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Discovery and characterization of the *N*-phenyl-*N*′-naphthylurea class of p38 kinase inhibitors

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ABSTRACT

An effort aimed at exploring structural diversity in the *N*-pyrazole-*N*′-naphthylurea class of p38 kinase inhibitors led to the synthesis and characterization of *N*-phenyl-*N*′-naphthylureas. Examples of these compounds displayed excellent inhibition of TNF-α production in vitro, as well as efficacy in a mouse model of lipopolysaccharide induced endotoxemia. In addition, perspective is provided on the role of a sulfonamide functionality in defining inhibitor potency.

Elevated levels of pro-inflammatory cytokines, most notably tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are associated with a number of inflammatory diseases.[1] Biologics directed at TNF-α and IL-1β, have been approved and marketed for diseases such as rheumatoid arthritis.[2]

The p38α mitogen activated protein kinase (p38 hereafter) plays a central role in the signal transduction pathway leading to the production of a variety of cytokines, including TNF-α and IL-1β.[3] Therefore intense research has been devoted to the discovery of p38 inhibitors, as these would be anticipated to have similar therapeutic benefit to the anti-cytokine biologics, with the convenience of oral dosage, and lower cost.[4]

We previously described the discovery of the *N*-*N*′-diarylurea inhibitors of p38, which culminated in the clinical compound **BIRB 796** (doramapimod; **1**).[5] We subsequently reported further elaboration of this class of compounds by modifications in the adenine/ATP binding site, as exemplified by compound **2** (Fig. 1).[6]

The binding mode of these diarylureas to p38 has been described. Figure 2 graphically summarizes the key interactions using compound **2** as an example. The pyrazole *tert*-butyl substituent takes advantage of a lipophilic binding site made available by movement of the side chain of Phe 169 on the kinase activation loop ('Phe out' mode).[5]

In an effort to expand the structural diversity of this class of inhibitors, we investigated modifications on the side of the urea associated with the 'Phe out' binding mode. Since the pyrazole nitrogen atom does not appear to hydrogen bond to the enzyme, we hypothesized that this heterocyclic ring acts primarily as a scaffold to orient the appended pharmacophores, and that it could be replaced by a six-membered aromatic ring. We therefore investigated *N*-phenyl-*N*′-naphthylurea inhibitors of p38.[7]

We prepared compounds **5** and **6** as the first examples (Table 1) for comparison to the methyl pyrazole analogues **3** and **4** and the more potent tolyl pyrazole analogues **1** and **2**. Because of the intrinsic high affinity and slow binding kinetics of members of this class of compounds to p38, we relied on a thermal denaturation assay as a means of assessing relative binding affinity.[8] The higher the denaturation temperature (T_m) of the enzyme-inhibitor complex, the tighter the binding of the inhibitor to the enzyme. The T_m values for compounds **5** and **6** were comparable though somewhat lower than the pyrazole analogues **3** and **4**, and as expected were substantially lower than the more elaborated pyrazole analogues **1** and **2**. Interestingly, we observed a greater difference in cell potency between compounds **5** and **6** than we observed between compounds **1** and **2** or **3** and **4**. Compound **6** proved essentially equipotent to compound **2** in the THP-1 and whole blood cell

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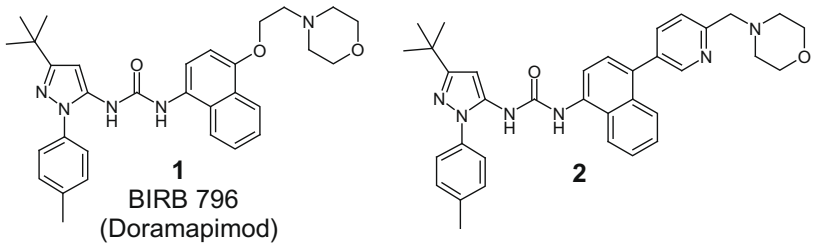


Figure 1. Structures of *N*-pyrazole-*N*′-naphthylurea inhibitors of p38.

