Biochemical Pharmacology 218 (2023) 115860

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The prohibitin-binding compound fluorizoline induces the pro-inflammatory cytokines interleukin-8 and interleukin-6 through the activation of JNK and p38 MAP kinases

Ismael Sánchez-Vera [a], José Saura-Esteller [a], Sonia Núñez-Vázquez [a], Ana M. Cosialls [a,b], Ouldouz Ghashghaei [c], Rodolfo Lavilla [c], Gabriel Pons [a], Joan Gil [a,1,*], Daniel Iglesias-Serret [b,d,1]

[a] Departament de Ciències Fisiològiques, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona, Oncobell-IDIBELL (Institut d'Investigació Biomèdica de Bellvitge), L'Hospitalet de Llobregat, Barcelona, Spain
[b] Departament d'Infermeria Fonamental i Clínica, Facultat d'Infermeria, Universitat de Barcelona, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
[c] Laboratory of Medicinal Chemistry. Faculty of Pharmacy and Food Sciences and Institute of Biomedicine (IBUB), University of Barcelona, Spain
[d] Facultat de Medicina, Universitat de Vic-Universitat Central de Catalunya (UVic-UCC), Vic, Barcelona, Spain

ARTICLE INFO

Keywords:
Fluorizoline
Prohibitin
Apoptosis
P38
JNK
Cytokines
IL-8
IL-6

ABSTRACT

Fluorizoline is a prohibitin (PHB)-binding compound that induces apoptosis in several cancer cell lines as well as in primary cells from hematologic malignancies. In this study, we show that fluorizoline treatment triggers the activation of the stress-activated kinases c-Jun N-terminal kinase (JNK) and p38 prior to caspase activation in human cell lines. However, the blockage of p38 and JNK activity with chemical inhibitors or siRNA-mediated downregulation of *MAPK14* (p38) does not prevent fluorizoline-induced apoptosis, suggesting that the activation of these kinases plays an alternative role in the cell response to fluorizoline treatment. Here, we describe that fluorizoline treatment leads to the secretion of pro-inflammatory cytokines interleukin-8 (IL-8) and interleukin-6 (IL-6). Importantly, we demonstrate that the activation of the stress-activated kinases JNK and p38 mediates the secretion of both IL-8 and IL-6. This study shows novel insights into the pro-inflammatory role exhibited by a compound that binds to PHB, thus supporting the potential of PHBs as anti-inflammatory proteins.

1. Introduction

In previous studies, we described the synthesis of a new family of compounds, fluorinated thiazolines, which have the capacity to induce apoptosis regardless of the mutational status of p53 [1]. Among this family of compounds, we focused on the analysis of fluorizoline, due to its higher pro-apoptotic activity. Fluorizoline treatment led to apoptosis in different cancer cell lines with different origin, in the nematode *Caenorhabditis elegans* and, notably, in primary cells from different hematological malignancies [2–6]. Through an affinity-based proteomic screening using polymer-coated magnetic nanobeads, we identified prohibitin 1 (PHB1) and prohibitin 2 (PHB2), key proteins involved in the maintenance of cell viability, as the targets for these compounds [1]. Furthermore, depletion of PHBs in mouse embryonic fibroblasts blocked the induction of apoptosis by the compound [2]. Our research is focused on investigating the potential of fluorizoline as an anticancer agent. To achieve this goal, we investigate the functions of fluorizoline molecular targets. PHBs are homologous and conserved proteins that are ubiquitously expressed within the cell, but they are mainly located in the mitochondria. Interestingly, fluorizoline represents a potent tool for the easily modulate PHB function. As a potential anticancer agent, it is essential to understand how fluorizoline-mediated modulation of PHB

Abbreviations: ASK1, activation of the apoptosis signal-regulating kinase 1; AP1, activator protein 1; APC, allophycocyanin; JNK, c-Jun N terminal kinase; DMEM, Dulbecco's Modified Eagle Medium; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; HRI, haem regulated eIF2α kinase; IL-6, interleukin-6; IL-8, interleukin-8; IRE1, inositol-requiring enzyme-1; ISR, integrated stress response; MAPK, mitogen-activated protein kinase; MKK6, MAP kinase kinase 6; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; PERK, PKR-like ER kinase; PHB, prohibitin; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-alpha.

* Corresponding author at: Departament de Ciències Fisiològiques, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona, Oncobell-IDIBELL (Institut d'Investigació Biomèdica de Bellvitge), L'Hospitalet de Llobregat, Barcelona, Spain.
E-mail address: jgil@ub.edu (J. Gil).
[1] These authors share senior authorship.

https://doi.org/10.1016/j.bcp.2023.115860
Received 26 July 2023; Received in revised form 9 October 2023; Accepted 16 October 2023
Available online 24 October 2023

function can lead different molecular and phenotypic effects, with the ultimate aim of attaining specificity in a potential treatment. Treatment with fluorizoline results in marked mitochondrial fragmentation and mitochondrial cristae disruption, resembling the effect of PHB down-regulation [2,7]. Moreover, this compound activates the intrinsic pathway of apoptosis mainly through the induction of the BH3-only protein NOXA, along with other BH3-only proteins depending on the specific cell type [2,5,6,8].

Our findings have revealed that fluorizoline elicits the activation of the Integrated Stress Response (ISR) signalling pathway in various cell lines. The ISR is a central and evolutionarily conserved adaptative network, which is activated in response to different extracellular or intracellular stresses that can be harmful for cell survival. The phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) is the central event of this pathway, which causes a general reduction in protein synthesis by inhibiting the initiation of 5′ cap dependent translation [9], meanwhile some mRNAs containing upstream open reading frames (uORFs) can still be translated, including mRNAs of activating transcription factor 4 (ATF4) [9,10]. In order to restore cell homeostasis, the upregulation of ATF4 can lead to pro-survival or pro-apoptotic mechanisms, depending on the duration or intensity of the stress [9]. Recently, we have elucidated that the induction of apoptosis resulting from fluorizoline treatment is mediated by the ISR, triggered by a fluorizoline-induced mitochondrial stress sensed by the haem regulated eIF2α kinase (HRI) in HeLa and HAP1 cells [8,11]. Conversely, in HEK293T and U2OS cells, the activation of this pathway has shown a protective role in response to fluorizoline treatment [12].

