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2-Arylidenedihydroindole-3-ones: Design, synthesis, and biological activity on bladder carcinoma cell lines

Bastien Gerby,[a] Ahcène Boumendjel,[b,*] Madeleine Blanc,[b] Pierre Paul Bringuier,[c] Pierre Champelovier,[a] Antoine Fortuné,[b] Xavier Ronot[a] and Jean Boutonnat[a]

[a]*Laboratoire de Dynamique Cellulaire, EPHE, UMR-CNRS 5525, IFRT 130, Université Joseph Fourier, Pavillon Taillefer, 38706 La Tronche Cedex, France*
[b]*Département de Pharmacochimie Moléculaire, UMR-CNRS 5063, Faculté de Pharmacie de Grenoble, 5, avenue de Verdun, BP 138, 38243 Meylan, France*
[c]*Laboratoire d'Anatomie pathologique, Hôpital Edouard Herriot, 69437 Lyon, France*

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Abstract—2-Arylidenedihydroindole-3-ones were assayed for their antiproliferative and apoptotic abilities as potential drug candidates to treat bladder tumor. These compounds were tested on cell lines obtained from bladder tumors of various stages [superficial (pTa and pT1) *vs.* invasive (⩾pT2)]. The most active compound (**3c**) inhibited the proliferation, induced apoptosis, and decreased the expression of *p*-Stat5 and *p*-Pyk2 in DAG-1 and RT112 lines in which the FGFR3 is either mutated or overexpressed. Knowing that FGFR3 is involved in cell proliferation, differentiation, and migration through cell signaling pathways including *p*-Stat5 way via *p*-Pyk2, let us assume that compound **3c** may probably act through FGFR3 pathway.

Bladder cancer is the fourth most common malignancy in men and the ninth most common in women in the Western world. More than 90% of bladder tumors are urothelial cell carcinomas (UCC).[1] The carcinoma can be classified in two development stages [superficial (pTa and pT1) vs invasive (⩾pT2)] and three grades (G1 to G3).[2] The clinical course of superficial (pTa and pT1) papillary urothelial cell carcinoma is characterized by a high risk of recurrence (>70%) and a propensity to progress in invasive tumors (10–15%). Muscle-invasive tumors (⩾pT2) have a poorer prognosis because 50% of these patients will relapse with metastatic disease within 2 years of treatment.[3–5]

Fibroblast growth factor receptors (FGFRs) are tyrosine kinase receptors that integrate many different intracellular signals affecting cell growth, differentiation, migration, and angiogenesis. Activation of FGFR3 induces phosphorylation residues in the intracellular domain and causes cellular proliferation and tumor development. Depending on the cellular context, these effects are activated through various signal transduction pathways.[6] FGFR3 can interact with various proteins such as SH2-B, *p*-Stat5 through *p*-Pyk2 (a member of the focal adhesin kinase family), and PI3kinase.[7–9] FGFR3 mutations and surexpression of wild type FGFR3 are consequently frequent events in low stage UCC and superficial bladder tumors.[10]

Currently, drugs available for treating UCC-related cancer need to be taken in high doses to be efficient. In this context, molecules targeting cells where FGFR3 is mutated or overexpressed can be of high interest as potential drug candidates for treating bladder carcinoma. In this regard, several molecules acting as FGFR3 inhibitors have been recently reported. Such inhibitors include: PKC412, benzimidazole quinolinones, derivatives of arylaminooxazole, CHIR-258, and imidazolidinediones.[11–15]

The naturally occurring flavonoids are known to either induce apoptosis or to modulate cell proliferation of UCC.[16] Flavonoid-rich nutrition is highly recommended for preventing bladder cancer. In earlier studies, it was demonstrated that aurones, a flavonoid-subgroup, were able to interact with ATP-binding site of P-glycoprotein

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* Corresponding author. Tel.: +33 4 76 04 10 06; fax: +33 4 76 04 10 07; e-mail: Ahcene.Boumendjel@ujf-grenoble.fr

