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Sulfasalazine decreases soluble fms-like tyrosine kinase-1 secretion potentially via inhibition of upstream placental epidermal growth factor receptor signalling

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ABSTRACT

Objectives: Preeclampsia is a hypertensive disorder of pregnancy with no available medical treatment. We recently reported sulfasalazine, an anti-inflammatory medication, to be a candidate therapeutic for preeclampsia. We showed sulfasalazine decreases placental secretion of soluble Fms-like tyrosine kinase-1 (sFlt-1), an anti-angiogenic factor strongly implicated in the pathogenesis of preeclampsia. However, the cellular mechanism(s) by which sulfasalazine reduces placental sFlt-1 are yet to be determined. Recently we also reported that both the mitochondria and the epidermal growth factor receptor (EGFR) signalling pathways regulate secretion of placental sFlt-1. In this study we sought to assess directly whether sulfasalazine's capacity to reduce sFlt-1 secretion may be mediated via EGFR or the mitochondria.

Methods and results: Using primary cytotrophoblast cells, we confirmed sulfasalazine reduced sFlt-1 secretion. Interestingly, when we measured the mRNA expression of EGFR, we found a reduction in EGFR expression which closely mirrored the changes in sFlt-1 secretion. At the protein level, sulfasalazine significantly reduced phosphorylated and active EGFR (phosphorylated/total) expression. Additionally, sulfasalazine significantly reduced the protein expression of ERK1/2 and STAT3 which are key adaptor molecules downstream of EGFR. Next, we assessed mitochondrial respiration following sulfasalazine treatment and found no effect on basal respiration, ATP production, proton leak or maximal respiration.

Conclusion: Sulfasalazine reduces EGFR and down-stream signalling molecule expression coincident with reduced sFlt-1 secretion. EGFR signalling is a potential mechanism by which sulfasalazine decreases placental secretion of sFlt-1. Further interrogation of the EGFR may identify new candidate treatments for preeclampsia.

1. Introduction

Preeclampsia is a common and serious hypertensive disorder of pregnancy characterized by maternal endothelial dysfunction and end organ damage. Currently, there are no medical therapeutics available for preeclampsia, leaving delivery as the only definitive treatment [1]. The anti-angiogenic factor, soluble Fms-like tyrosine kinase-1 (sFlt-1) is strongly implicated in the pathogenesis of this disease [2,3]. Poor placental perfusion and resulting placental hypoxia is thought to drive excess placental secretion of sFlt-1 into the maternal circulation, where it antagonises vascular endothelial growth factor (VEGF) and ultimately disrupts vascular homeostasis [2,4]. Given the effects of sFlt-1, there has been growing interest in exploring the potential of repurposing therapeutics which are already used during pregnancy, for their ability to mitigate excess sFlt-1 release to treat or prevent preeclampsia.

One therapeutic of interest is sulfasalazine, an anti-inflammatory medication used to treat inflammatory arthritis and inflammatory bowel disease. Sulfasalazine was first suggested as a candidate therapeutic for preeclampsia when our team discovered its ability to decrease placental secretion of sFlt-1, whilst upregulating the pro-angiogenic molecule placental growth factor (PlGF) and rescuing vascular endothelial cell dysfunction [5]. Following these laboratory investigations into sulfasalazine we have progressed this concept to a phase I clinical trial investigating its safety and efficacy for treating

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Table 1
Antibodies and dilutions.

Antibody	Catalog #	Dilution
Anti-EGFR (Santa Cruz Biotechnology, Texas USA)	373,746	1:200
Anti-phospho-EGFR (Cell Signalling Technology)	7707s	1:1000
Anti-ERK1/2 (Promega)	V1141	1:1000
Anti-phospho-ERK1/2 (Cell Signalling Technology)	4370	1:2500
Anti-STAT-3 (Cell Signalling Technology)	4904	1:1000
Anti-phospho-STAT-3 (Cell Signalling Technology)	9138	1:500
Anti-AMPK (Abcam)	80,039	1:750
Anti-phospho- AMPK (Cell Signalling Technology)	2535	1:750
Anti-SIRT-1 (Sigma)	SAB1404972	1:1000
Anti-PCG1α (Origene)	TA326711	1:700
Anti-GAPDH (Cell Signalling Technology)	3683S	1:5000
Anti-rabbit, secondary (Promega)	W401	1:2500
Anti-mouse, secondary (Sigma)	W402B	1:10,000

preeclampsia (trial number ACTRN12617000226303). The mechanism by which it reduces placental secretion of sFlt-1 is unclear. Sulfasalazine induces the anti-oxidant enzyme heme oxygenase-1 (HO-1), which others have previously suggested to be involved in the regulation of sFlt-1 [6]. However, we have shown that HO-1 does not directly regulate placental sFlt-1 [7], nor does HO-1 mediate the effect of sulfasalazine on the placental secretion of sFlt-1 [5].