Furthermore, we have also reported ER stress upon fluorizoline treatment, denoted by PKR-like ER kinase (PERK) and inositol-requiring enzyme-1 (IRE1) activation in different cell lines [11,12]. Alternatively to the ISR signalling pathway, it has been described that ER stress can also induce apoptosis downstream of the activation of c-Jun N terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) through different processes, including NOXA upregulation [13–15]. Interestingly, the activation of JNK and p38 in the presence of endoplasmic reticulum (ER) stress is mainly mediated by the activation of the apoptosis signal-regulating kinase 1 (ASK1), which is activated by IRE1 [16,17]. Consequently, the activation of JNK and p38 stress-activated kinases emerges as a viable alternative mechanism for triggering apoptosis in these cell lines.

Indeed, previous studies have linked modulations of PHB activity to increased JNK and p38 activity. Downregulation of PHB1 results in increased JNK and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activity in liver cancer cell lines [18]. In a similar way, PHB1 overexpression prevented p38 activation through inhibition of MAP kinase kinase 6 (MKK6) [19]. Moreover, flavaglines, which also target PHBs, protect against tumour necrosis factor-alpha (TNF-α)-induced reactive oxygen species (ROS) production and apoptosis and this effect is mediated by increased p38 activity [20].

In addition to JNK and p38 involvement in apoptosis, important roles of these kinases in the induction of an inflammatory response have been reported [21,22]. Concretely, the activation of JNK and p38 are able to regulate a subset of cytokines through their downstream transcription factors activator protein 1 (AP1) and NF-κB, respectively [22–25]. Furthermore, a growing body of studies have reported anti-inflammatory roles of PHBs [26].

Therefore, as JNK and p38 could be involved in both biological processes, apoptosis and inflammation, here we have investigated whether the PHB-binding compound fluorizoline could modulate the activity of these kinases to induce either apoptosis or the secretion of pro-inflammatory cytokines in HEK293T and U2OS cells. In this regard, we describe the activation of both JNK and p38 after fluorizoline treatment, which are not directly involved in the apoptotic process. Nevertheless, we demonstrate their involvement in mediating the secretion of the pro-inflammatory cytokines IL-8 and IL-6 after fluorizoline treatment.

2. Materials and methods

2.1. Cell lines

HEK293T and U2OS cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 100 ng/mL gentamycin and 2 mM L-glutamine (all from Biological Industries, Israel). Cells were cultured at 37° C in a humidified atmosphere containing 5 % carbon dioxide.

2.2. Reagents

The synthesis of fluorizoline and compound 2a were synthesized as previously described [1]. Fluorizoline doses (10 – 20 μM) used in this study were selected based on previous studies of the group [12]. Rocaglamide A was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Q-VD-OPh was from R&D systems (Minneapolis, Minnesota, USA). IN-8 and SB203585 were from Calbiochem (Merck KGaA, Darmstadt, Germany).

2.3. Cell viability

Cell viability was assessed by measuring phosphatidylserine exposure by annexin V-Allophycocyanin (APC) (88-8007-74, Invitrogen) staining and analysed by flow cytometry using FACSCanto™ and FACSDiva™ software (Becton Dickinson, NJ, USA). Cells were collected and incubated for 15 min in the dark with annexing V-APC and annexin binding buffer before analysis. Data represent the percentage of non-apoptotic cells (Annexin V-negative).

2.4. siRNA transfection

HEK293T cells were transfected with siRNA of *MAPK14* (VHS40416), using Lipofectamine® RNAiMax reagent, both from Thermo Fisher Scientific (Waltham, Massachussets, USA). Complexes were added dropwise into growing cells in antibiotic-free DMEM, followed by an overnight incubation. Then, cells were washed with PBS buffer and fresh DMEM was added. Downregulation efficiency was assessed by western blot.

2.5. Western blot

Protein extracts were obtained by lysing whole-cell samples with Laemmli sample buffer, and protein quantification was carried out using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, Illinois, USA). Protein extracts (20–40 μg) were subjected to reducing conditions, loaded onto a polyacrylamide gel and then transferred to Immobilon-P membranes from Millipore (Billerica, Massachusetts, USA). One hour after blocking with 5 % (w/v) non-fatty milk in Tris-buffered saline solution with Tween® 20, membranes were incubated with the following specific primary antibodies: β-actin (AC-15, Sigma-Aldrich), Cleaved caspase 3 (Asp175, #9661, Cell Signaling), p-Hsp27 (p-HSP25 (Ser86), #44-536G, Invitrogen), p-c-Jun (Ser63) (#9261, Cell Signaling), JNK (SAPK) (#9252, Cell signalling) p-JNK (Thr183/Tyr185) (#9251, Cell signaling), NOXA (#114C307, Abcam), p38 (#9218, Cell signalling), p-p38 (Thr180/Tyr182) (#9211S, New England Bio Labs), PARP (#9542, Cell Signaling). Antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase, and the enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).

2.6. Cytokine array

Cytokine array was performed with the Proteome Profiler Human Cytokine Array Kit according to the manufacturer's procedures (#ARY005B, R&D Systems). In brief, supernatants from U2OS cells pre-

treated with Q-VD-OPh for 1 h and then treated for 24 h with DMSO, fluorizoline or TNF-α were collected. Samples were centrifugated to clarify the medium, which was diluted and mixed with the biotinylated detection antibody cocktail. Then, the mixture was incubated with the Human Cytokine Array membranes. After thorough washing to remove unbound components, streptavidin-horseradish peroxidase and chemiluminescent detection reagents were sequentially introduced. The resulting cytokine array was visualized through autoradiography.

2.7. IL-8 and IL-6 enzyme-linked immunosorbent assay (ELISA)

IL-8 and IL-6 levels in the cell culture supernatants were measured with a quantitative enzyme-linked immunosorbent assay according to each manufacturer's procedures. (Human IL-8/CXCL8 or IL-6 DuoSet ELISA; #DY208 and #DY206, respectively; R&D Systems). In brief, U2OS cells were pre-treated with Q-VD-OPh and with DMSO, IN-8 or SB203585 inhibitors for 1 h, and then treated with fluorizoline for 24 h. Cell supernatants were collected as in the Cytokine Array and IL-8 and IL-6 levels were analysed with DuoSet ELISA kit.

