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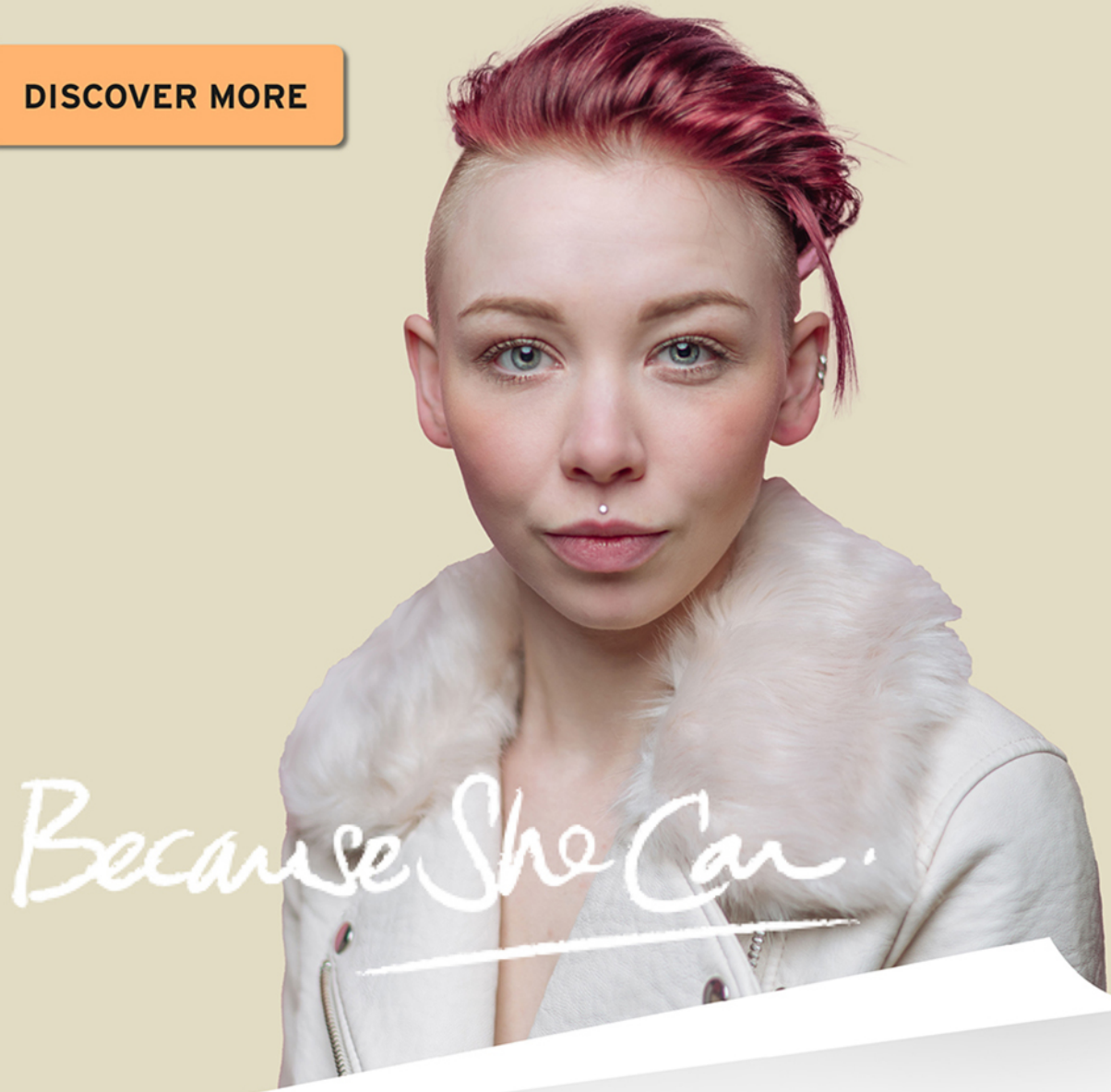

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RESEARCH ARTICLE

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Fluoxetine exerts anti-inflammatory effects on human epidermal keratinocytes and suppresses their endothelin release

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Abstract

Fluoxetine is a safe antidepressant with remarkable anti-inflammatory actions; therefore, we aimed to investigate its effects on immortalized (HaCaT) as well as primary human epidermal keratinocytes in a polyinosinic-polycytidylic acid (p(I:C))-induced inflammatory model. We found that a non-cytotoxic concentration (MTT-assay, CyQUANT-assay) of fluoxetine significantly suppressed p(I:C)-induced expression and release of several pro-inflammatory cytokines (Q-PCR, cytokine array, ELISA), and it decreased the release of the itch mediator endothelins (ELISA). These effects were not mediated by the inhibition of the NF-κB or p38 MAPK pathways (western blot), or by the suppression of the p(I:C)-induced elevation of mitochondrial ROS production (MitoSOX Red labeling). Instead, unbiased activity profiling revealed that they were most likely mediated via the inhibition of the phosphoinositide 3-kinase (PI3K) pathway. Importantly, the PI3K-inhibitor GDC0941 fully mimicked the effects

Abbreviations: AD, atopic dermatitis; FX, fluoxetine; p(I:C), polyinosinic-polycytidylic acid; PI3K, phosphoinositide 3-kinase; SSRI, selective serotonin reuptake inhibitor; TLR, toll-like receptor.

of fluoxetine (Q-PCR, ELISA). Although fluoxetine was able to occupy the binding site of GDC0941 (in silico molecular docking), and exerted direct inhibitory effect on PI3K (cell-free PI3K activity assay), it exhibited much lower potency and efficacy as compared to GDC0941. Finally, RNA-Seq analysis revealed that fluoxetine deeply influenced the transcriptional alterations induced by p(I:C)-treatment, and exerted an overall anti-inflammatory activity. Collectively, our findings demonstrate that fluoxetine exerts potent anti-inflammatory effects, and suppresses the release of the endogenous itch mediator endothelins in human keratinocytes, most likely via interfering with the PI3K pathway. Thus, clinical studies are encouraged to explore whether the currently reported beneficial effects translate in vivo following its topical administration in inflammatory and pruritic dermatoses.

KEYWORDS
atopic dermatitis, COVID-19, endothelin, fluoxetine, inflammation, itch, keratinocyte, phosphoinositide 3-kinase, polyinosinic-polycytidylic acid, toll-like receptor 3

1 | INTRODUCTION

Human skin is not only an indispensable protective barrier,[1] but also an active neuroendocrinoimmune organ.[2,3] Epidermal keratinocytes are central players in the formation of the physicochemical barrier of the skin,[1] and may thus be considered as 'universal sensors'. Indeed, they are capable of detecting various external signals (including, but not limited to for example, temperature, osmolarity, mechanical stimuli, UV light, presence of various microbes, etc.).[4–7] Moreover, following appropriate 'processing' of the incoming information, they are able to release several pro- and anti-inflammatory cytokines and chemokines, itch mediators (e.g. endothelin 1, 2 and 3),[8–11] anti-microbial peptides, etc. Thus, keratinocytes can 'translate' these signals to other cell types, including sensory neurons and professional immune cells.[12–14] Indeed, altered keratinocyte functions, as well as pathological release patterns of certain keratinocyte-derived mediators were shown to be involved in the pathogenesis of multiple inflammatory and/or pruritic dermatoses (including e.g. atopic dermatitis [AD]). Therefore, targeting keratinocytes may represent a promising therapeutic opportunity in different skin conditions.[15–20]

Fluoxetine (FX) is a selective serotonin reuptake inhibitor (SSRI) that has been in clinical use as an antidepressant agent since 1988.[21] Importantly, besides its 'core' effects on the central nervous system, it has been shown to exert remarkable anti-inflammatory actions in various systems, including the skin.[22–26] Moreover, it has clinically relevant anti-pruritic effects,[27,28] it appears to promote (re)pigmentation of microdissected human hair follicles,[29] it improves wound healing following topical application in diabetic mice[30] as well as in humans with infected wounds,[31] and it was demonstrated to ameliorate AD-like skin lesions in a murine model of AD.[32] Moreover, recent studies demonstrated that, most likely due to direct antiviral and immune regulatory effects, FX administration beneficially influenced the clinical outcome in SARS-CoV-2 infection as well.[33–35] Importantly, certain pieces of evidence argue that the anti-inflammatory effects of FX might be independent of classical serotoninergic signalling,[34] and may rather be coupled to interactions with other pathways, including NLRP3-mediated inflammasome activation,[36] nitric oxide production,[37] or the phosphoinositide 3-kinase (PI3K) pathway.[38–41]

Considering the remarkable safety profile of FX[42,43] as well as the aforementioned growing body of evidence supporting its additional beneficial biological effects, it is not surprising that repositioning it to treat various diseases and pathological conditions has already been suggested.[30,31,44,45] Thus, in the current study, we aimed to further explore its putative beneficial effects on human epidermal keratinocytes.