Epidermal growth factor receptor (EGFR) signalling and mitochondrial function are two key regulators of cellular function [8,9]. We have previously reported both EGFR signalling and mitochondrial function to be upregulated in placental tissue obtained from women with preeclampsia and importantly, inhibiting either pathway reduces placental secretion of sFlt-1 [10]. Additionally, other therapeutics that we and others have reported to reduce sFlt-1 including metformin [11], pravastatin [12] and esomeprazole [13] – all modulate EGFR signalling and/or mitochondrial function [10]. Together, these findings implicate both EGFR and mitochondrial function in the regulation of placental sFlt-1 secretion and suggest they may be unifying mechanisms by which therapeutics alter sFlt-1 secretion. Therefore, in this study we set out to examine whether sulfasalazine alters EGFR signalling and/or mitochondrial function as a mechanism for reducing sFlt-1 secretion.

2. Materials and methods

2.1. Isolation of primary cytotrophoblasts

Primary cytotrophoblast cells were isolated as previously described from women undergoing elective caesarean section at term [14]. Cells were plated in DMEM containing 10% FCS at 80–90% confluency. Primary cytotrophoblasts were maintained in a humidified incubator at 8% O2 (normoxia) and 5% CO2 for the duration of the experiments. The day after plating, cells were treated with increasing doses of sulfasalazine (250, 500, 750 μM) or vehicle control. 48 h after treatment, culture media and cell lysates were collected for protein or mRNA extraction, and subsequently stored at −20 or −80 °C respectively.

2.2. ELISA analysis

Total sFlt-1 concentrations were measured in culture media using the Human VEGF/R1 Duoset Enzyme-Linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, USA) according to manufacturer's instructions. Media from cytotrophoblast cells was diluted in PBS, 1:10, to ensure samples were within the range of the standard curve.

2.3. Reverse transcription polymerase chain reaction

Total RNA was extracted from primary trophoblast using the RNeasy mini kit (Qiagen, Valencia, CA). RNA quality and quantity were assessed using a Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE), with a A260/A280 ratio of 1.8–2.0 considered acceptable for cDNA conversion. RNA from each triplicate well was converted to cDNA using Applied Biosystems high capacity cDNA reverse transcription kit (Life Technologies) as per manufacturer guidelines. Quantitative PCR was performed using Taqman gene