2.8. Statistical analysis

The results are shown as the mean ± standard error of the mean (SEM) of values obtained in three or more independent experiments. Statistical analysis was performed using the Student's *t*-test (two-tailed) for single comparisons or ANOVA with a Tukey *post hoc* test for multiple comparisons, by using GraphPad Prism 8.0c Software Inc. Differences were considered significant at p values below 0.05 ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

3.1. Fluorizoline induces the phosphorylation of JNK and p38 kinases

First, we analyse whether fluorizoline treatment affected the activation status of JNK and p38. Fluorizoline treatment of HEK293T and U2OS cells resulted in increased phosphorylation of both JNK (Thr183/Tyr185) and p38 (Thr180/Tyr182) (Fig. 1A and B). These increases of both JNK and p38 phosphorylation could be observed at the early time

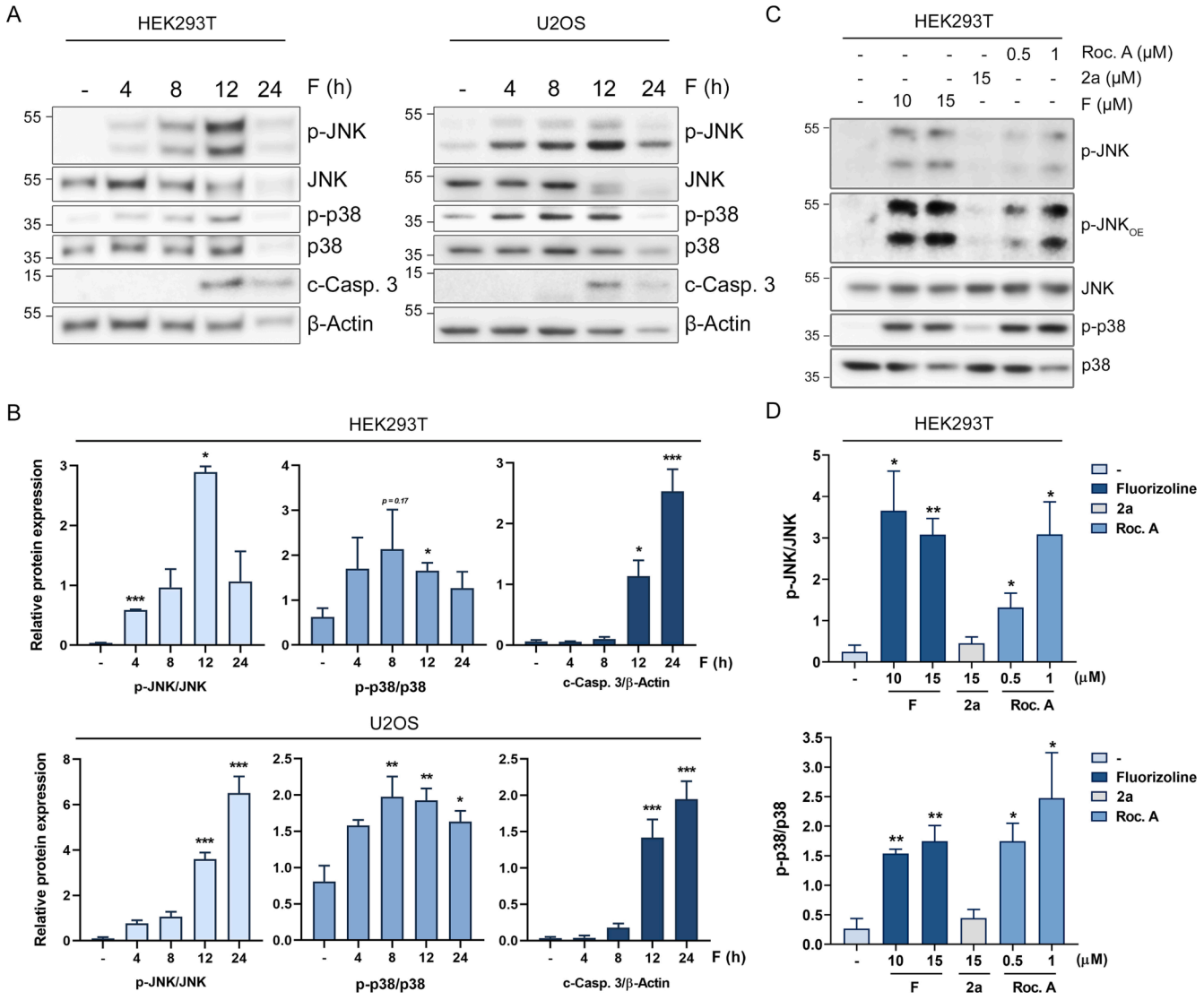

Fig 1. Fluorizoline induces the phosphorylation of JNK and p38 kinases. (A and B) HEK293T and U2OS cells were treated with 15 and 20 μM fluorizoline (F), respectively, for the indicated times. (C and D) HEK293T cells were treated with 10 and 15 μM fluorizoline (F), 15 μM compound 2a (2a) and 0.5 and 1 μM rocaglamide A (Roc. A). (A – D) Protein levels were analysed by western blot. β-actin was used as a loading control. (B and D) Quantification of p-JNK, p-p38 and c-Casp.3 (cleaved Caspase 3) relative to JNK, p38 and β-Actin band intensity, respectively. Data show the mean ± SEM (n = 3) of the relative band intensity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of 4 h. This effect reached its peak after 8 h of treatment in both cell lines. Interestingly, this increase seemed to precede the cleavage of caspase 3, which was observed mainly after 12 h of treatment (Fig. 1A and B).

It has been reported that other PHB-binding compounds, such as flavaglines, activate JNK and p38 in different cellular contexts [27]. Therefore, to test whether the activation of these kinases was due to fluorizoline binding to PHBs, we used two different control treatments. On the one hand, we used another PHB-binding compound from the flavaglines family, rocaglamide A. On the other hand, we used the fluorizoline derivate compound 2a, which lacks three fluors on its structure, leading to its inability to bind PHBs and to induce apoptosis [1,6]. HEK293T cells were treated with different doses of fluorizoline, compound 2a and rocaglamide A for 6 h. As expected, we observed significant increases in the phosphorylation of JNK and p38 after both fluorizoline and rocaglamide A treatments (Fig. 1C and D). Importantly, treatment with the inactive compound 2a failed to induce the phosphorylation of both kinases (Fig. 1C and D). These data point out that JNK and p38 activation is produced due to the binding of fluorizoline to PHBs.

These results indicate that fluorizoline binding to PHBs leads to the activation of JNK and p38 in HEK293T and U2OS cell lines.

3.2. Fluorizoline-induced JNK and p38 phosphorylation is not due to caspase activation

Although the observed increases in p38 and JNK phosphorylation after fluorizoline treatment were previous to caspase activation we sought to determine whether these modulations were independent of caspase activity. We had previously shown that pre-treatment with the pan-caspase inhibitor Q-VD-OPh prevented fluorizoline-induced apoptosis and it blocked the cleavage of caspase 3 and PARP produced by this compound in HEK293T and U2OS cells [12]. We pre-treated HEK293T and U2OS cells with Q-VD-OPh for 1 h and then we evaluated whether caspase inhibition had any effect in the JNK and p38 phosphorylation induced by fluorizoline. Caspase inhibition efficiency was confirmed, as caspase 3 and PARP cleavage was totally blocked in the presence of Q-VD-OPh. As expected, we observed that pre-treatment with Q-VD-OPh did not prevent the effect of fluorizoline on JNK and p38 phosphorylation in both cell lines (Fig. 2A–D).