Although common wisdom considers it to be merely a receptor to detect viral infections, toll-like receptor (TLR)-3 appears to play a complex role in skin (patho)physiology. Indeed, it was shown to be upregulated in the lesional epidermis of itching (and hence, chronically scratched) skin of patients suffering from AD and other pruritic dermatoses.[46] Moreover, activation of TLR3 by polyinosinic-polycytidylic acid (p(I:C)) was shown to have two important and synergistic effects in relation to non-histaminergic itch. On the one hand it stimulated the release of the well-known itch mediator[8–11,47] endothelin 1 from keratinocytes,[46] and on the other hand it upregulated TLR3 expression as well. These two effects together strongly support the hypothesis that TLR3 signalling may contribute to the development of the vicious circle of the itch-scratch cycle, where scratching-induced cellular damage and the subsequent release of self-RNA may maintain, or even worsen, itching by activating TLR3.[46] Based on these data, we decided to test the effects of FX in our previously optimized p(I:C)-induced inflammatory model[48] in order to simultaneously assess the anti-inflammatory efficiency of FX in a model system in which it has never been tested before, and to obtain some hints about its putative anti-pruritic activity as well.

2 | METHODS

Primary normal human epidermal keratinocytes (NHEKs) were isolated from dermatologically healthy individuals undergoing surgical interventions following written informed consent adhering to Helsinki guidelines, and after obtaining Institutional Research Ethics Committee's and Government Office for Hajdú-Bihar County's permission (document IDs: IX-R-052/01396–2/2012, IF-1647-2/2016, IF-1647-9/2016, IF-778-5/2017, 61566–5/2021/EÜIG, DE RKEB/IKEB 4988–2018). Relevant anamnestic data of the donors can be found in Table 1. Description of the applied materials and cell culture techniques to cultivate HaCaT keratinocytes and NHEKs, as well as of the various molecular biology and other assays, including the MTT- and CyQUANT-assays, measurement of mitochondrial ROS production (MitoSOX Red labelling), quantitative, real-time polymerase chain reaction (Q-PCR), RNA-Seq analysis, cytokine array, ELISA, western blot, BioMAP® Diversity PLUS® profile analysis, in silico molecular docking analysis, as well as of the assessment of PI3K-activity together with the description of the applied statistical tests can be found in the Supplementary Methods section.

3 | RESULTS

3.1 | Up to 14 μM, FX does not decrease viability and cell count of human epidermal keratinocytes

First, we intended to identify the highest concentration of FX that exerts no detrimental effects on the viability of the keratinocytes in course of 24-h treatments. As shown on Figure S1, we challenged the keratinocytes by using several concentrations of FX (up to 100 μM). Appropriate curve fitting indicated that, in course of 24-h treatments, half-cytotoxic concentration of FX was ~25.43 μM, whereas the highest concentration that did not decrease signal intensity below the level of the vehicle control was 14 μM (Figure S1). Next, in order to confirm that FX does not induce cytotoxic effects at 14 μM, we investigated its effects on the cell count by using another technique (CyQUANT-assay) as well. This assay confirmed that, up to 14 μM, FX did not decrease the number of cells as compared to the vehicle-treated control group over the course of a 24-h treatment (Figure S2), while it greatly reduced it when applied at 100 μM. Thus, we concluded that FX can be used at 14 μM in our planned short- (3-h) and medium-term (24-h) experiments without the risk of obvious cytotoxicity.

3.2 | FX suppresses p(I:C)-induced up-regulation of several pro-inflammatory cytokines in human epidermal keratinocytes

Next, in order to investigate the putative anti-inflammatory effects of FX, we used our previously established pro-inflammatory model system,[48] and administered the TLR3 activator p(I:C) (20 μg/mL). As expected, p(I:C) greatly increased the mRNA expression of several pro-inflammatory cytokines (interleukin [IL]-1α, IL-1β, IL-6, and IL-8 [a.k.a. CXCL8]) over the course of 3-h treatments. Importantly, simultaneous administration of FX (14 μM) was able to significantly suppress the effect in case of IL-1α, IL-1β and IL-8 (CXCL8), and tended to decrease the expression of IL-6 ($p = 0.1615$) as well (Figure 1A-D).

3.3 | FX suppresses p(I:C)-induced release of IL-8 from human epidermal keratinocytes

By using our optimized readout parameters, we found that the test concentration of FX may be efficient in alleviating pro-inflammatory effects of p(I:C); therefore, we next wanted to extend our investigation to the protein level. In order to get a deeper insight into the actions of FX, we first assessed its effects in a pilot experiment by using a commercially available cytokine array kit. We found that 3-h treatment with p(I:C) (20 μg/mL) was able to influence the release of several immunologically active molecules. More precisely, we found that release of 13 molecules was increased, whereas production of 4 molecules was reduced by p(I:C) (Figure S3; Table S1; note that we considered ≥1.5-fold change compared to the control signal intensity as relevant alteration). Of great importance, all of the said alterations appeared to be decreased upon the co-administration of FX (14 μM; Figure S3) arguing that its anti-inflammatory effect previously seen at the mRNA-level may translate to the level of the released cytokines/chemokines as well. In order to challenge this hypothesis, we assayed the supernatants of keratinocytes following 3- and 24-h treatments to monitor the inflammatory stimulus-induced immediate release of the already available cytokine pool (3 h) as well as of the secretion of the de novo synthesized cytokines (24 h). We found that, in line with our Q-PCR data, although FX tended to suppress the p(I:C)-induced release of IL-6, the effect failed to reach statistical significance at either time point, whereas it significantly suppressed the release of IL-8 (CXCL8) at 24 h (Figure 1E–H).

TABLE 1 Relevant anamnestic data of the NHEK donors.

ID	Sex	Age (y)	Region	Passage No.	Colour code on Figure 1J
17–26	Female	48	Abdomen	3	
17–62	Female	42	Abdomen	3	
17–48	Male	69	Abdomen	3	
17–43	Female	41	Abdomen	3	
16–86	Female	35	Abdomen	2	

Note: Each color represents data of cells of an independent donor.