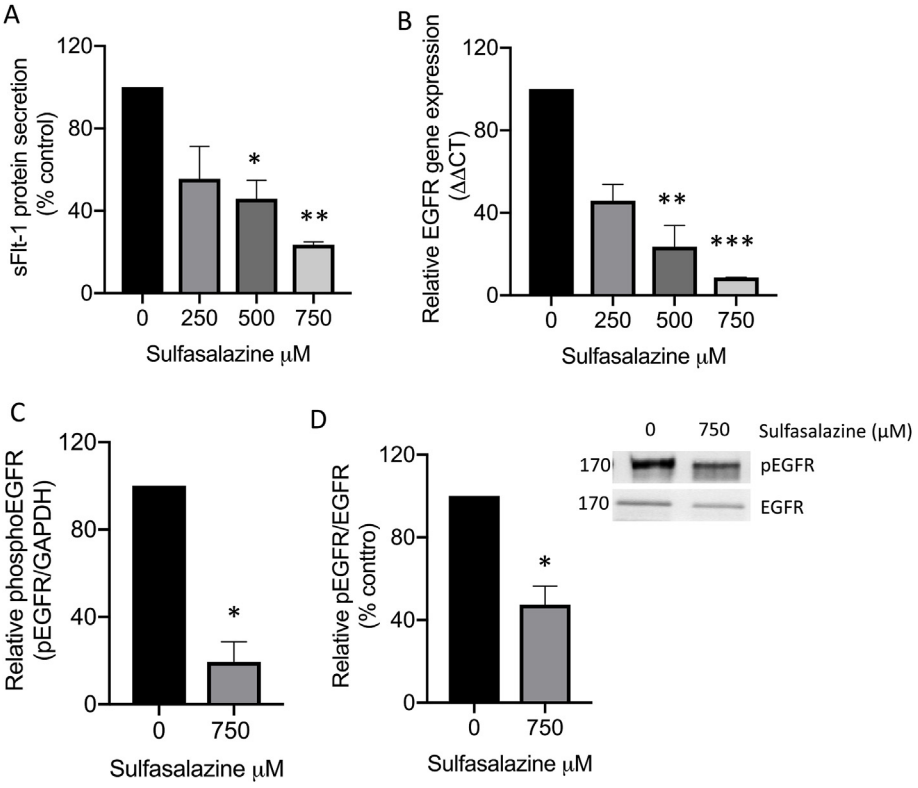

Fig. 1. Sulfasalazine reduces EGFR signalling. To assess the effects of sulfasalazine on EGFR signalling, primary cytotrophoblasts were treated with increasing doses (250, 500, 750 μM) of sulfasalazine and EGFR expression measured. (A) Demonstrates primary cytotrophoblast dose response to sulfasalazine (0–750 μM) significantly and dose dependently reduced sFlt-1 secretion and (B) EGFR mRNA expression. (C–E) Sulfasalazine significantly reduced the protein expression of phosphorylated, total nand active EGFR (phosphorylated/total EGFR) expression. (F) Representative western blots of protein expression shown. Data are mean ± SEM of 4–5 independent experiments in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001.