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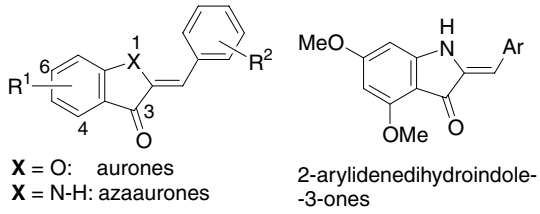
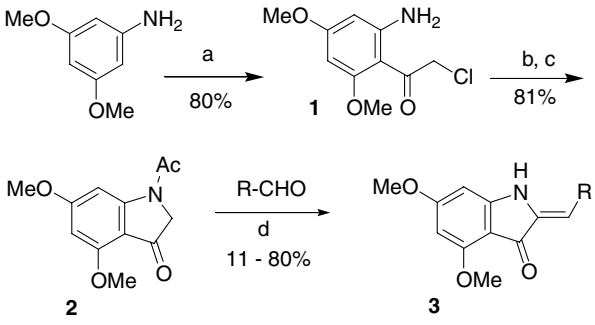


Figure 1. Structures of investigated molecules.



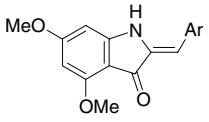
Scheme 1. Reactions and conditions: (a) Cl–CH_2–CN, $ZnCl_2$/BCl_3, CH_2Cl_2 (reflux) then HCl (2 N), reflux; (b) CH_3CO–Cl, 85 °C; (c) K_2CO_3, acetone, reflux; (d) KOH, H_2O/MeOH, 50 °C.

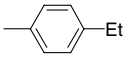
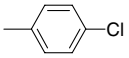
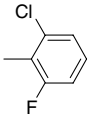
and to inhibit kinases.[17,18] Therefore, we tested aurone family members for their antiproliferative effect on UCC, because no prior studies had investigated them as agents for UCC treatment. Especially, we studied 2-arylidene-2,3-dihydro-1*H*-indole-3-ones (azaaurones) as structural analogs of the naturally occurring aurones (Fig. 1). In this series, we maintained the 4,6-dimethoxy-2,3-dihydro-1*H*-indole-3-one moiety and varied the arylidene group. The arylidene moieties were chosen on the basis of literature reports, where halogenated aryls and imidazoles were used as structural features in a number of kinase inhibitors.[19–22]

Azaaurones **3** were prepared from indolin-3-one **2** by base-catalyzed condensation with an arylaldehyde (Scheme 1). The condensation affords exclusively *Z*-azaaurones as confirmed by ^{1}H NMR.[23] Oxindole **2** was prepared starting from 3,5-dimethoxyaniline and chloroacetonitrile as previously reported.[24]

In this study, we have used three bladder tumour cell lines that mimic the bladder tumour progression process. These lines were obtained from bladder tumors of various grades and various stages (RT112 was from a pT1G2, DAG-1[25] from a pT1G3 and J82 from a pT2G3 tumors). DAG-1 cells featured the S249C mutation on exon 7 and J82 cells the K650E mutation on exon 15, whereas RT112 line overexpressed the wild type FGFR3.[26] Flow cytometry analysis showed a more important FGFR3 expression in RT112 (39 ± 2 a.u.) cells than in DAG-1 (28 ± 3 a.u.) and J82 (19 ± 4 a.u.) cells (data not shown). A preliminary screening using an in vitro test to assess the ability of a set of aurones

Table 1. The effect of azaaurones (at 10 μM) on cell viability in DAG-1, RT112, and J82 cell lines after 72 h incubation



Compound (10 μM)	Ar	Cell lines		
		DAG-1	RT112	J82
3a		98 ± 3	97 ± 3	99 ± 4
3b		99 ± 4	93 ± 3	94 ± 4
3c		58 ± 2	48 ± 3	97 ± 4
3d		91 ± 3	93 ± 5	95 ± 4
3e		90 ± 5	92 ± 3	89 ± 4
3f		89 ± 4	92 ± 4	90 ± 3
Control	Without compound	100 ± 3	100 ± 2	100 ± 2

Results are expressed in percentage of viable cells.

analogs to effect cell viability on DAG-1, RT112, and J82 cell lines allowed us to retain 2-arylidene-4,6-dimethoxy-2,3-dihydro-1*H*-indole-3-ones (azaaurones) as potential inhibitors of FGFR3 pathway (Table 1).