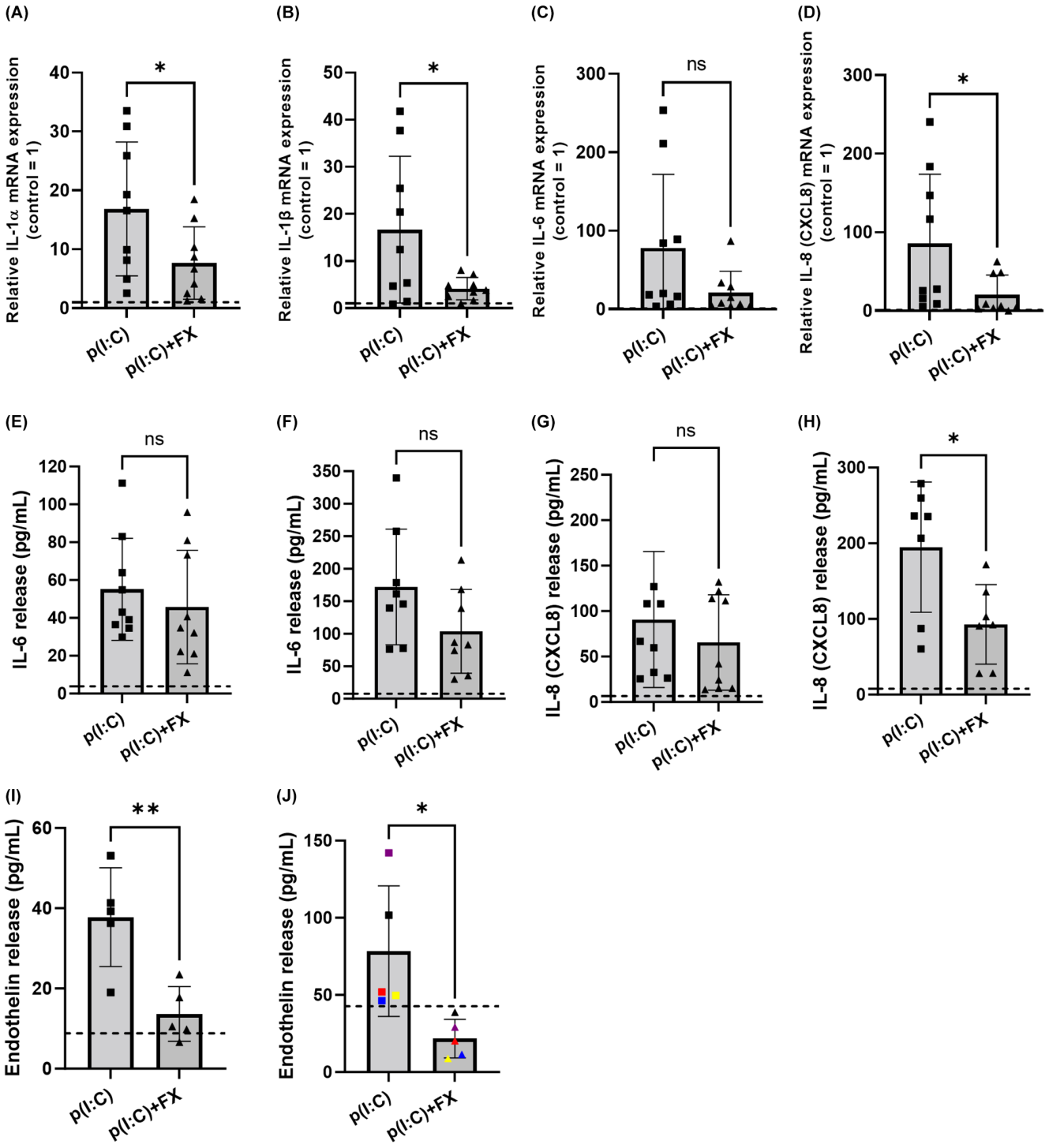

FIGURE 1 FX exerts anti-inflammatory action, and suppresses p(I:C)-induced endothelin release in human keratinocytes. (A–D) mRNA expression of IL-1α (A), IL-1β (B), IL-6 (C), and IL-8 (CXCL8) (D) was assessed by Q-PCR following the indicated 3-h treatments of HaCaT keratinocytes. Data are presented by using the ΔΔCT method regarding 18S RNA-normalized mRNA expressions of the vehicle-treated control group as 1 (dashed line). Data are expressed as mean ± SD of $N = 9$ biological replicates (each determined as mean of three technical replicates). (E–H) ELISA. HaCaT keratinocytes were treated as indicated for 3 (E, G) or 24 (F, H) hours. Supernatants were collected and subsequently assayed by using specific ELISA kits to detect IL-6 (E and F) and IL-8 (CXCL8) (G and H). Data are expressed as mean ± SD of $N = 7$–9 biological replicates (each determined as mean of three technical replicates), as indicated. (I, J) Endothelin ELISA. HaCaT keratinocytes (I) as well as primary human epidermal keratinocytes (J) isolated from 5 donors were treated as indicated for 24 h. Supernatants were collected and subsequently assayed by using a specific ELISA kit to detect endothelin release. Data are expressed as mean ± SD of $N = 5$ biological replicates (each determined as mean of three technical replicates; HaCaT keratinocytes) (I) or 5 donors (primary epidermal keratinocytes; each determined as mean of three technical replicates) (J). Mean of the vehicle-treated control cultures is shown as a dashed line in both cases. In case of primary epidermal keratinocytes, each colour represents an independent donor. * and ** $p < 0.05$, and 0.01, respectively, as indicated. ns, not significant. FX, fluoxetine (14 μM); IL, interleukin; p(I:C), polyinosinic-polycytidylic acid (20 μg/mL).

3.4 | FX suppresses p(I:C)-induced release of the itch mediator endothelins from immortalized (HaCaT) as well as primary human epidermal keratinocytes

Having demonstrated that FX may exert potentially clinically relevant anti-inflammatory effects in our model system, we aimed to further explore its putative beneficial actions. As mentioned above, itch is one of the most common symptoms of a wide-variety of dermatological conditions,[16,18,19,49] and, via the release of different pruritogens, epidermal keratinocytes are important players in its development.[16,18] Notably, endothelin 1 (together with the much less studied, structurally similar endothelin 2 and 3) is a key keratinocyte-derived itch-promoting mediator.[8–11,47] Indeed, endothelin 1 was found to be upregulated in the lesional epidermis of AD patients,[50] and its plasma level was not only elevated in AD patients during exacerbation of the disease, but it also showed a positive correlation with the severity of the symptoms.[51] Because TLR3 was recently found to be upregulated in several pruritic dermatoses, and its activation by p(I:C) greatly increased endothelin-release from primary human epidermal keratinocytes,[46] we intended to investigate how FX can influence this process. First, in a pilot experiment, we demonstrated that, similar to the primary epidermal keratinocytes, endothelin release peaks at 24 h upon 20 μg/mL p(I:C) treatment in HaCaT keratinocytes (Figure S4). In our subsequent experiments, we challenged the keratinocytes by using 20 μg/mL p(I:C) in course of 24-h treatments. We found that simultaneous administration of FX (14 μM) was able to efficiently abrogate the p(I:C)-induced endothelin-release in HaCaT keratinocytes (Figure 1I), as well as in primary human epidermal keratinocytes (Table 1; Figure 1J). Thus, our data argue that FX may not only exert anti-inflammatory effects, but might have the potential to alleviate certain types of itch as well, and its effects are likely to develop in a donor-independent manner.

3.5 | FX does not influence the p(I:C)-induced phosphorylation of p38 and IκBα, and does not prevent the elevation of mitochondrial production of reactive oxygen species (mtROS)

Stimulation of TLR3 may lead to the activation of the NF-κB pathway[52] via the phosphorylation (and hence inactivation) of the inhibitory regulator IκBα. Moreover, TLR3 can also activate the p38 mitogen-activated protein kinase (MAPK) pathway.[53] Data obtained in a preliminary experiment, in which we compared the effects of 10-, 20-, 30-, and 60-min p(I:C)-treatments (20 μg/mL), indicated that p(I:C) induced the most robust phosphorylation of IκBα and p38 following 60 minutes (western blot; *data not shown*). Thus, in our subsequent experiments, we decided to employ 60-min treatments. As shown on Figure S5A–D, we found that co-administration of FX (14 μM) did not influence p(I:C)-induced phosphorylation of IκBα or p38 (western blot), indicating that its anti-inflammatory action may develop without direct interaction with the NF-κB or p38 MAPK activity.

Besides its effects on the NF-κB and p38 MAPK pathways, activation of TLR3 was also shown to elevate mitochondrial production of reactive oxygen species (mtROS).[54] However, as shown on Figure S5E, although co-administration of FX tended to suppress p(I:C)-induced mtROS production (MitoSOX Red labelling; 30-min treatments), the difference failed to reach statistical significance ($p = 0.142$).

3.6 | Activity profiling reveals remarkable similarity between the biological effects of FX and the PI3K-inhibitor GDC0941

Having excluded three of the most obvious pro-inflammatory signalling pathways,[52–54] we decided to opt for a different, more general method to unveil the mechanism of the effects of FX. In order to follow a perfectly unbiased approach, we performed activity profiling using BioMAP® Diversity PLUS® panel of 12 primary human cell-based systems with 148 protein-based and functional readouts,[55–57] including assessment of proliferation, viability, production of several key cytokines and chemokines (e.g. IL-2, IL-6, IL-8 [CXCL8], IL-10, IL-17A, IL-17F, tumour necrosis factor [TNF]-α, etc.), expression of selected biomarkers (e.g. CD40, etc.) and various other molecules (e.g. urokinase plasminogen activator surface receptor [uPAR], P-selectin [Psel], vascular cell adhesion molecule 1 [VCAM-1], monocyte chemoattractant protein-1 [MCP-1], plasminogen activator inhibitor-1 [PAI-1], secreted immunoglobulin G [sIgG], etc.) that are relevant for the investigated cell types (the complete list of all readout parameters can be found in Table S2).