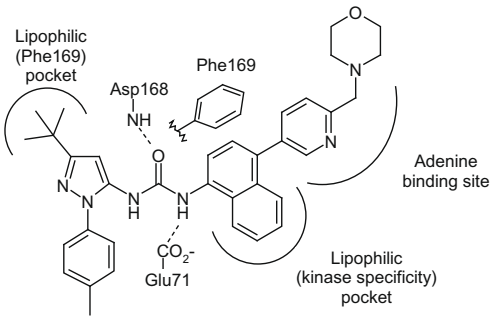
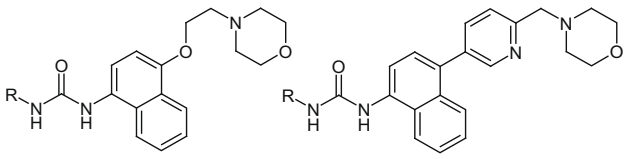
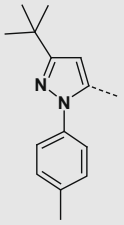
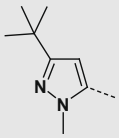
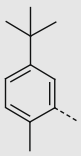


Figure 2. Graphical representation of key interactions between **2** and p38.

Table 1
Effect of changes on the *N*-aryl portion of the *N*-aryl-*N*′-naphthylurea

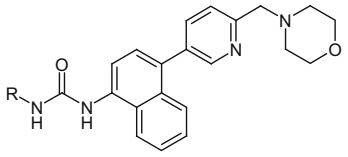


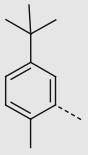
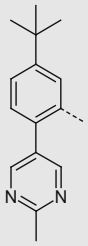
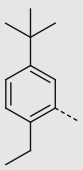
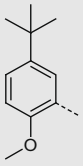
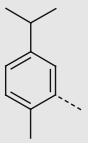
Compound	R		T_m[a] (°C)	THP-1[b] IC_{50} (nM)	HWB[c] IC_{50} (nM)
1		A	63.7	18	780
2		B	63.6	8.0	220
3		A	54.5	140	10,000
4		B	57.9	29	230
5		A	52.7	380	>10,000
6		B	54.6	9.4	180

[a] Denaturation temperature of the unphosphorylated human p38:inhibitor complex; standard deviation for $n > 2$ experiments typically 0.1–0.5 °C.
[b] Values are means of 2–4 experiments, standard deviations for $n > 2$ experiments typically ±50% of reported value.
[c] Values are geometric means from 4–12 donor experiments, standard deviation typically ±50% of reported value.

assays despite having a 9° lower T_m.[9] This result was even more incongruous considering that the relatively weak cell potencies observed for compound **5** fell in line with expectations based on its T_m value. Intrigued by this result with compound **6**, we made other phenyl analogs with naphthylurea **B**, focusing initially on changes at the methyl and *tert*-butyl positions (Table 2).

Table 2
Effect of substitutions on *N*-phenyl group



Compound	R	T_m[a] (°C)	THP-1[b] IC_{50} (nM)	HWB[c] IC_{50} (nM)
6		54.6	9.4	180
7		56.6	29	350
8		53.9	8.8 (2)	700
9		57.9	5.5	140
10		52.3	76	Not tested

[a,b,c]See notes from Table 1.

To determine whether the increased potency associated with the tolyl group over the methyl group in pyrazole-based inhibitors would translate to the new phenyl-based inhibitors, we prepared compound **7**. We selected a methyl pyrimidine over a tolyl group since the methyl pyrimidine had previously been identified in the pyrazole series as an equipotent but less lipophilic tolyl replacement.[6] Compound **7** did have a modestly higher T_m than compound **6**, but its cell potency was more in line with what might have been predicted based on the T_m-THP-1 trends for compounds **1-5**.

Confirmation that the favorable THP-1 cell potency observed for compound **6** was not a unique occurrence came from the comparable cell potencies obtained for structurally related analogues **8** and **9**. A comparison of compound **6** and **10** confirmed that the three methyls of the *tert*-butyl group are still required for potency, consistent with what we observed for the pyrazole-based inhibitors. Since the methoxy derivative **9** provided the best combination of T_m and cell potency, we selected it as a frame of reference for further SAR.