These experiments show that fluorizoline treatment causes an increase of JNK and p38 phosphorylation independently of caspase activity.

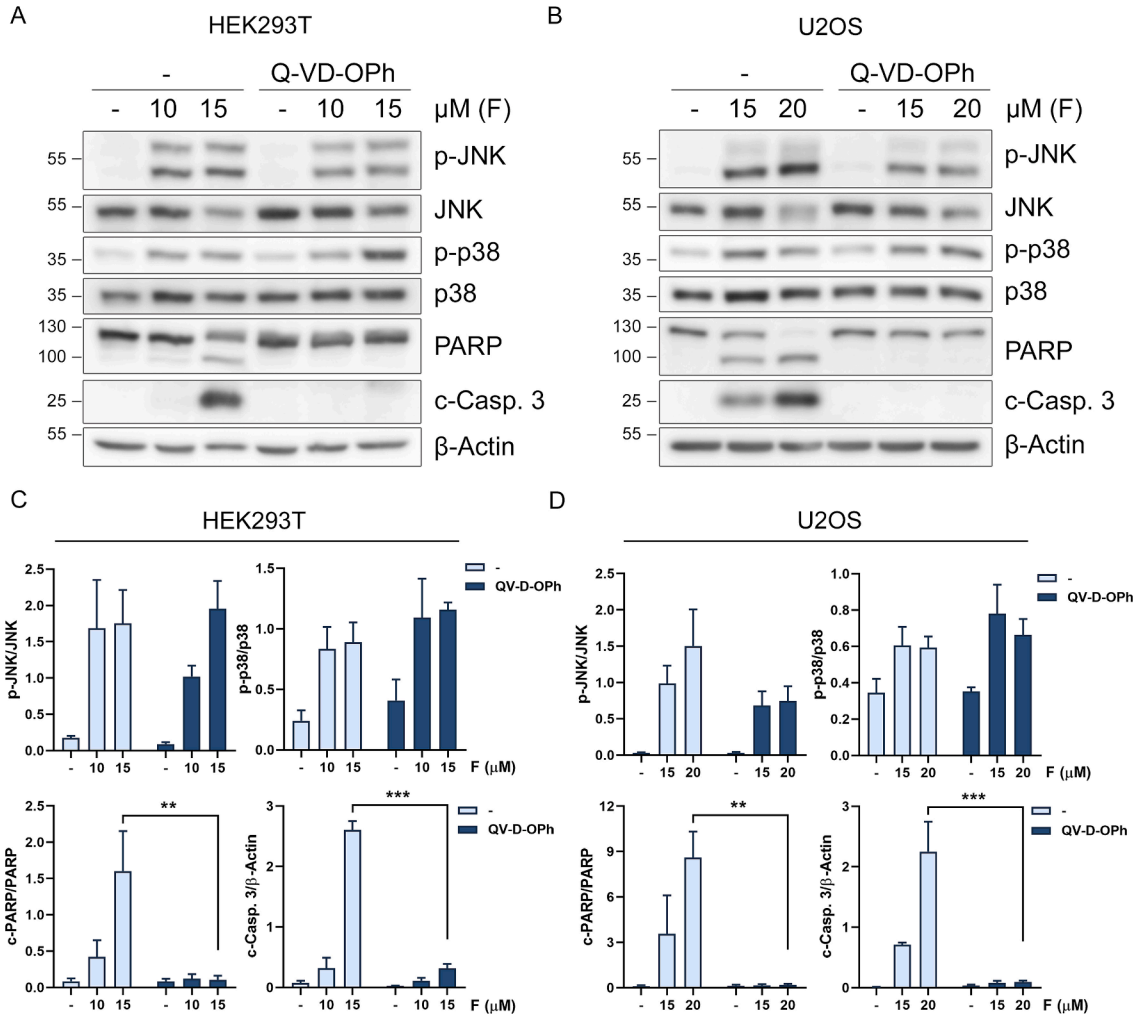

Fig 2. Fluorizoline-induced JNK and p38 phosphorylation are not due to caspase activation. (A) HEK293T and (B) U2OS cells were pre-treated with 20 µM Q-VD-OPh for 1 h and then treated with the indicated doses of fluorizoline (F) for 24 h. Protein levels were analysed by western blot. β-actin was used as a loading control. (C and D) Quantification of p-JNK, p-p38, c-PARP and c-Casp.3 relative to JNK, p38, PARP and β-Actin band intensity, respectively. Data show the mean ± SEM (n = 3) of the relative band intensity. ** $p < 0.01$, *** $p < 0.001$.

3.3. JNK and p38 kinases are not involved in the fluorizoline-induced apoptosis

As stated above, the activation of JNK and p38 signalling pathways can lead to the BH3-only member NOXA induction to trigger apoptosis [13–15]. Moreover, we have also reported important roles of NOXA in the induction of apoptosis after fluorizoline treatment in HEK293T and U2OS cells [6]. Once we have determined that JNK and p38 phosphorylation upon fluorizoline treatment was prior to caspase activation, we wanted to analyse whether these kinases could participate in the induction of NOXA and apoptosis after fluorizoline treatment. In order to block the activity of these two kinases, we used the chemical inhibitors of JNK and p38 activity, IN-8 and SB203580 [28,29], respectively.

First, we assessed JNK involvement in fluorizoline-induced apoptosis. Fluorizoline-induced activation of JNK was confirmed as its substrate, c-Jun, was subsequently phosphorylated in HEK293T (Fig. 3A and C) and U2OS cells (Fig. 3D and F). Pre-treatment with IN-8 efficiently blocked JNK activity, as it prevented the phosphorylation of c-Jun in HEK293T (Fig. 3A and C) and U2OS cells (Fig. 3D and F). As expected, JNK phosphorylation was not affected by IN-8, as showed by the ratio pJNK/JNK. However, after JNK inhibition, NOXA protein levels remained induced compared to control cells, especially in U2OS cells (Fig. 3A, C, D and F). In concordance with NOXA analysis, chemical inhibition of JNK activity did not prevent fluorizoline-induced apoptosis in the cell lines analysed (Fig. 3B and E).