Evaluation of the carefully selected, standardized set of readout parameters in every model results in a unique 'activity fingerprint' that reflects the characteristic biological effects of the agent to be tested. This 'fingerprint' is then mathematically compared to the activity profiles of other benchmark compounds in the database. Such comparison with compounds that have well-characterized target spectra is therefore able to highlight signalling pathway(s) that are influenced by the tested concentration of the compound of interest. This analysis revealed that, when applied at 14 μM, the activity profile of FX exhibited remarkable similarity based on Pearson's correlation coefficient to the one of GDC0941 (a.k.a. pictilisib; 370 nM), a well-known inhibitor[58] of the PI3K pathway (Table 2; Figure 2A).

Notably, although FX is known as an SSRI that primarily acts by elevating serotonin tone, serotonin was not among the top hits (Pearson's correlation coefficient (r) = 0.354 when compared to 90 μM serotonin), suggesting that at its potent anti-inflammatory concentration, FX exerts its effects independently from 'classical' serotoninergic signalling. This was further confirmed by the cluster analysis of the data. This analysis clearly demonstrated that while lower (520 and 1600 nM) concentrations of FX clustered away from the higher doses (and closer to serotonin), its higher (4.7 and 14 μM) concentrations formed a distinct cluster that exhibited great similarity to the effects of GDC0941 (Figure 2B). This suggested that anti-inflammatory and itch mediator suppressing

TABLE 2 Activity profiling of the potent anti-inflammatory concentration of FX reveals remarkable similarity to the biological actions of the PI3K-inhibitor GDC0941.

Test agent	Target agent	Z-score	Pearson's Score	Common Readouts	Mechanism
Fluoxetine (14 μM)	GDC0941 (370 nM)	19.631	0.926	148	PI3K Inhibitor
	Amitriptyline (30 μM)	18.973	0.918	148	Noradrenaline/Serotonin Reuptake Inhibitor
	Cysteamine Hydrochloride (680 μM)	18.967	0.918	148	Cystine Modulator

effects of 14 μM FX were most likely independent of its classical targets in the serotoninergic signalling system, and were rather dependent on the direct or indirect interference with the pro-inflammatory PI3K signalling.[59–62]

3.7 | GDC0941 mimics the anti-inflammatory as well as endothelin suppressing effects of FX

As mentioned above, PI3K is known to be a key orchestrator of inflammatory processes.[59–62] Notably, FX was already shown to inhibit phosphorylation (and hence activation) of PI3K in vivo in diabetic rats,[38] in murine BV-2 microglia cells,[39] as well as in macrophages,[41] whereas others reported a concentration- and treatment duration-dependent indirect, bi-phasic effect on PI3K activity.[40]

We found that, when applied at 370 nM, GDC0941 could mimic the effects of FX in human keratinocytes. Indeed, just like FX (14 μM), it prevented the p(I:C)-induced up-regulation of IL-1α, IL-1β, and IL-8 (CXCL8), and it had no significant effect on the mRNA expression of IL-6 (Figure 3A–D; Q-PCR, 3-h treatments). Moreover, it significantly suppressed p(I:C)-induced elevation of the levels of IL-6, IL-8 (CXCL8), and endothelins in course of 24-h treatments (Figure 3E–G; ELISA). Thus, our data indicated that beneficial anti-inflammatory and pruritogen-release suppressing effects of FX applied at 14 μM may indeed be mediated via the direct or indirect inhibition of the PI3K pathway.

3.8 | In silico molecular docking data suggest that GDC0941 and FX may occupy the same binding site on PI3K

As mentioned above, several lines of recently published evidence indicate that FX can modulate the activity of the PI3K pathway.[38–41] However, to the best of our knowledge, it has never been examined whether FX directly or indirectly inhibits the activity of PI3K. Thus, to test this hypothesis, we decided to use in silico molecular docking to explore the potential of FX to bind PI3K in an ATP-competitive manner, that is, in a similar way as GDC0941 does.

The docking protocol was optimized using the coordinates of the GDC0941-PI3K complex (PDB ID:3DBS).[63] The semi-empirical free energy scoring function[64] within AutoDock 4.2[65] was found to consistently reproduce the experimental pose of GDC0941, with a top-ranked pose which differed only by 0.81 Å from the experimentally observed binding mode (Figure S6A). Notably, key molecular interactions that mediate binding were successfully reproduced, including the hydrogen bonds that the indazole moiety of GDC0941 forms with the carbonyl group of D841 and the phenol oxygen of Y867. The same holds true for the H-bonds formed between the morpholine moiety and the main chain of V882, and for the sulfonyl group at the piperazine ring of the ligand which addresses the side chain of K802 (denoted by blue dashed lines on Figure S6A).

Docking of FX within the ATP binding cleft of PI3K using our validated protocol yielded a top-scored docking pose, where the nitrogen of the propan-1-amine and the oxygen of the 4-(trifluoromethyl)phenoxy moieties address the side chains of D841 and Y867 through H-bonds, respectively (Figure S6B). These residues are also experimentally addressed by indazole moiety of GDC0941. Moreover, the (trifluoromethyl)benzene and phenyl moieties of FX partially fill the hydrophobic cavity defined by W812, I831, I881, F961 and I963, which is experimentally addressed by the hydrophobic thienopyrimidine core of GDC0941.[63] Notably, the 5 best ranked poses of GDC0941 had superior binding energies than the 5 best ranked poses of FX (Table S3). This could account for the smaller size of FX in comparison to GDC0941, which experimentally engages in additional interactions by extending its 4-methanesulfonyl-piperazin-1-ylmethyl arm towards the side chain of K802 and M804.

3.9 | FX exerts minor inhibition on the activity of PI3K in cell-free enzyme activity assay

Next, in order to provide definitive proof, we probed whether FX is able to directly inhibit activity of PI3K in a cell-free assay. Although high concentration of FX could significantly inhibit activity of PI3K (Figure S7A), the observed inhibition was much weaker as compared to the one seen in case of GDC0941 (Figure S7B). Thus, our data argue that, although at sufficiently high concentrations, FX may directly inhibit the activity of PI3K, its anti-inflammatory effects are most likely mediated via indirect interference with the PI3K pathway, and not through direct inhibition of the enzyme. Alternatively, there may be other factors that contribute to the in silico predicted FX binding in whole cells that were not recuperated in a cell-free environment.

