intermediate inhibition", and 44 kinases show "no inhibition." GDNF has been shown to induce RET-dependent phosphorylation of the extracellular signal-regulated protein kinases 1/2 (ERK1/2) in serum-starved MCF-7 breast cancer cells.[20] MCF-7 cells were grown for 24 h in low-serum medium (1% fetal bovine serum; FBS) and then exposed to increasing concentrations of **7a** for 30 min prior to the addition of 10 ng/mL GDNF. The cells were then incubated for a further 45 min and processed for Western blotting. Addition of GDNF resulted in a rapid increase in ERK1/2 phosphorylation (Figure 3A).

Further experiments have demonstrated that **7a** inhibits GDNF-induced phosphorylation of ERK1/2 at concentrations as low as 100 nM (Figure 3A). To confirm that the decreased phosphorylation of ERK1/2 is due to selective inhibition of the RET kinase by **7a**, we compared its effect on ERK1/2 phosphorylation in TPC1 papillary thyroid carcinoma cells, which express a constitutively active RET fusion protein (RET/PTC1),[21] and HCT116 colon cancer cells, which do not express the receptor kinase. In these experiments, **7a** partially suppressed ERK1/2 phosphorylation in TPC1. However, in HCT116 cells, the ERK1/2 phosphorylation was not suppressed, which suggests that the inhibition is RET-dependent (Figure 3B). Phosphorylation induces the translocation of ERK1/2 to the nucleus.[22] We observed a reduction in nuclear pERK1/2 in TPC1, but not HCT116 cells, exposed to 200 nM **7a** for 24 h (Figure 3C). Together, these findings demonstrate
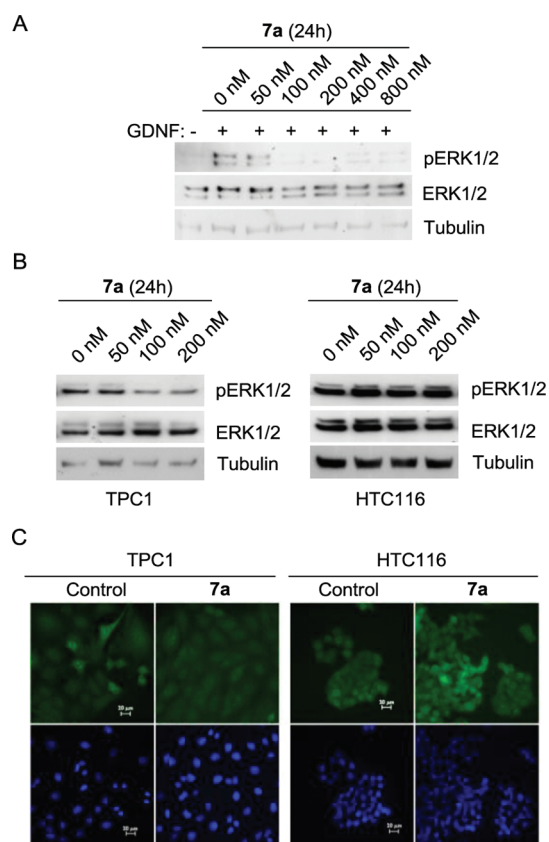
Figure 3. In vivo characterization of **7a**. (A) MCF-7 cells were grown for 24 h in low-serum medium and then exposed to the indicated doses of **7a** for 30 min prior to addition of GDNF. The cells were then incubated for a further 45 min. Total cell lysates were resolved by SDS-PAGE, and membranes were probed with antibodies directed against pERK1/2, ERK1/2, and tubulin. (B) HCT116 and TPC1 cells were exposed to the indicated concentrations of **7a** for 24 h. Total cell lysates were treated as in Figure 4A. (C) TPC1 and HCT116 cells grown on coverslips were exposed to 200 nM of **7a** for 24 h, fixed, and stained with antibodies directed against pERK1/2. Following staining with a fluorescein isothiocyanate-conjugated secondary antibody, cells were examined by immunofluorescence microscopy. Scale bar: 20 μm.

Figure 4. Induced fit dockings of **7a**, **7b**, **7g**, **7h**, **7m**, and **9** into the crystal structure of the RET kinase (2IVV).

that **7a** is cell-permeable and a potent RET inhibitor that could be useful for studying RET-dependent signaling.