In parallel, we investigated the involvement of p38 activity in fluorizoline-induced apoptosis. The inhibitor of p38, SB203580, efficiently inhibited p38 phosphorylation in HEK293T (Fig. 4A and B) and U2OS cells (Fig. 4D and E). Furthermore, the activation of p38 by fluorizoline was confirmed through the phosphorylation of its substrate HSP27 in U2OS cells (Fig. 4D and E). Indeed, the p38 inhibitor could efficiently inhibit the phosphorylation of HSP27, which evidences the proper p38 activity inhibition (Fig. 4D and E). Nevertheless, p-HSP27 was not detected in HEK293T cells. Chemical inhibition of p38 activity did not prevent fluorizoline-induced apoptosis in the cell lines analysed (Fig. 4C and F). Despite this, we observed that pre-treatment with SB203580 resulted in a reduction of fluorizoline-induced NOXA upregulation in both cell lines analysed (Fig. 4A, B, D and E).

To determine whether this reduction in NOXA increase after fluorizoline treatment was specific to the inactivation of p38 produced by SB203580 inhibitor we downregulated *MAPK14* (p38) using siRNA technology. HEK293T cells were efficiently downregulated, as shown by p38 protein levels in the western blot and quantification (Fig. 4G and H). As observed with the SB203580 pre-treatment, the downregulation of *MAPK14* did not prevent fluorizoline-induced apoptosis in HEK293T cells (Fig. 4I). Interestingly, we neither observed a reduction of NOXA induction by fluorizoline after RNA interference (Fig. 4G and H). This observation indicates that the reduction of fluorizoline-induced NOXA protein expression by SB203580 is not due to p38 inhibition.

Altogether, these data indicate that neither JNK nor p38 pathways are responsible for fluorizoline-induced apoptosis.

3.4. Fluorizoline induces IL-8 and IL-6 pro-inflammatory cytokines through JNK and p38

It has been reported that JNK and p38 activation can lead to a pro-inflammatory outcome [21,23]. Since we have not observed any effect on apoptosis execution after JNK and p38 inhibition, we hypothesized that this activation of JNK and p38 could mediate pro-inflammatory cytokines secretion. To test this, we performed a proteome profiling of 36 human cytokines using a cytokine array.

We analysed U2OS and HEK293T cells supernatants after 24 h of fluorizoline treatment in the presence of the pan-caspase inhibitor Q-VD-OPh. This approach was used to mitigate potential non-specific effects resulting from the apoptotic outcome. Among all 36 cytokines assessed, we observed significant increases of 5.4-fold for IL-8 and 2.5-fold IL-6 in U2OS cells, which are pro-inflammatory cytokines (Fig. 5A and B). However, no significant cytokine release was detected in HEK293T cells (data not shown). To validate these findings regarding IL-8 and IL-6, we used an enzyme-linked immunosorbent assay (ELISA) technique to quantify the concentration of these specific cytokines in the supernatants of U2OS cells. In accordance with the array results, we observed significant secretion of IL-8 and IL-6, with IL-8 exhibiting higher secretion levels compared to IL-6 (Fig. 5C and E).

To investigate the potential involvement of JNK and p38 activation in the observed cytokine increases, we pre-treated cells with the specific inhibitors of JNK and p38. In addition, we used the pan-caspase inhibitor Q-VD-OPh to mitigate any non-specific effects resulting from apoptosis execution by caspases. Remarkably, the secretion of IL-8 induced by fluorizoline was significantly diminished upon inhibition of JNK or p38 activity (Fig. 5D). Furthermore, in the case of IL-6, JNK inhibition did not affect its secretion, whereas the inhibition of p38 activity completely abrogated the fluorizoline-induced secretion of IL-6 (Fig. 5F). We used TNF-α treatment as a positive control of ILs secretion, as it is known to induce robust secretion of IL-8 and IL-6, mediated by JNK or p38 activation [30–32]. The secretion of IL-8 and IL-6 induced by TNF-α was higher compared to the one induced by fluorizoline. Nevertheless, IL-8 and IL-6 secretion pattern in response to TNF-α was similar to those observed with fluorizoline treatment, with significant reductions in IL-8 secretion by both inhibitors and IL-6 secretion only by the inhibition of p38 activity (Fig. 5D and F). These results demonstrate that fluorizoline treatment leads to the release of IL-8 and IL-6 pro-inflammatory cytokines through the stress-activated kinases JNK and p38, independently of the execution of apoptosis.

4. Discussion

In this study, we describe that the prohibitin-binding compound fluorizoline induces the phosphorylation of JNK and p38. The activation of both kinases by fluorizoline precedes caspase activation. However, we demonstrate that JNK and p38 do not play a direct role in fluorizoline-induced apoptosis. Interestingly, our findings demonstrate that fluorizoline induces the secretion of the pro-inflammatory cytokines IL-8 and IL-6. Concretely, IL-8 secretion is mediated by both JNK and p38, while IL-6 is mainly regulated by p38.

Considering the established associations of IL-6 and IL-8 inflammatory cytokines with apoptosis resistance, tumour growth, invasion, and metastasis [33–35], the potential use of this PHB-binding compound as an anti-tumorigenic agent could present a limitation. To address this concern in the future, it may be crucial the combination of fluorizoline with IL-6 and IL-8 inhibitory strategies.

Fluorizoline treatment causes an increase of JNK and p38 phosphorylation, which was observed early on time in HEK293T and U2OS cell lines. Interestingly, the fluorizoline derivate compound 2a, which is unable to bind PHBs [1], failed to activate JNK and p38, indicating that the specific binding of fluorizoline to PHBs leads to the activation of these kinases. Furthermore, the activation of these kinases precedes caspases activation. Despite this, neither JNK nor p38 are involved in the induction of NOXA and apoptosis by fluorizoline. Conversely, it was described that another family of PHB-binding compounds, flavaglines, cause p38 activation to induce apoptosis in NT2/D1 pluripotent cancer cells [36,37], thus we cannot completely exclude p38 involvement in fluorizoline-induced apoptosis in other cancer cells. Therefore, although the molecular pathways involved in apoptosis may differ between fluorizoline and flavaglines, at least the activation of JNK and p38 may represent a shared feature among these PHB-interacting drugs.