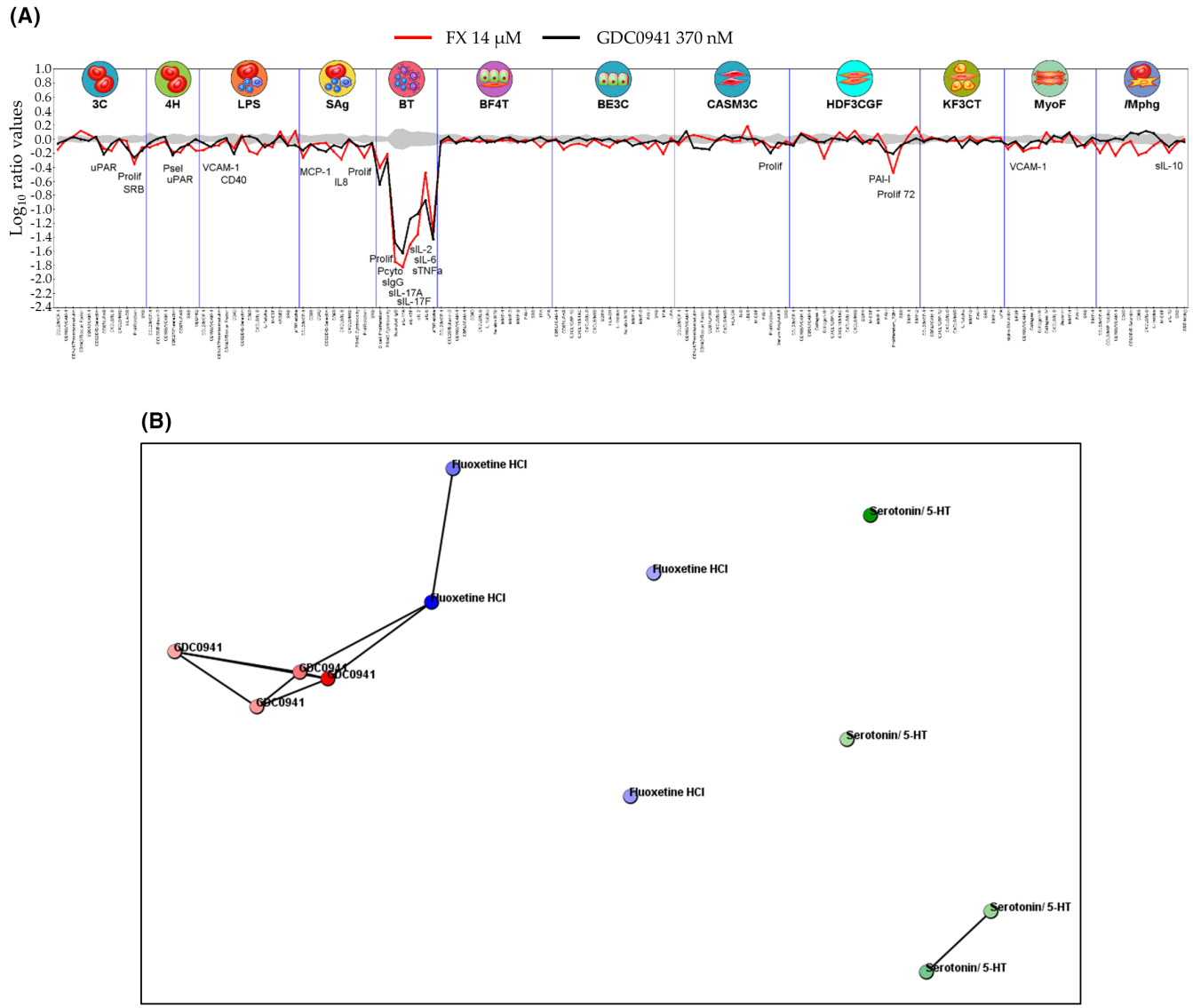

FIGURE 2 Unbiased profiling of FX reveals that when applied at 14 µM, its biological effects exhibit remarkable similarities to that of the PI3K-inhibitor GDC0941 (370 nM). Note that the profiles (A) of FX (14 µM) and the PI3K-inhibitor GDC0941 (370 nM) greatly overlap. The grey shadow indicates significance envelope calculated using historical vehicle control data at a 95% confidence interval. There are 23 common activities that are annotated within the following systems: 3C (uPAR, Prolif, SRB), 4H (P-selectin, uPAR), LPS (VCAM-1, CD40), SAg (MCP-1, IL-8, Prolif), BT (Prolif, Pcyto, sIgG, sIL-17A, sIL-17F, sIL-2, sIL-6, sTNFα), CASM3C (Prolif), HDF3CGF (PAI-1, Prolif 72), MyoF (VCAM-1) and IMphg (sIL-10). Common biomarker readouts are annotated when the readout for both profiles is outside of the significance envelope with an effect size >20% ($|\log_{10} ratio| > 0.1$) in the same direction. There are 32 differentiating activities (not shown) within the following systems: 3C (TF, HLA-DR), 4H (MCP-1, VEGFR2), LPS (CD69, IL-8 [CXCL8], IL-1α, M-CSF, $sPGE_2$, sTNFα), SAg (E-selectin), BF4T (tPA), BE3C (IL-8 [CXCL8], HLA-DR, PAI-1, tPA), CASM3C (VCAM-1, TM, TF, uPAR, LDLR, SAA), HDF3CGF (Collagen III, TIMP-2), KF3CT (IL-1α), MyoF (Collagen I, Collagen III, PAI-1), and IMphg (MCP-1, VCAM-1, CD69, IL-8 [CXCL8]). Differentiating biomarkers are defined when one profile has a readout outside of the significance envelope with an effect size >20% ($|\log_{10} ratio| > 0.1$), and the readout for the other profile is either inside the envelope or in the opposite direction. Importantly, comparative analysis (B) of multiple concentrations of FX (0.52, 1.6, 4.7, and 14 µM), GDC0941 (14, 41, 120 and 370 nM), and serotonin (3.3, 10, 30 and 90 µM) demonstrates that serotonin clearly clusters away from FX and GDC0941. Darker colours indicate higher concentrations. Dots are connected if their Pearson's correlation coefficient is greater than 0.7. CD, cluster of differentiation; FX, fluoxetine; HLA-DR, class II histocompatibility antigen; IL: interleukin; LDLR, low density lipoprotein receptor; MCP-1: monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; PAI-1, plasminogen activator inhibitor-1; Pcyto, PBMC cytotoxicity; PBMC, peripheral blood mononuclear cell; Prolif, proliferation; Prolif 72, proliferation at 72 h; SAA, serum amyloid A; sIgG, secreted immunoglobulin G; sIL, soluble interleukin; sPGE2, soluble prostaglandin E_2; SRB, sulforhodamine B; TF, tissue factor; TM, thrombomodulin; sTNFα, soluble tumour necrosis factor alpha; TIMP-2, tissue inhibitor of metalloproteinases 2; tPA, tissue plasminogen activator; uPAR, urokinase plasminogen activator surface receptor; VCAM-1, vascular cell adhesion molecule 1; VEGFR2, vascular endothelial growth factor receptor 2.