Compound **9** did not possess ideal physicochemical properties (pH 7.4 aqueous solubility <0.5 μg/mL). Consequently, in addition to exploring the SAR of these new phenyl-based inhibitors, we also wanted to identify opportunities to improve their physicochemical properties. A single crystal X-ray diffraction of compound **9**-unphosphorylated human p38 complex revealed that the 6-position on the phenyl ring is exposed to solvent. Therefore, we explored simple polar functionality at this position with the initial intention of establishing if substitutions would be tolerated at this position (selected examples shown in Table 3).

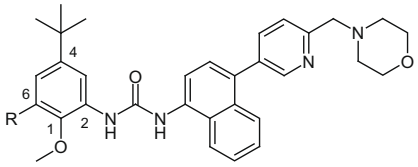
Unexpectedly the modifications shown in Table 3 had a far greater effect on T_m than they did on inhibitor aqueous solubility which was marginally improved at best versus compound **9**. Several R groups improved T_m 5–7 °C. Intriguingly, the series of compounds in Table 3 displayed a wide range of T_ms ranging ~9 °C from lowest to highest. In contrast, potency in the THP-1 cell and whole blood assays remained essentially invariant.

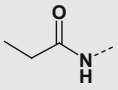
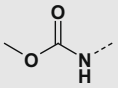
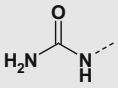
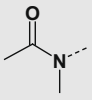
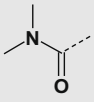
Despite the apparent T_m-THP-1 assay disconnects, compound **17** emerged as having the best combination of improved whole blood potency over compounds **1** and **2** together with hydrolytic stability in vivo that avoided formation of aniline **11**. Compound **17** was profiled in a mouse model of lipopolysaccharide-induced endotoxemia. An oral dose of 3 mg/kg resulted in 57% inhibition of TNFα production. BIRB 796 (**1**) did not show significant efficacy at this dose.[10]

While we had successfully identified phenyl-based p38 inhibitors surpassing our best pyrazole-based inhibitors, we were left pondering the apparent disconnects between the T_m and THP-1 cell assay results. Compound **6** and close analogues have relatively low T_m values but good cell potency while derivatives in Table 3 can have substantially higher T_m values with little impact on cell potency. Of course one should not always expect good correlations between molecular and cellular potency based on the impact of molecular structure on a compound's ability to reach efficacious concentrations inside a cell. However, we had seen reasonable T_m-THP-1 correlations within the pyrazole series. In addition, the physicochemical properties of the new phenyl based inhibitors were not substantially different from those of the pyrazole series.

It is conceivable that the thermodynamics of binding to p38 for phenyl-based inhibitors differs significantly from pyrazole-based inhibitors. This could impact the relationship between T_m and K_d enough to make inter-series T_m comparisons less meaningful. This thermodynamic argument could also apply to inhibitors within a series, especially if structural modifications create new or different interactions with p38. This argument could explain the lack of impact of increasing T_m on cell potency for the various compounds in Table 3. In addition, reaching the limit of differentiation in the THP-1 cell assay could be another possible explanation for this disconnect. We have never observed an IC_{50} below 5 nM in this assay with ours or competitor's compounds.

Table 3
Effect of 6-position substituents



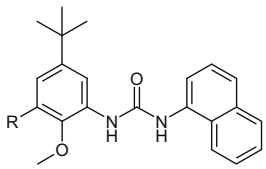
Compound	R	T_m[a] (°C)	THP-1[b] IC_{50} (nM)	HWB[c] IC_{50} (nM)
9	H-	57.9	5.5	140
11		61.5	5.0	22
12		63.6	9.0	35
13		58.7	5.1	71
14		58.1	8.5	110
15		56.7	12	110
16		64.9	15	120
17		63.3	10	46
18		65.5	9.4	74
19		65.6	10	97
20		56.2	12	97

[a,b,c]See notes from Table 1.

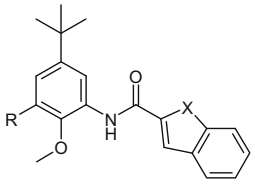
Given these T_m–THP-1 assay disconnects, we became particularly interested in verifying whether the presence of a sulfonamide group truly improves binding affinity (compound **9** versus **17**), since the phenyl sulfonamide portion of **17** became a key piece in subsequent inhibitor series.[11] This was not straightforward to test.