We have previously described that the activation of the ISR in HEK293T and U2OS cells plays a pro-survival role in the fluorizoline-induced apoptotic mechanism [12]. Here, we show that the stress-activated MAP kinases JNK and p38 are also not involved in fluorizoline-induced apoptosis. Hence, these findings may suggest an

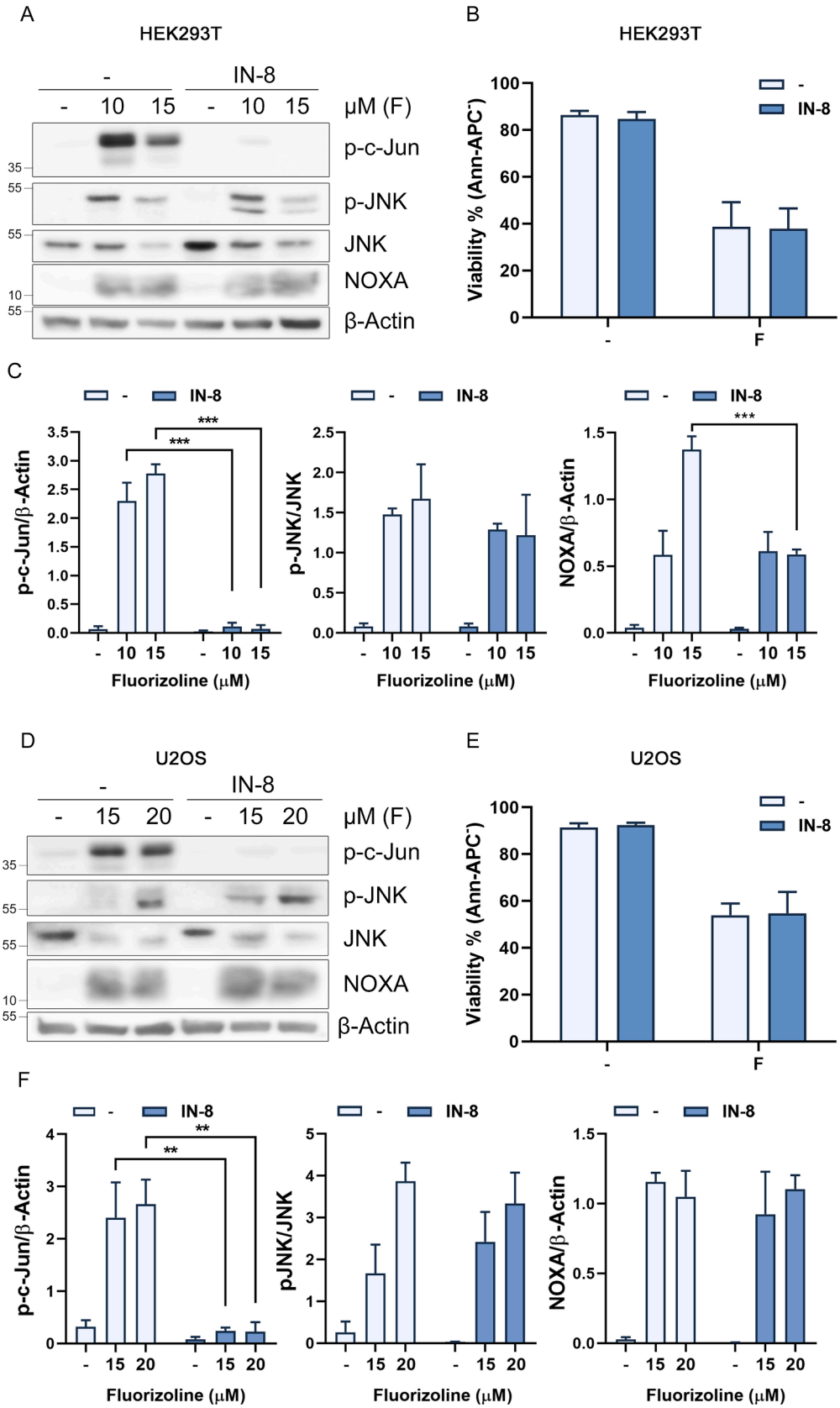

Fig 3. Fluorizoline-induced NOXA and apoptosis are not mediated by the JNK signalling pathway. (A - C) HEK293T and (D - F) U2OS cells were pre-treated or not with 0.5 μM IN-8 for 1 h and then treated with the indicated doses of fluorizoline (F) for 24 h. (A and D) Protein levels were analysed by western blot. β-actin was used as a loading control. (C and F) Quantification of p-JNK relative to JNK and p-c-Jun and NOXA relative to β-Actin band intensity. Data show the mean ± SEM (n = 3) of the relative band intensity. ** $p < 0.01$, *** $p < 0.001$. (B and E) Viability was measured by flow cytometry, and it is expressed as the mean ± SEM (n = 3) of the percentage of non-apoptotic cells (annexin V-negative).