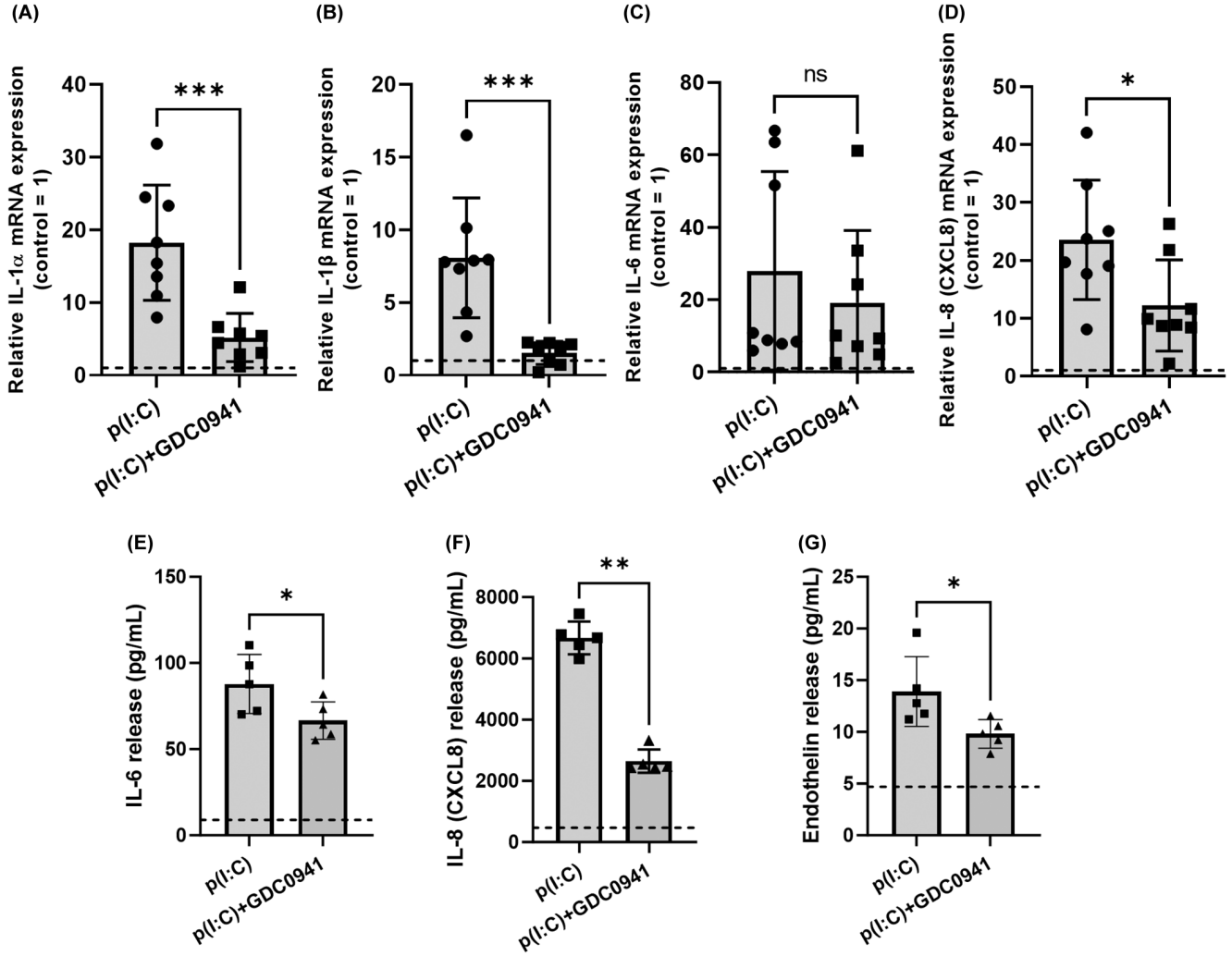

FIGURE 3 When applied at 370 nM, GDC0941 mimics key cellular effects of FX. (A–D) Q-PCR. HaCaT keratinocytes were treated as indicated for 3 h. Data are presented by using the ΔΔCT method regarding 18S RNA-normalized mRNA expressions of the vehicle control group as 1 (dashed line). Data are expressed as mean ± SD of $N = 8$ biological replicates (each determined as mean of three technical replicates), as indicated. GDC0941 was efficient in preventing p(I:C)-induced up-regulation of IL-1α (A), IL-1β (B), and IL-8 (CXCL8) (D), and tended to suppress up-regulation of IL-6 (C). (E–G) ELISA. HaCaT keratinocytes were treated as indicated for 24 hours. Supernatants were collected and assayed by using specific ELISA kits to detect IL-6 (E), IL-8 (CXCL8) (F), and endothelins (G). Data are expressed as mean ± SD of $N = 5$ biological replicates (each determined as mean of three technical replicates), as indicated. Mean of the vehicle-treated control cultures is shown as a dashed line. *, **, and ***$p < 0.05$, 0.01, and 0.001, respectively, as indicated. ns: not significant. IL: interleukin; p(I:C): polyinosinic-polycytidylic acid (20 μg/mL).

3.10 | FX greatly modulates p(I:C)-induced gene expression pattern

Finally, in order to get a deeper insight to the exact nature of the anti-inflammatory effect of FX on human keratinocytes, we collected RNA samples following 24-h treatments with p(I:C) (20 μg/mL), p(I:C) + FX (14 μM), or appropriate vehicles. First, principal component analysis revealed that co-administration of FX resulted in a distinct cluster as compared to the p(I:C)-treated cultures, arguing that FX may have a major impact on the p(I:C)-induced transcriptional response of human keratinocytes (Figure 4A). In a perfect agreement with these findings, pathway analysis of the significantly up-regulated (>1.5 fold-change; $p < 0.05$; p(I:C) vs. control) genes using g:Profiler toolset[66] indicated that, as expected, p(I:C)-treatment led to the enrichment of GO: molecular function terms related to inflammation (e.g. 'cytokine activity', 'cytokine receptor binding', 'chemokine activity', 'CXCR chemokine receptor binding', or 'chemokine receptor binding'; the top ten GO: molecular function terms are shown on Figure 4B). Importantly, analysis of the genes that were significantly (>1.5 fold-change; $p < 0.05$) down-regulated by FX (p(I:C) + FX vs. p(I:C)) yielded very similar results ('CXCR chemokine receptor binding', 'chemokine activity', or 'chemokine receptor binding'; Figure 4C) highlighting that FX can indeed alleviate pro-inflammatory action of p(I:C). Moreover, analysis of the differentially expressed genes (Excel S1) revealed that FX was able to abrogate the p(I:C)-induced up-regulation of several key pro-inflammatory molecules and potential