The effect of **7a** on HCT116 and TPC1 proliferation was also investigated (Figure 2S, SI). **7a** inhibited HCT116 and TPC1 proliferation, with EC$_{50}$ values of 2.4 and 3.3 μM, respectively. TPC1 cells however, were considerably more sensitive to 1 μM **7a** than were HCT116 cells. Furthermore, **7a** and sorafenib inhibited TPC1 proliferation to similar degrees (EC$_{50}$: 2.4 and 2.6 μM, respectively; Figure 1S, SI). At the concentrations used (≤10 μM), no overt cytotoxicity was observed in any of the cell lines tested.

Compounds **7a**, **7b**, **7g**, **7h**, **7m**, and **9** were docked into the crystal structure of the RET-KD kinase (2IVV), using induced fit dockings (Schrödinger's Glide) (Figure 4). The docking results show that compounds (**7a**, **7b**, **7g**, **7h**, **7m**, and **9**) utilize the hydrophobic back pocket, even though compound **7m**, with a more sterically demanding substituent, is positioned further out in the ATP binding site (Figure 4E).

CONCLUSIONS

Taken together, the data presented here indicate that **7a** is, indeed, a potent RET kinase inhibitor, and the strong cell-free
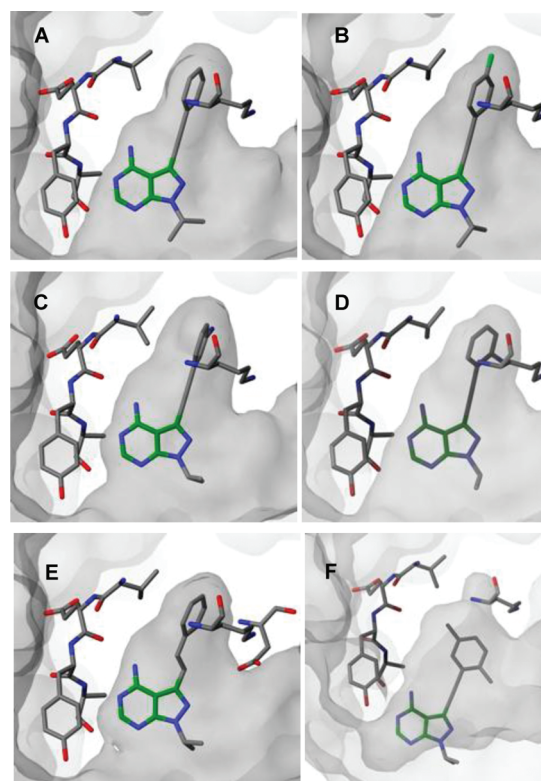
inhibition (8 nM) suggests that the compound binds more strongly to the hydrophobic pocket than does **1**. Compound **7a** should be a useful tool for studying and understanding the complex molecular mechanisms that underlie resistance to endocrine therapy.

EXPERIMENTAL SECTION

General. See SI for details. All final compounds were confirmed to be of >95% purity, except **7f** (76%), based on HPLC analysis.

General Procedure A for the Synthesis of 7a−p. Compound **6** (1 equiv), aryl acetylene (5 equiv), Pd(PPh$_3$)$_4$ (2 mol %), Amberlite IR-67 (5 equiv), and CuI (20 mol %) in THF was heated for 15 min at 120 °C in the microwave. After cooling to ambient temperature, the reaction mixture was filtered through a pad of Celite. The solvent was removed in vacuo, and the crude product was purified by flash column chromatography on silica gel.

General Procedure B for the Synthesis of 8a and 8b. The starting material (1 equiv) was dissolved in ethanol (10 mL, 99.9%). Pd/C (10% w, 10 mg) was added, and the reaction mixture was put under H$_2$ atmosphere. The solution was carefully heated to 40 °C for 48 h. The reaction mixture was filtered through a pad of Celite, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on silica gel (MeOH:CHCl$_3$ = 1:20).

ASSOCIATED CONTENT

Ⓢ Supporting Information

Preparation and characterization of compounds **7−9**, ^{1}H- and ^{13}C NMR spectra, HPLC purity, and effects of our RET inhibitors in human cancer cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

TK, tyrosine kinases; RTK, receptor tyrosine kinases; RET, rearranged during transfection; GDNF, glial-derived neurotrophic factor; MEN2, multiple endocrine neoplasia 2 syndromes; FMTC, familial medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; DMF, dimethylformamide; THF, tetrahydrofuran

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