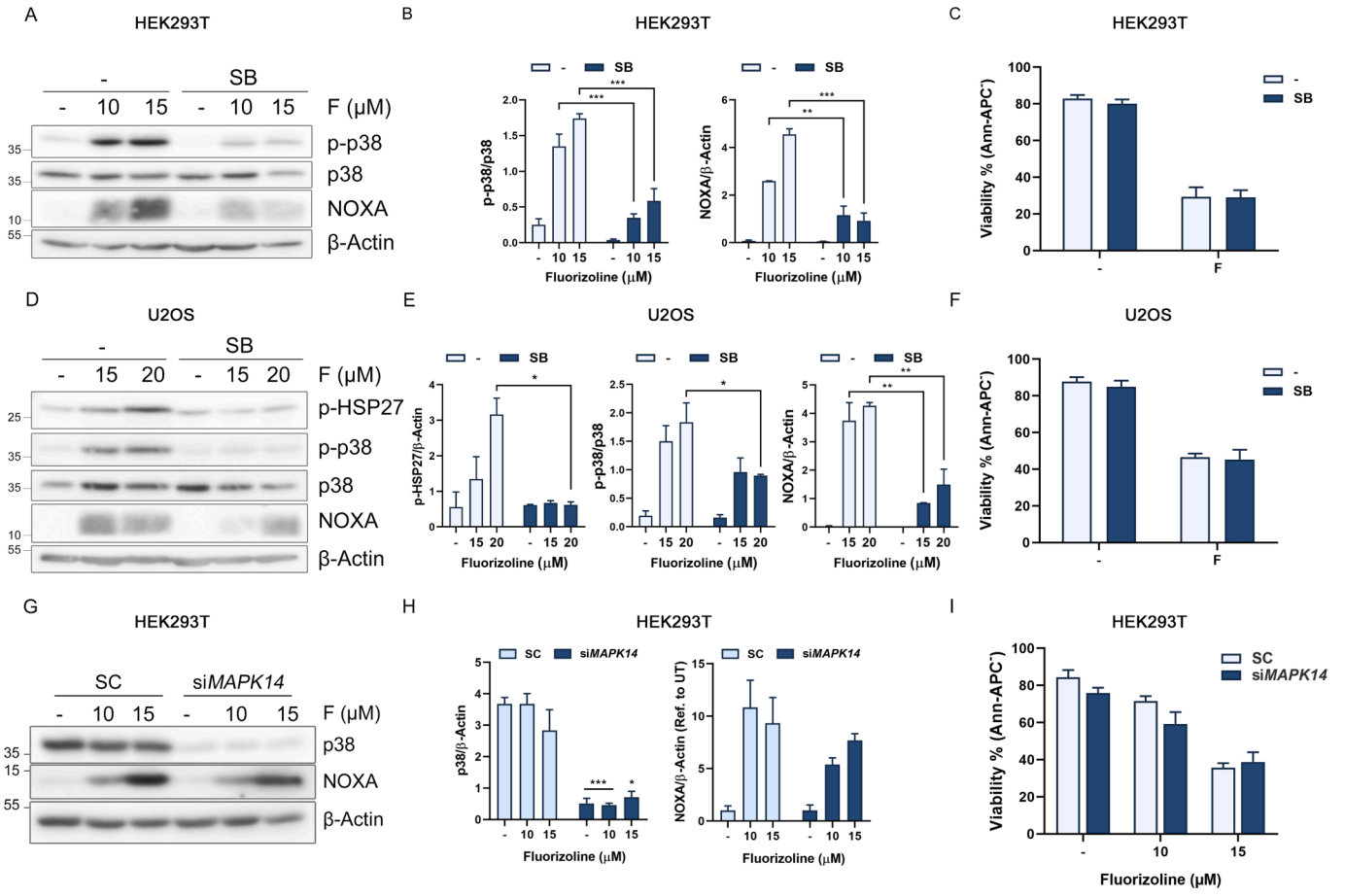

Fig 4. Fluorizoline-induced NOXA and apoptosis are not mediated by p38 signalling pathway. (A - F) HEK293T and U2OS cells were pre-treated with or without 10 μM SB203580 (SB) for 1 h and then treated with the indicated doses of fluorizoline (F) for 24 h. (G - I) HEK293T cells were transfected with scramble (SC) or *MAPK14* (p38) siRNA (si*MAPK14*) for 48 h and then treated with the indicated doses of fluorizoline (F). (A, D and G) Protein levels were analysed by western blot. β-actin was used as a loading control. (B, E and H) Quantification of p-p38 relative to p38 and p-HSP27, NOXA and p38 relative to β-Actin band intensity. Data show the mean ± SEM (n = 3) of the relative band intensity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C, F and I) Viability was measured by flow cytometry and it is expressed as the mean ± SEM (n = 3) of the percentage of non-apoptotic cells (annexin V-negative).

alternative pathway leading to apoptosis upon fluorizoline treatment in these specific cell types, which should be further investigated.

Interestingly, in this study, we describe that fluorizoline treatment leads to the secretion of the pro-inflammatory cytokines IL-8 and IL-6 through JNK and p38 kinases. Furthermore, our findings indicate that IL-8 secretion is partially regulated by both JNK and p38, while IL-6 is only mediated by p38. Indeed, the remaining levels of IL-8 upon JNK or p38 inhibition suggest a cumulative effect on the secretion of this cytokine. These results are consistent with previous studies describing IL-8 and IL-6 secretion via JNK and p38 signalling pathways [21,31,32,38,39]. For instance, p38 exerts regulatory control over the expression of pro-inflammatory cytokines by modulating various transcription factors, including NF-κB, as well as through MAPK activated protein kinases (MAPKAPK) 2 or MAPKAPK3, which facilitate the stabilization or translation of pro-inflammatory mRNAs such as IL-6, IL-1β, IL-8, or TNF-α [22,23]. Furthermore, JNK has also been closely related to pro-inflammatory cytokine secretion of IL-8, IL-6 and TNF-α, through AP1 transcription factor [23–25]. Taken together, these data further support the involvement of JNK and p38 in the fluorizoline-induced secretion of IL-6 and IL-8, which may depend on the cell type.

The mechanism by which JNK and p38 are activated upon fluorizoline treatment is still unknown. However, it is consistently described that upon ER stress, IRE1 can activate ASK1 protein to phosphorylate JNK and p38 [16,40]. Furthermore, ASK1 is a protein that is present in the ER membrane where is able to act as a molecular switch for redox imbalances, such as increases in ROS [41]. Interestingly, we previously showed that fluorizoline treatment resulted in increased cytosolic and mitochondrial ROS production, together with ER stress and IRE1 activation [2,12]. Furthermore, we have also reported that ROS inhibition did not prevent fluorizoline-induced apoptosis in HEK293T and U2OS cells. This finding is consistent with the results described in this article, as we observed that the activation of JNK and p38, which may be a consequence of ROS production, is not involved in fluorizoline-induced apoptosis. Importantly, in this work we describe that the activation of these kinases leads to the release of the pro-inflammatory cytokines IL-8 and IL-6, a finding that has been closely related to inflammatory responses upon ER stress and/or ROS production [30,42,43]. Additionally, it has been described the secretion of inflammatory cytokines is linked to the mitochondrial innate immune response. Nevertheless, the secretion of cytokines in response to fluorizoline is significantly reduced upon p38 or JNK inhibition, which implies that the primary molecular pathway leading to the production of these cytokines should involve the activation of p38 and JNK. However, given that PHBs are located in the mitochondria, we cannot rule out the possibility that a mitochondrial-based innate immune response may contribute to the residual cytokine release observed upon p38 or JNK inhibition. Furthermore, p38/NFκB inflammatory signalling pathway induced by mtROS is closely related to the mitochondrial-based innate immune response [44,45], suggesting that the release of IL-6 and IL-8 induced by fluorizoline could be a consequence of the mitochondrial-based innate immune response.