We attempted to calculate dissociation constants for compounds **9** and **17** using exchange curve binding kinetic analyses. We previously used this method to generate a K_d for compound **1** (0.1 nM).[12] However, the inherent long off rates of our potent 'Phe out' p38 inhibitors often impeded accurate off rate extrapolation. This undermined the general utility of this method for determining K_d values (the half life for dissociation of compound **1** from p38 was on the order of 24 h). Nevertheless, compound **9** returned

Table 4
The effect of sulfonamide group on truncated inhibitors



C



D: X = O
E: X = S

	Compound	R	p38α IC_{50}[d] (nM)	T_m[a] (°C)
C	**21**	H-	3900	50.3
	22	$MeSO_2NH$-	79	56.3
D	**23**	H-	>10,000	46.2[e]
	24	$MeSO_2NH$-	4100	48.8
E	**25**	H-	>10,000	46.9[e]
	26	$MeSO_2NH$-	2700	51.4

[a] See notes from Table 1.
[d] Assay measured inhibition of ATF2 phosphorylation by p38 (LANCE) after 4 h pre-incubation of p38 with inhibitor (value average of two determinations ±40%).
[e] Result from one test.

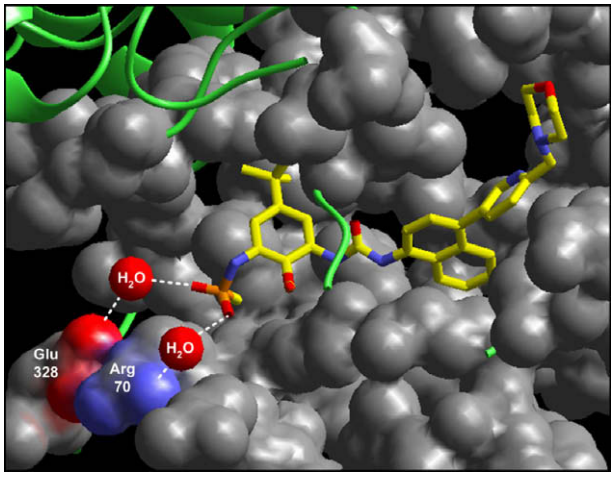


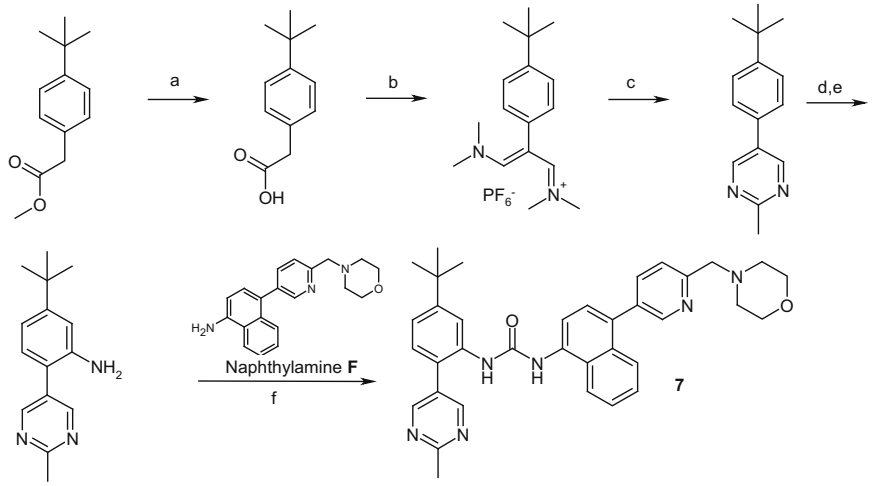
Figure 3. Single crystal X-ray diffraction analysis of the compound **17**- unphosphorylated human p38 complex, at 2.4 Å resolution, highlighting the interactions of the sulfonamide group with water bound at the surface of the enzyme.[13]

a K_d value of 1.8 nM, approximately 10-fold weaker than **1** and consistent with their relative T_ms. Unfortunately we could not generate a reliable K_d for compound **17** due to an inability to accurately extrapolate its off rate. However, the experiment at least suggested that compound **17** bound tighter to p38 than compound **9**.