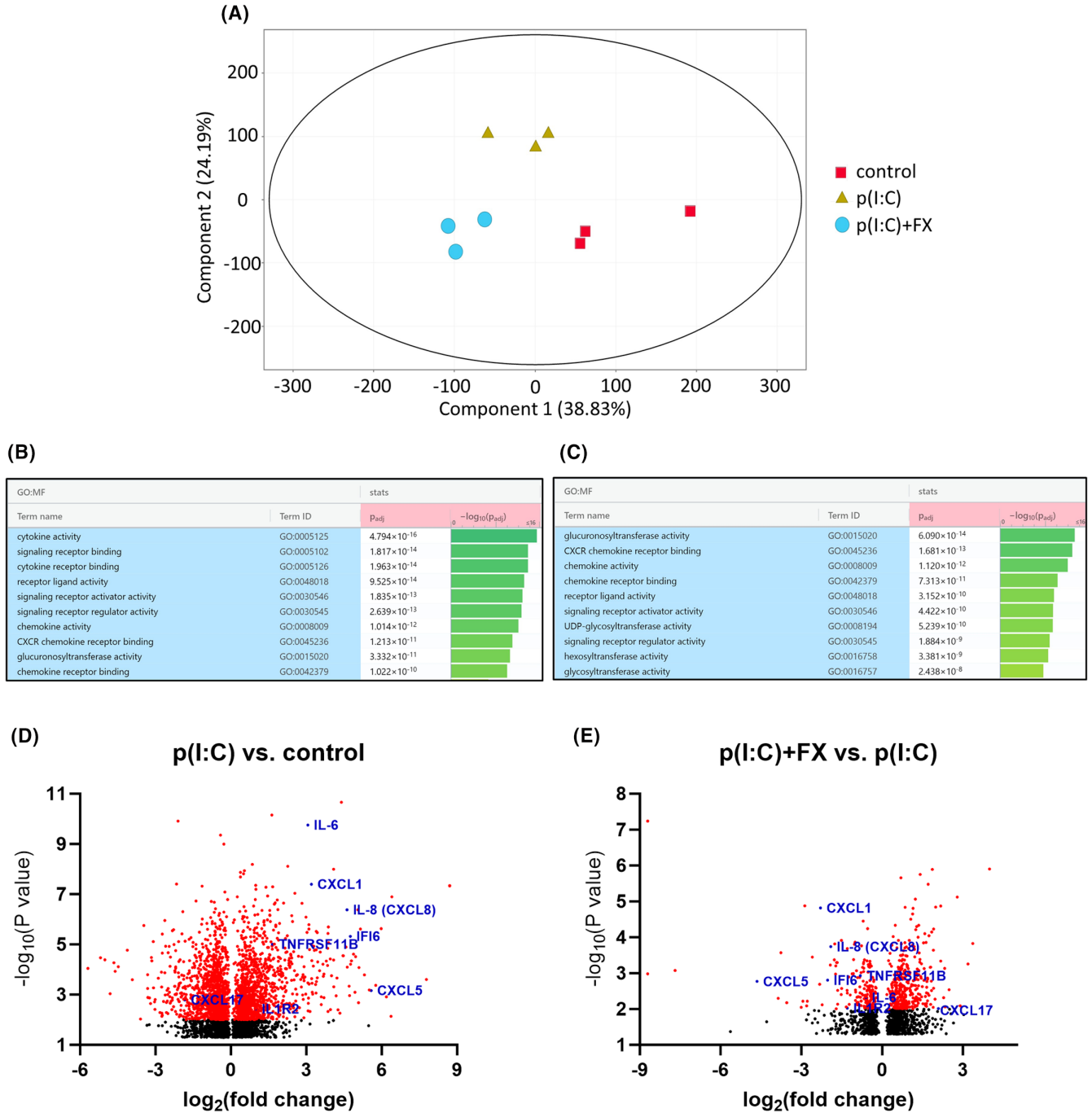

FIGURE 4 FX profoundly impacts on the p(I:C)-induced pro-inflammatory response in human keratinocytes. RNA-Seq analysis. HaCaT keratinocytes were treated as indicated for 24 h. (A) Principal component analysis (PCA) was performed by using the normalized gene expression values of all genes. PCA plot represents the relationship of the examined samples to each other. The replicates in the same treatment group show high similarity to each other and the three different treatment groups are clearly separated. Each symbol represents a biological replicate. (B–C) Pathway analysis of the significantly (>1.5 fold-change; $p < 0.05$) up-regulated (p(I:C) vs. control) (B) or down-regulated (p(I:C) + FX vs. p(I:C)) genes (C) using g:Profiler toolset. Enrichment analysis was performed against the GO: molecular function pathways database, and the top ten GO: molecular function terms are shown. (D, E) Volcano plots created by using all genes exhibiting significant ($p < 0.05$) alterations. Red dots highlight genes exhibiting more significant ($p < 0.01$) alterations. Genes of the most important inflammatory regulators as well as itch mediators are highlighted as blue dots, and are named next to the corresponding dots. FX: fluoxetine (14 μM); CXCL: C-X-C motif chemokine ligand; IFI6: interferon alpha inducible protein 6 (G1P3); IL: interleukin; IL1R2: interleukin-1 receptor type 2; p(I:C): polyinosinic-polycytidylic acid (20 μg/mL); TNFRSF11B: tumour necrosis factor receptor superfamily member 11b (osteoprotegerin).

itch mediators. Indeed, without being exhaustive, co-administration of FX suppressed the expression of IL-6, IL-8 (CXCL8), GROα (a.k.a. CXCL1),[67] CXCL5 (a.k.a. ENA-78),[68] interferon alpha inducible protein 6 (IFI6, a.k.a. G1P3),[69,70] tumour necrosis factor (TNF) receptor superfamily member 11b (TNFRSF11B, a.k.a. osteoprotegerin),[71] and interleukin-1 receptor type 2 (IL1R2),[72] and up-regulated the rather immunosuppressive CXCL17 (Figure 4D,E).[73] Collectively, these data strongly argue that FX may exert a profound anti-inflammatory effect in human epidermal keratinocytes.

4 | DISCUSSION

FX is a widely used antidepressant agent that belongs to the SSRI family.[21] It possesses a remarkable safety profile,[42,43] and it was also shown to exert anti-inflammatory,[23–26] anti-pruritic,[27,28] pro-wound healing,[30,31] and anti-AD[32] effects. Thus, it is not surprising that it has already been recommended to treat various diseases and pathological conditions outside of its current indication as an antidepressant.[30,31,44,45] Hence, within the confines of this study, we aimed to further investigate its putative beneficial actions by using human epidermal keratinocytes.

4.1 | FX exerts anti-inflammatory effects on human epidermal keratinocytes

We found that a non-cytotoxic concentration (14 μM; Figures S1, S2) of FX could abrogate the upregulation of several important pro-inflammatory cytokines (IL-1α, IL-1β, and IL-8 [CXCL8]) induced by the TLR3 activator p(I:C), whereas it only tended to suppress expression of IL-6 in course of 3-h treatments (Q-PCR; Figure 1A-D). Moreover, FX was able to significantly suppress p(I:C)-induced release of IL-8 (CXCL8) (ELISA; 24-h treatments; Figure 1H), a key regulator of cutaneous inflammatory processes that has been suggested as a biomarker for monitoring therapeutic efficiency in AD.[74]

Because itch is a key symptom of several inflammatory epidermal skin diseases, including AD,[16,18,49] and the itch-scratch cycle is known to contribute to the pathogenesis of these diseases,[19] we next intended to extend our investigations towards the highly AD-relevant, keratinocyte-derived itch mediator endothelin 1. Endothelin 1 was not only demonstrated to be elevated in the lesional epidermis of AD patients,[50] but its plasma level was found to correlate with the severity of the symptoms.[51] Importantly, endothelin 1 release appears to operate under the control of TLR3 signalling. Indeed, via activating TLR3, p(I:C) was shown to increase the release of endothelin 1 from primary human epidermal keratinocytes. Moreover, TLR3 itself was found to be upregulated in the chronically scratched, lesional epidermis of patients suffering from pruritic dermatoses (namely AD, prurigo nodularis, and psoriasis) compared to the skin of healthy individuals (all three conditions) and compared to the symptom-free, perilesional skin of the patients (prurigo nodularis).[46] Interestingly, in case of prurigo nodularis and psoriasis (but not of AD), TLR3 protein expression was found to be elevated in the perilesional, symptom-free epidermis as well when compared to the skin of healthy donors.[46] Because p(I:C) treatment also upregulated TLR3 expression in human epidermal keratinocytes, it was hypothesized that TLR3 as well as the keratinocyte-derived endothelin 1 release guided by pathological TLR3-signalling may be significant contributors to the itch-scratch cycle.[46] Because besides endothelin 1, endothelin 2 and endothelin 3 were also shown to be produced by epidermal keratinocytes, as well as to evoke itch,[8–11,47] we decided to use a pan-endothelin-specific ELISA kit. Importantly, we found that the above potent anti-inflammatory concentration (14 μM) of FX was able to suppress p(I:C)-induced endothelin release in HaCaT keratinocytes as well as in primary human epidermal keratinocytes (Figure 1I,J). These data argue that FX may have the potential to exert both anti-inflammatory as well as clinically relevant anti-pruritic effects. Further discussion of the putative beneficial effects of FX can be found in the Supplementary Discussion section.

Here, it is important to note that, according to the available scant evidence, concentration of FX in the skin may be less than half (ca. 41%) of its concentration in the plasma.[75] Thus, considering the usual plasma levels (i.e. ~0.4–2 μM)[76,77] of FX, it seems highly unlikely that one can achieve a sufficiently high cutaneous concentration following systemic application at non-toxic doses.[75] Importantly, however, 14-μM concentration could easily be achieved using appropriate topical formulations. Such limited, local FX administration would most likely not lead to relevant systemic FX exposure, and hence to systemic (side) effects.[45]

4.2 | FX exerts its anti-inflammatory actions most likely via interfering with the activity of the pro-inflammatory PI3K signalling

Next, we intended to unveil the mechanism of the aforementioned beneficial actions. Here, it should be emphasized that the test concentration of FX (i.e. 14 μM) used in our study is much higher than its reported K_i value (i.e. 3 nM) at the serotonin transporter (SLC6A4),[78] and, as mentioned above, also exceeds its usual plasma levels (i.e. ~0.4–2 μM)[76,77] found in FX-treated patients. This, together with the aforementioned reports,[34,36–41] argued that the effects of FX may be mediated via the modulation of unexpected cellular targets. Having excluded three potential anti-inflammatory mechanisms (i.e. inhibition of p(I:C)-induced NF-κB and p38 MAPK activation, and suppression of mtROS production; Figure S5A–E), in order to unveil its mechanism of action, we decided to follow an unbiased approach, and performed activity profiling using a BioMAP® Diversity PLUS® panel of 12 human primary cell-based systems (Table S2; Figure 2A,B). We found that, when applied at 14 μM, the activity profile of FX, which is based on the modulated levels of 148 biomarkers, greatly overlapped with the ones of amitriptyline (30 μM; a norepinephrine/serotonin reuptake inhibitor that was also shown

to exert anti-inflammatory effects in various model systems;[79–85] Table 2), and of GDC0941 (a.k.a. pictilisib; 370 nM), a well-known inhibitor[58] of the PI3K enzymes (Table 2; Figure 2A). Importantly, serotonin was not among the top hits (Pearson's correlation coefficient (r) = 0.354 when compared to 90 μM serotonin), suggesting that the observed effects of 14 μM FX were most likely independent from 'classical' serotoninergic signalling. Indeed, although lower (520 and 1600 nM) concentrations (i.e. concentrations that are normally achieved in the plasma of FX-treated patients)[76,77] of FX clustered closer to serotonin, higher concentrations (4.7 and 14 μM) clearly clustered away from it, and grouped rather together with GDC0941 (Figure 2B). Thus, our data indicated that, at 14 μM, FX most likely interferes with the PI3K signalling either by direct or indirect inhibition of the PI3K enzymes, or by direct or indirect inhibition of some of its key down-stream effectors. Importantly, the activity profile reveals the phenotypic effects of each test agent on the cell(s) in the system rather than informing on the underlying mechanism. If a test agent has higher similarity (Pearson's correlation coefficient (r) > 0.7) or clusters test agents with well-characterized mechanisms, this indicates that there is phenotypic similarity most likely due to shared mechanisms.