The preliminary test aimed to evaluate the effect of our compounds on the cell viability shows clearly that the fluorobenzylidene analog (**3c**) is the only compound able to decrease cell viability in DAG-1 and RT112 cells, but not in J82 line. These results indicate that on one hand, the effect of **3c** was not related to either grade or stage of bladder tumors and on the other hand, this effect was not simply a direct consequence of the increase of FGFR3 expression but likely to be related to the type of mutation. The results shown in Table 1 for compound **3c** were confirmed

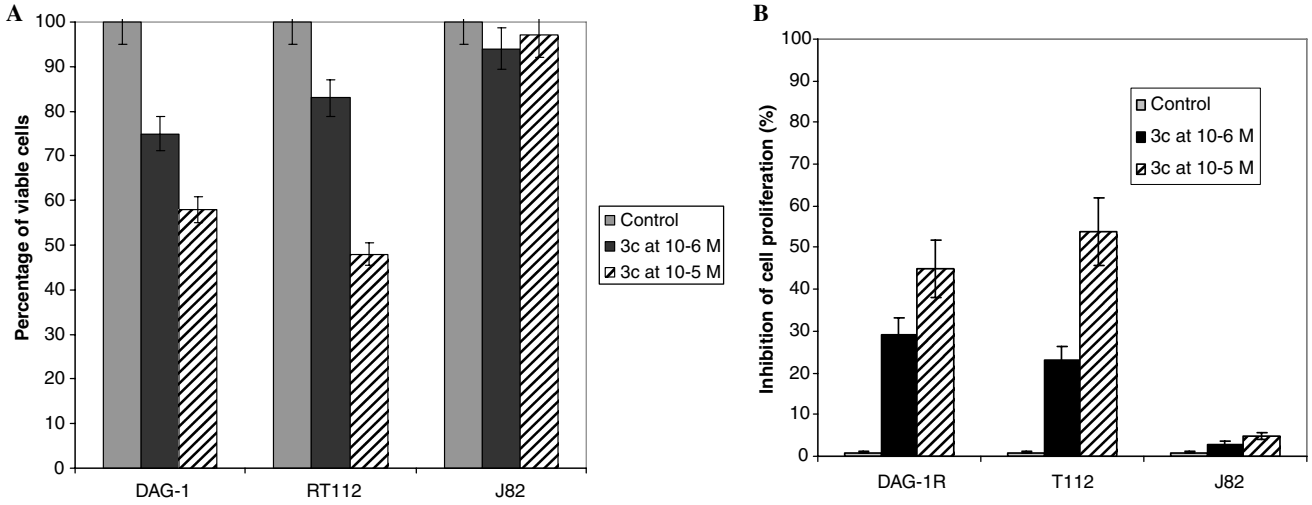


Figure 2. Effect of **3c** on cell viability and cell proliferation. DAG-1, RT112, and J82 cells were incubated for 72 h at 10^{-5} and 10^{-6} M **3c**. Then, cells were trypsinized and counted. Cell viability (A) and the inhibition of proliferation percentage (B) were calculated using the formula [(Nc − Nf)/Nc] × 100%.