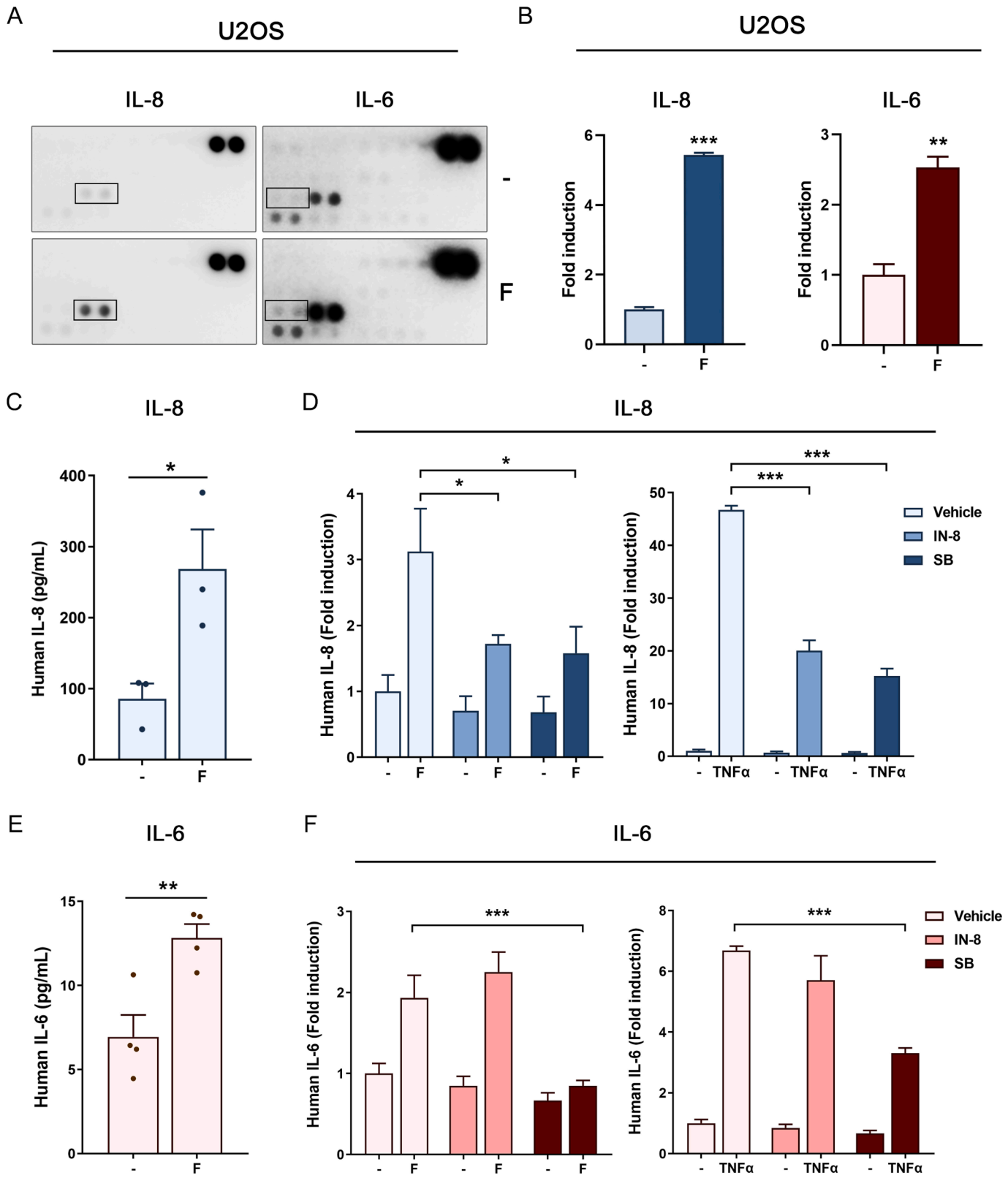

Fig 5. Fluorizoline treatment induces the pro-inflammatory cytokines IL-8 and IL-6 through the activated p38 and JNK kinases. (A and B) U2OS cells were pre-treated with 20 μM Q-VD-OPh for 1 h and then, cells were either untreated (–) or treated with 20 μM fluorizoline (F) for 24 h. (A) Cytokine array analysis of U2OS cells supernatants of untreated (–) or fluorizoline-treated cells (F). Squares in the plot indicate the respective detection dots of IL-8 and IL-6. (B) Quantification of IL-8 and IL-6 dots related to the reference dots intensity (upper right corner of the blot). Data show the mean ± SEM (n = 3) of the relative band intensity. ** $p < 0.01$, *** $p < 0.001$. (C–F) U2OS cells were pre-treated with 20 μM Q-VD-OPh and with DMSO (Vehicle), 0.5 μM IN-8 or 10 μM SB203580 (SB) for 1 h. Then, cells were treated with either DMSO (Vehicle), 20 μM fluorizoline (F) or 20 ng/mL TNF-α. Enzyme-linked immunosorbent assay (ELISA) quantification of IL-8 and IL-6 levels in U2OS cells supernatants of each condition. (C and E) Data show the mean raw data ± SEM of ELISA quantification. (D and F) Data show the mean referred to control condition (vehicle) ± SEM of ELISA quantification. ((C and D) n = 3; (E and F) n = 4) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Interestingly, PHBs have mainly shown anti-inflammatory roles in different settings. For instance, PHB1 downregulation in hepatocellular carcinoma leads to increased IL-8 production through the JNK and p38 downstream transcription factors AP-1 and NF-κB [18]. Transgenic mice overexpressing PHB1 in intestinal epithelial cells demonstrate reduced pro-inflammatory NF-κB signalling in the colonic mucosa after TNF-α treatment, indicating this anti-inflammatory role of PHB1 [46]. Additionally, heterozygous PHB1$^{+/-}$ mice show increased sensitivity to liver insults and inflammatory aggression compared to wild-type animals [47]. Conversely, the PHB-binding compound FL3 (flavagline family) has shown anti-inflammatory effects in Crohn's disease mediated by FL3-induced PHB increases, by reducing inflammatory stress in the intestinal epithelial cells [20]. Thus, the secretion of pro-inflammatory cytokines upon fluorizoline treatment might be a common trait of targeting PHBs function by either chemical or molecular approaches.

In conclusion, we show that fluorizoline treatment results in the activation of the stress-activated kinases JNK and p38 in human cell lines. Interestingly, we have found that their activation is not essential for the fluorizoline-induced apoptotic mechanism of action. Instead, these kinases play a role in the secretion of the pro-inflammatory cytokines IL-8 and IL-6 following fluorizoline treatment. These results provide the first insight into the pro-inflammatory effects of a PHB-binding compound, supporting the potential role of PHBs as anti-inflammatory proteins. These findings may contribute to the general knowledge of PHBs and their potential involvement in inflammatory processes, although further research is necessary to fully understand their implications in this context.

CRediT authorship contribution statement

Ismael Sánchez-Vera: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **José Saura-Esteller:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Sonia Núñez-Vázquez:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Ana M. Cosialls:** Investigation, Writing – review & editing. **Ouldouz Ghashghaei:** Resources. **Rodolfo Lavilla:** Resources. **Gabriel Pons:** Investigation, Writing – review & editing. **Joan Gil:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Daniel Iglesias-Serret:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by a grant from the Ministerio de Ciencia e Innovación (Spain) and European Regional Development Fund (ERDF) (PID2020-120537RB-I00 to J.G. and PID2022-139180OB-I00 to R.L.). I. S.-V. is recipient of a research fellowship from Ministerio de Ciencia e Innovación. We thank the Scientific and Technological Centers of the Bellvitge Campus at the University of Barcelona.

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