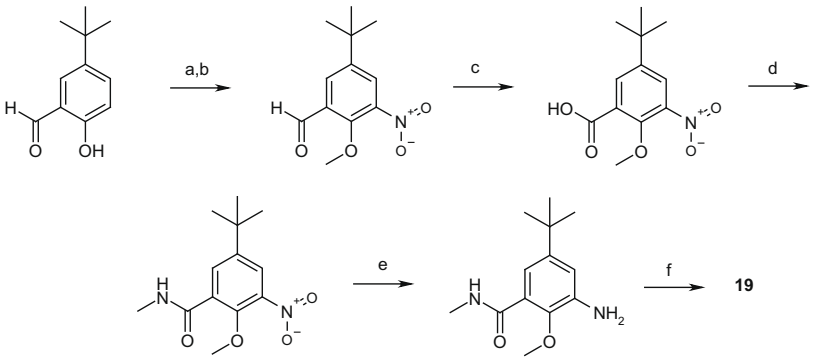
The intrinsic high affinity and slow binding kinetics of these 'Phe out' inhibitors also complicated the use of conventional kinase assays for accurately assessing relative potency. The low concentrations of both inhibitor and enzyme required for assessing subnanomolar potency coupled with inherently slow on rates necessitates preincubation times of 24 h or more. Enzyme stability and the general reproducibility of the assay became issues with this lengthy preincubation. Since the need for extensive preincubation diminishes as the potency of the compound decreases, we assessed the relative potency of three pairs of sulfonamide and non-sulfonamide containing compounds lacking the adenine binding site moiety (Table 4). These truncated analogues were expected to be significantly less potent such that reliable IC_{50}s could be expected with a 4 h pre-incubation. Compounds **21** and **22** were the obvious selections from the *N*-phenyl-*N*′-naphthylurea class of p38 inhibitors. We also evaluated two pairs of truncated compounds from the benzofuran (**23** and **24**) and benzothiophene (**25** and **26**) series previously described.[11a] Although the truncated benzofuran and benzothiophene-based inhibitors were inherently weak p38 inhibitors, in all three cases the sulfonamide containing inhibitors produced lower IC_{50}s than the corresponding non-sulfonamide analogues. These experiments helped support the results of the T_m assay and substantiated that the sulfonamide group does increase affinity to p38.

An X-ray co-crystal structure of compound **17** bound to unphosphorylated human p38 revealed interactions that could help explain the role of the sulfonamide group (Fig. 3).[13] The sulfonamide methyl group situates itself into a small lipophilic pocket below the plane of the phenyl ring. In addition, the sulfonamide oxygens partake in water mediated hydrogen bonding interactions with Glu 328 and Arg 70 side chains.

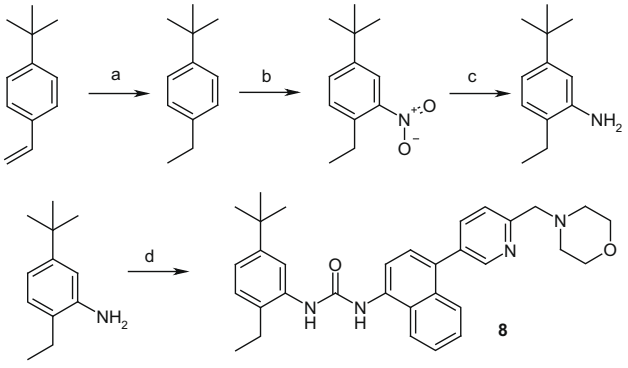
The synthesis of the naphthylamine portion of the inhibitors contained in this paper has been described previously.[5,6] The substituted anilines required to prepare **6**, **9** and **10** are commercially available. The synthesis of the sulfonamide containing *tert*-butyl aniline in compound **17** has been detailed previously starting from commercially available 4-*tert*-butyl-2,6-dinitroanisole.[11a] The syntheses of anilines contained in compounds **7**, **8**, and **19** are



Scheme 1. Reagents and conditions: (a) $LiOH{\cdot}H_2O$, $MeOH–H_2O$, rt; (b) $POCl_3$, DMF, 0–110 °C, then $NaPF_6$; (c) $CH_3C(NH)NH_2{\cdot}HCl$, NaOEt, EtOH, reflux; (d) NO_2BF_4, CH_3CN, 0 °C; (e) HCO_2NH_4, Pd/C, EtOH, reflux; (f) $COCl_2$, satd aq $NaHCO_3$, CH_2Cl_2, 0 °C; then naphthylamine **F**, THF, rt.