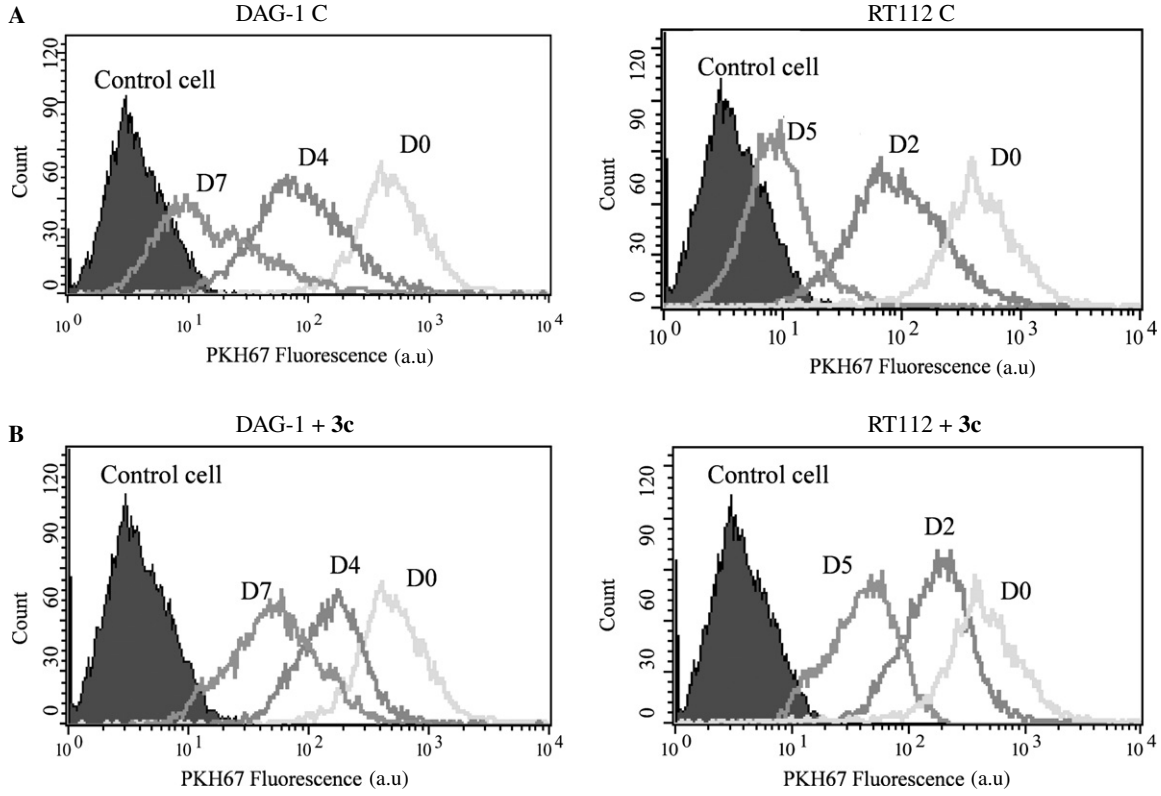


Figure 3. Effect of **3c** on cell division. DAG-1 and RT112 cells were labeled with PKH67 and treated or not with **3c** at 10^{-5} M. The cell proliferation index was determined by calculating the ratio of the daily means of fluorescence intensities compared with Day 0 (MIF_{D0}/MIF_{Dx}). The cell proliferation index was compared at D4 and D7 for DAG-1 cells (A), and at D2 and D5 for RT112 cells (B).