Dysregulation of cutaneous PI3K signalling may lead to severe pathological conditions characterized by uncontrolled cutaneous proliferation and inflammation,[59,86] and FX was already shown to interfere with this pathway.[38–41] Thus, our findings argued that, at least when applied at 14 μM, FX may exert its beneficial actions by directly or indirectly inhibiting the PI3K pathway. In order to challenge this hypothesis, we repeated our experiments by using the aforementioned PI3K inhibitor (GDC0941; 370 nM). We found that GDC0941 mimicked the effects of FX, that is, it prevented the p(I:C)-induced upregulation of IL-1α, IL-1β, and IL-8 (CXCL8), while it had no significant effect on the expression of IL-6 (Q-PCR; 3-h treatments; Figure 3A–D), but suppressed the p(I:C)-induced endothelin and IL-8 (CXCL8) release (ELISA; 24-h treatments; Figure 3E–G). Intriguingly, we also observed a subtle difference as compared to FX, since, unlike FX, GDC0941 significantly decreased p(I:C)-induced release of IL-6 (ELISA; 24-h treatments; Figure 3E).

Despite the aforementioned minor difference, our data collectively argued that FX might inhibit PI3K-coupled signalling in human keratinocytes. Although the idea that (at certain concentrations) FX is capable of influencing the activity of the PI3K is not unprecedented in the literature (see e.g. references[38–41]), to the best of our knowledge, it is still unknown whether this is exerted via direct or indirect (or maybe combined, i.e. direct and indirect) inhibition of PI3K. In order to answer this question, first, we performed in silico docking analysis. This revealed that FX may have favourable shape complementary and cavity filling potential towards the ATP binding site of PI3K, that is, towards the site where GDC0941 binds (Figure S6A,B). However, it is also important to emphasize that the 5 best ranked poses of GDC0941 had superior binding energies than the 5 best ranked poses of FX (Table S3), indicating that, even if it can indeed bind to PI3K enzymes, FX may have inferior potency and efficacy as compared to the genuine PI3K-inhibitor GDC0941. Next, having these data in hand, we probed the ability of FX to directly inhibit PI3K activity by using a cell-free activity assay. Importantly, in a perfect agreement with our in silico data, we found that a sufficiently high concentration of FX was indeed able to significantly inhibit PI3K activity (Figure S7A); however, its action was much weaker as compared to GDC0941 (Figure S7B). Thus, our data argue that, albeit its actions might be complemented by direct enzyme inhibition, FX most likely indirectly inhibits the PI3K-coupled pro-inflammatory signalling pathway in human epidermal keratinocytes. On one hand, this could be possible via modulating one of its up-stream or feedback regulators,[87] e.g. the 3-phosphoinositide-dependent protein kinase 1 (PDK1)-related endogenous negative feedback loop that was shown to regulate PI3K expression and hence activity in certain cell types.[88] On the other hand, FX may also interfere with some down-stream targets of the PI3K-coupled pro-inflammatory signalling. In either case, our data highlight the possibility that appropriate chemical modification of the FX backbone may result in new compounds capable of addressing more of the residues at the ATP binding site of PI3K. Thus, our current findings may inspire and guide intelligent drug design studies,[89,90] resulting in new FX-derivatives with improved potency/efficacy towards PI3K.

4.3 | FX profoundly influences the p(I:C)-induced pro-inflammatory response in human keratinocytes

Finally, our RNA-Seq data revealed that FX had a great impact on the pro-inflammatory action of p(I:C) in human keratinocytes, as demonstrated by the results of principal component analysis, pathway analysis as well as the assessment of the differentially expressed genes (Excel S1; Figure 4A-E).

Indeed, without being exhaustive, FX suppressed the p(I:C)-induced up-regulation of neutrophil chemoattractant chemokines GROα (a.k.a. CXCL1)[67] and CXCL5 (a.k.a. ENA-78).[68] It is noteworthy that GROα (CXCL1) may also act as an itch mediator,[67] and our data indicated that FX may be able to suppress its release as well (cytokine array; Figure S3). Moreover, FX was efficient in suppressing p(I:C)-induced up-regulation of IFI6 (G1P3) that was found to be upregulated in lesional and non-lesional skin of psoriasis patients.[69,70] Furthermore, FX down-regulated TNFRSF11B (osteoprotegerin), a well-known decoy receptor for receptor activator of NF-κB (RANK) ligand (RANKL). Because epidermally expressed RANKL was found to induce immunosuppression via increasing the number of regulatory T cells,[71] lowering TNFRSF11B expression may add an additional layer to the anti-inflammatory effects of FX in vivo. Likewise, FX decreased the p(I:C)-induced up-regulation of IL1R2, a decoy receptor for IL-1α and IL-1β. Because high level of IL1R2 is thought to play a role in the impaired migration of Langerhans cells in aged skin,[72] normalization of its expression may help restoring the physiological cutaneous immune surveillance (Excel S1; Figure 4D,E).

Finally, it is also noteworthy that while p(I:C) down-regulated CXCL17, co-administration of FX could significantly suppress this

action (Excel S1; Figure 4D,E). CXCL17 was recently found to attenuate cutaneous inflammation via recruiting myeloid-derived suppressor cells and regulatory T cells in an imiquimod-induced mouse model of psoriasis.[73] Obviously, it remains to be determined in future targeted studies whether FX-induced up-regulation of CXCL17 translates to the released cytokine level, and whether it is restricted to the p(I:C)-induced inflammatory response, or it is part of a more general anti-inflammatory activity. In either case, FX-induced up-regulation and release of CXCL17 may reinforce anti-inflammatory action of FX on the longer (>24 h) time scale in vivo.

Collectively, our data clearly demonstrate that FX may exert potent anti-inflammatory effects, and, by suppressing the release of the endogenous itch mediators such as endothelins (and maybe others, e.g. GROα [CXCL1]), its application may also lead to beneficial anti-pruritic activity, most likely via interfering with the pro-inflammatory PI3K pathway. Considering its well-known safety profile, clinical studies are encouraged to explore whether the currently reported beneficial effects translate in vivo in inflammatory and pruritic skin diseases following its administration in appropriate topical formulations ensuring a sufficiently high local, cutaneous concentration.

AUTHOR CONTRIBUTIONS

Conceptualization: Attila Oláh, Tamás Bíró, and Christoph Abels; *Methodology*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, and Attila Oláh; *Validation*: Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, and Attila Oláh; *Formal analysis*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, and Attila Oláh; *Investigation*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Jennifer I. Drake, Attila Gábor Szöllősi, Szilárd Póliska, Alison O'Mahony, and Attila Oláh; *Resources*: Ana Kilić, and Michael Soeberdt; *Data curation*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, Attila Gábor Szöllősi, and Attila Oláh; *Writing—original draft preparation*: Kinga Fanni Tóth, and Attila Oláh; *Writing—review and editing*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Tamás Bíró, Jennifer I. Drake, Alison O'Mahony, Ana Kilić, Michael Soeberdt, Christoph Abels, Szilárd Póliska, Attila Gábor Szöllősi, and Attila Oláh; *Visualization*: Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, and Attila Oláh; *Supervision*: Tamás Bíró, Ana Kilić, Michael Soeberdt, Christoph Abels, and Attila Oláh; *Project administration*: Kinga Fanni Tóth, Dorottya Ádám, József Arany, Yesid A. Ramirez, Jennifer I. Drake, Szilárd Póliska, Alison O'Mahony, Ana Kilić, and Michael Soeberdt; *Funding acquisition*: Attila Oláh, Attila Gábor Szöllősi, Yesid A. Ramirez, and Christoph Abels. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw data of the RNA-Seq analysis are accessible in the NCBI SRA database (hyperlink to the data: http://www.ncbi.nlm.nih.gov/bioproject/1022043).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1.

Data S2.

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