Scheme 3. Reagents and conditions: (a) NO_2BF_4, CH_3CN, −35 °C to rt; (b) MeI, K_2CO_3, DMF, rt; (c) Jones' reagent, $(CH_3)_2CO$, rt; (d) EDC, $MeNH_2$, CH_3CN, rt; (e) Cyclohexene, Pd/C, CH_3CN, reflux; (f) $COCl_2$, satd aq $NaHCO_3$, CH_2Cl_2, 0 °C; then naphthylamine **F**, THF, rt.



Scheme 2. Reagents and conditions: (a) HCO_2NH_4, Pd/C, CH_3CN, reflux; (b) NO_2BF_4, CH_3CN, −35–0 °C; (c) HCO_2NH_4, Pd/C, MeOH, reflux; (d) $COCl_2$, satd aq $NaHCO_3$, CH_2Cl_2, 0 °C; then naphthylamine **F**, THF, rt.

exemplified in Schemes 1–3, respectively.[13] The anilines contained in compounds **12** to **15** were derived from 4-*tert*-butyl-2,6-diaminoanisole[11a] by treatment with either 1 equiv of the appropriate acyl chloride, isocyanate or chloroformate. Aniline intermediates were typically incorporated into the final compound by treatment with phosgene to form the isocyanate, followed by reaction with naphthylamine **F**. The synthesis of compounds **22**, **24**, and **26** have been detailed[11a] and the corresponding des-sulfonamide analogs **21**, **23**, and **25** were prepared in an analogous manner.

In summary, we identified a class of *N*-phenyl-*N*′-naphthylurea based inhibitors of p38 in which the tolyl pyrazole portion of our previous series has been replaced by substituted phenyl derivatives. The highlighted compound **17** containing a sulfonamide group provided potent in vitro and in vivo inhibition of TNF-α production. In addition we provided some perspective on the role that the sulfonamide group plays in defining inhibitor potency.

Acknowledgement

The authors thank Rolf Göggel for determining the IC_{50}s for compounds **21** to **26**.

References and notes

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7. While these studies were on-going, reports appeared describing *N*,*N*′-diphenylureas as kinase inhibitors, including p38. An *N*,*N*′-diphenylurea BAY 43-9006 has successfully been developed into an anticancer drug inhibiting Raf, VEGFR and PDGFR kinases. See: Lowinger, T. B.; Riedl, B.; Dumas, J.; Smith, R. A. *Curr. Pharm. Des.* **2002**, *8*, 2269.
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9. Experimental details for the cell assays of LPS-induced TNF production can be found in Ref. 5.
10. Compound **17** showed negligible inhibition of the following kinases when tested at or above 3 μg/mL: BTK, CDK1, CK1 & 2, DYRK1A, ERK, GSK3b, HGFR, IGF1R, IKK, IR, JAK3, JNK1A1, LCK, MAPKAPK1B & 2, MKK1, NIK, PDK1, PHK, PI3K, PKA, PKCa, PRAK, PTK2, ROCK II, RSK2, S6K1, SGK, SYK, TXK. The flowing table lists kinases showing measurable inhibition:

Kinase	IC_{50} (μM)
Abl	0.48
b-Raf	7.2
c-Raf	0.88
CSK	62 @ 3*
ECK	5.2
EGFR	5.4
FGFR	7.7
HEK	3.2
jnk2α 2	0.003**
Lyn	2.4
VEGFR	3.0
PKBα/Akt1	6.4
pp60src	8.2

* Data shown as percent inhibition of p38 at concentration in uM.
** Value determined by exchange curve binding kinetic analyses.[12]

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13. The coordinates of compound **17** bound to p38 have been deposited with the RCSB Protein Data Bank (RCSB ID code rcsb051887 and PDB ID code 3GI3).
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