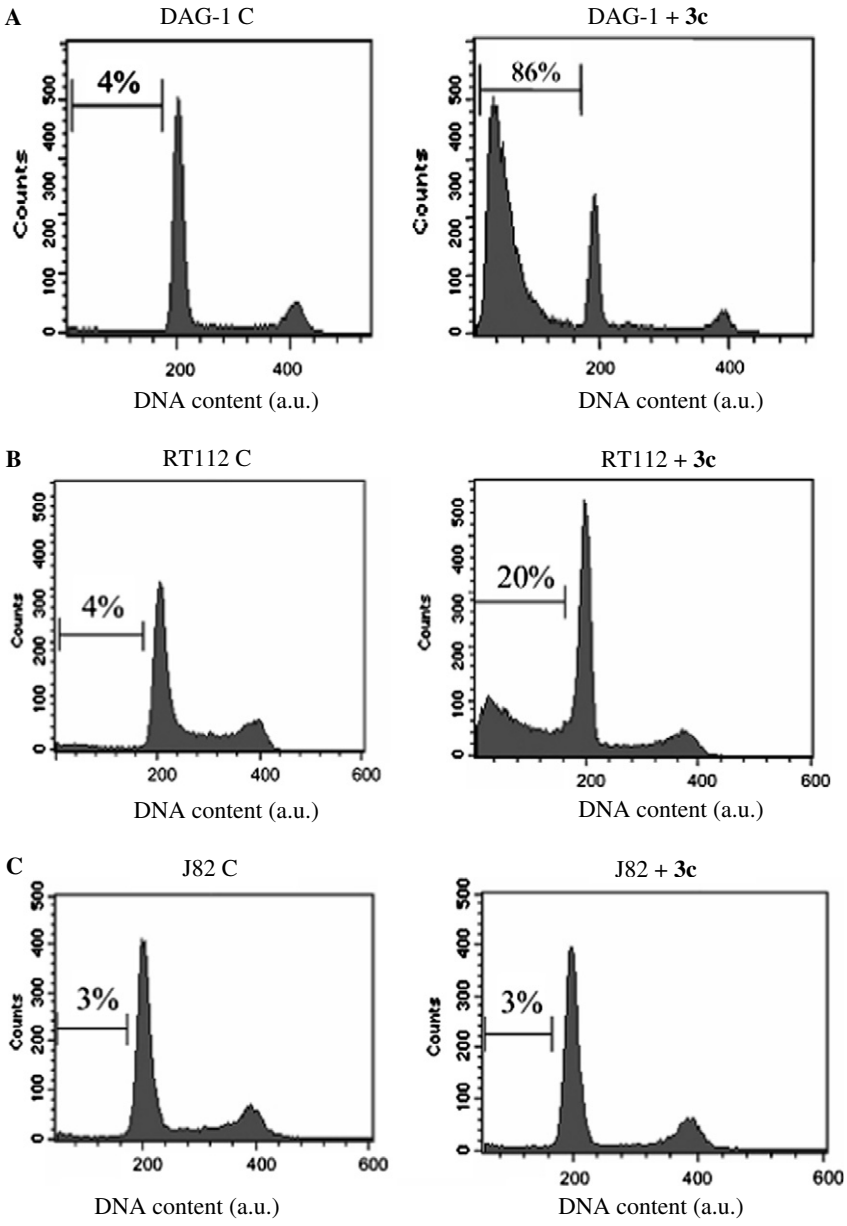


Figure 4. Flow cytometry analysis of apoptosis. DAG-1 (A), RT112 (B), and J82 (C) cells (5×10^5 cells/ml) were cultured without (C) or with **3c** at 10^{-5} M for 48 h. Cultures were then trypsinized, cells stained using either the DNA cycle test kit and analyzed by flow cytometry (FACScalibur).

at lower concentration 1 μM (Fig. 2). The position of the fluorine atom is crucial, because no significant activity was observed when this atom was moved to 3′ or 4′ positions (results not shown). In attempting to study the 2′,6′-difluoroanalog, we met with difficulties in synthesizing it because one of the two fluoro atoms was lost and we end up with **3c**.

In order to confirm the effect of **3c** on cell division, we proceeded for PKH67 labeling. The three cell lines were labeled with PKH67 and incubated with or without **3c** at 10 μM for various durations (D0, D4, and D7) for DAG-1 cells; (D0, D2, and D5) for RT112 and J82 cells. As shown in Figure 3, the cell proliferation index increased sensibly in DAG-1 and RT112 untreated cells but not in J82 (results not shown).

We also observed that **3c** induced apoptosis on FGFR-3-expressing cell lines. After treatment of DAG-1 and RT112 cells with 10 μM of **3c** for 48 h, adherent cells progressively detached, showing signs of an apoptotic process. The apoptosis of DAG-1 and RT112 cells was analyzed with the DNA cycle test kit. Flow cytometry showed that in presence of **3c**, the percentage of apoptotic and necrotic cells (subdiploid cells) increased up to $86 \pm 4\%$ and $20 \pm 3\%$, respectively, in DAG-1 and RT112 cells (Fig. 4). The compound did not induce an apoptotic process on the J82 cells.

The effect of **3c** on the *p*-Stat5 and *p*-Pyk2 antigen level was examined. We found that the inhibition of cellular growth and induction of apoptosis were associated with the inhibition of the FGFR3 cell signaling pathway. At

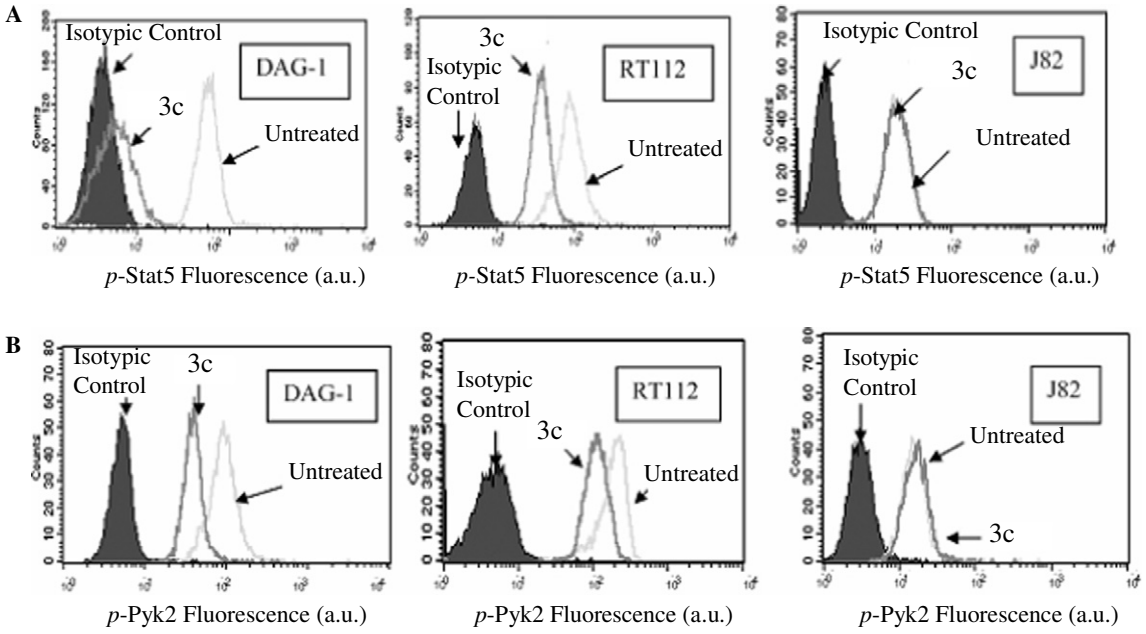


Figure 5. Flow cytometry analysis of *p*-STAT5 and *p*-Pyk2 antigens expression. DAG-1, RT112, and J82 cells were treated with **3c** (10^{-5} M) for 6 h. Then, *p*-Stat5 (A) and *p*-Pyk2 (B) antigen level expression was analyzed by flow cytometry.

10 μM, it inhibited the expression of phosphorylated Stat5 (*p*-Stat5) and phosphorylated Pyk2 (*p*-Pyk2) in both DAG-1 and RT112 cells in a time dependent manner. The compound caused a transient (from 0 to 6 h) decrease in the intracellular levels of both *p*-Stat5 and *p*-Pyk2 antigen expressions (9.5-fold for *p*-Stat5 and 2-fold for *p*-Pyk2 in DAG-1 cells) and (2.5-fold for *p*-Stat5 and 1.3-fold for *p*-Pyk2 in RT112 cells) (Fig. 5). However, no effect on the *p*-Stat5 and *p*-Pyk2 antigen levels in the J82 cells was observed.

We showed that the title compound (**3c**) modulates cell proliferation by decreasing FGFR3 protein signaling expression (*p*-Stat5 and *p*-Pyk2) in DAG-1 and RT112 cells. The fact that there is no modification of the expression of *p*-Pyk2 and *p*-Stat5 in J82 cells confirms that the FGFR3 signal could be mediated through *p*-Stat5 and *p*-Pyk2. In this study, we showed that FGFR3 acts by using Pyk2 and Stat5 signaling pathways like cytokines which can modulate cell proliferation by using several signaling pathways.[8]

FGFRs are known to activate Ras/MAPKinase and PI3Kinase pathways, to be associated with proteins involved in cell-to-cell interaction, and to contribute to the metastatic process.[1,4,25] Therefore, it would be of interest to determine the effect of **3c** on the two mechanisms related to cell signaling factors, such as cadherins, integrins, and MMPs repression. Experiments are in progress to investigate these hypotheses.

In conclusion, all data presented point out to the ability of (*Z*)-4,6-dimethoxy-2-(2′-fluorobenzylidene)-2,3-dihydro-1*H*-indole-3-one (**3c**) to inhibit bladder tumor cell proliferation probably through FGFR3-pathway and Stat5 cell-signaling pathway. This family of compounds may open a new and selective way to propose potential drug candidates for UCC treatment.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.09.057.

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