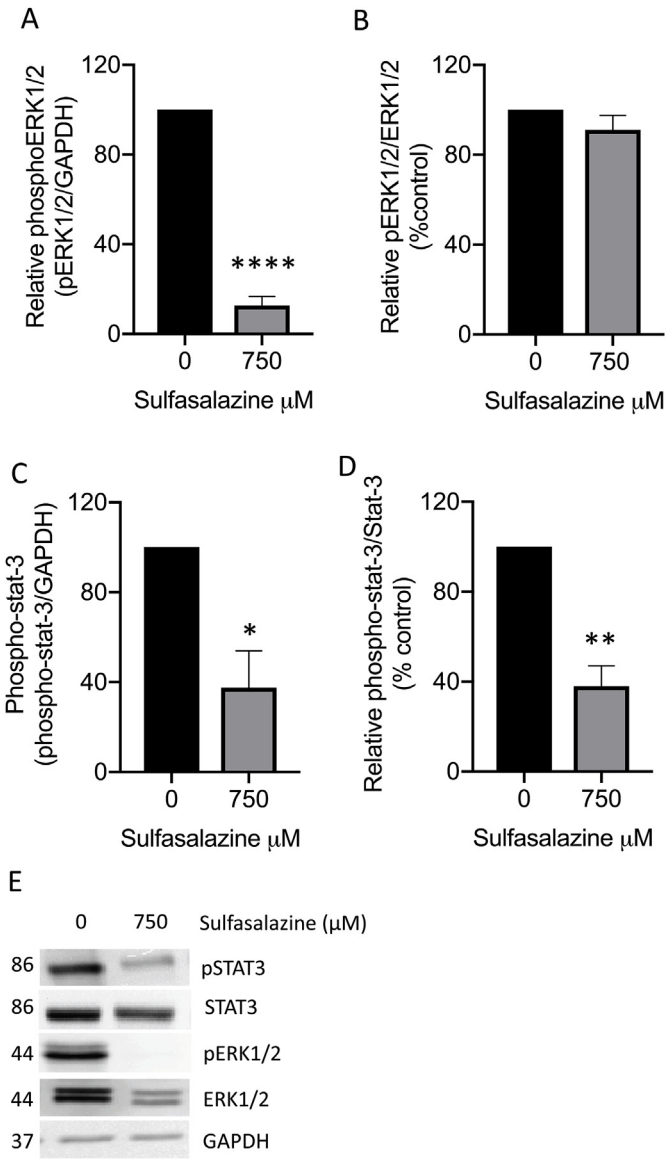

Fig. 2. Sulfasalazine reduces downstream EGFR signalling. To assess the effect of sulfasalazine on the key downstream signalling molecules ERK1/2 and STAT-3, primary cytotrophoblasts were treated with sulfasalazine and protein expression measured. (A–C) Primary cytotrophoblasts treated with sulfasalazine (750 μM) demonstrated significantly reduced phosphorylation of ERK/12 and total ERK1/2, active signalling was not altered. (D–F) Phosphorylation and activation of STAT-3 was significantly reduced by sulfasalazine. (G) Representative western blots of protein expression shown. Data are mean ± SEM of 4–5 independent experiments in triplicate. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression assays for EGFR (catalogue number: Hs01076090_m1). qRT-PCR was performed on the CFX 384 (Biorad, Hercules, CA) using Taqman universal PCR master mix (Life Technologies) with the following run conditions: 50 °C for 2 min; 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min (40 cycles). All data were normalized to the reference house-keeping gene, YWHAZ (Hs01122454_m1), as an internal control and calibrated against the average C_t of the control samples. The stability of the reference gene was confirmed as stable within each experiment. The results were expressed as fold change relative to controls. All samples were run in triplicate.

2.4. Western blot analysis

Placental tissue lysates and primary cytotrophoblast cells were extracted on ice in RIPA buffer (25 mM tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Pierce protease and phosphatase inhibitor), followed by homogenization and then centrifugation at 15,000g for 15 min at 4 °C to remove tissue debris. 20ug of protein (per sample) was separated by SDS–polyacrylamide gel electrophoresis (12 and 15%) and subsequently transferred onto PVDF membranes. After blocking, membranes were incubated with the primary antibody overnight at 4 °C. Targeted protein signal was detected using an ECL detection kit (Amersham Bioscience) and captured with ChemiDoc imaging system (BioRad). Images were analysed using ImageJ software (version 1.50i). Anti-GAPDH was used as a protein loading control, which remained stable across treatments. Antibodies and dilutions as shown in Table 1.

2.5. Mitochondrial respiration

Mitochondrial analysis was performed 24 h after cell treatments according to manufacturer instructions (Agilent XF24 Seahorse Analyser). Seahorse injector/probe plate was hydrated overnight at 37 °C prior to cellular respiration measurements. Treated cells were equilibrated in unbuffered DMEM supplemented with 1 mM Sodium pyruvate and 2 mM Glutamine for 1 h prior to port injections of 10uM Oligomycin, 10uM Carbonyl cyanide 4 (trifluoromethoxy)phenylhydrazone (FCCP) and 4.2uM Rotenone and Antimycin A. XF24 Seahorse Analyser was programmed to mix 3 min, wait 2 min, measure 3 min for 3 cycles prior to every injection. The mitochondrial respiration rate after each injection was used to analyse basal respiration, adenosine triphosphate (ATP) production, proton leak and maximal respiratory capacity.

2.6. Statistical analysis

A minimum of technical triplicates were performed for each biological replicate, with a minimum of three biological replicates (each from different patients) performed for each *in-vitro* study. When two groups were compared a *t*-test (parametric) or a Mann-Whitney test (non-parametric) was used and when three or more groups were compared a 1-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was used. Statistical analysis was done using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Sulfasalazine down regulates placental EGFR signalling

We treated primary cytotrophoblasts at doses we have previously reported not to affect placental cell viability [5], and were able to confirm a significant and dose dependent reduction of sFlt-1 secretion following treatment (Fig. 1A). Using mRNA collected from these cells we measured EGFR expression, and found a similar dose-dependent reduction in EGFR mRNA expression following sulfasalazine treatment (Fig. 1B). To determine whether this reduction in mRNA expression translated to altered protein expression, we measured phosphorylated EGFR protein expression via Western blot. Indeed, phosphorylated, total and activated EGFR (phosphorylated EGFR/total EGFR) protein expression was significantly reduced by sulfasalazine (Fig. 1C–E). Together this suggests that sulfasalazine reduces EGFR expression coincident with reduced sFlt-1 secretion.

3.2. Sulfasalazine down regulates placental EGFR downstream signalling

Activation of the EGFR ignites a plethora of downstream signalling cascades, of which we have previously shown ERK1/2 and STAT-3 are

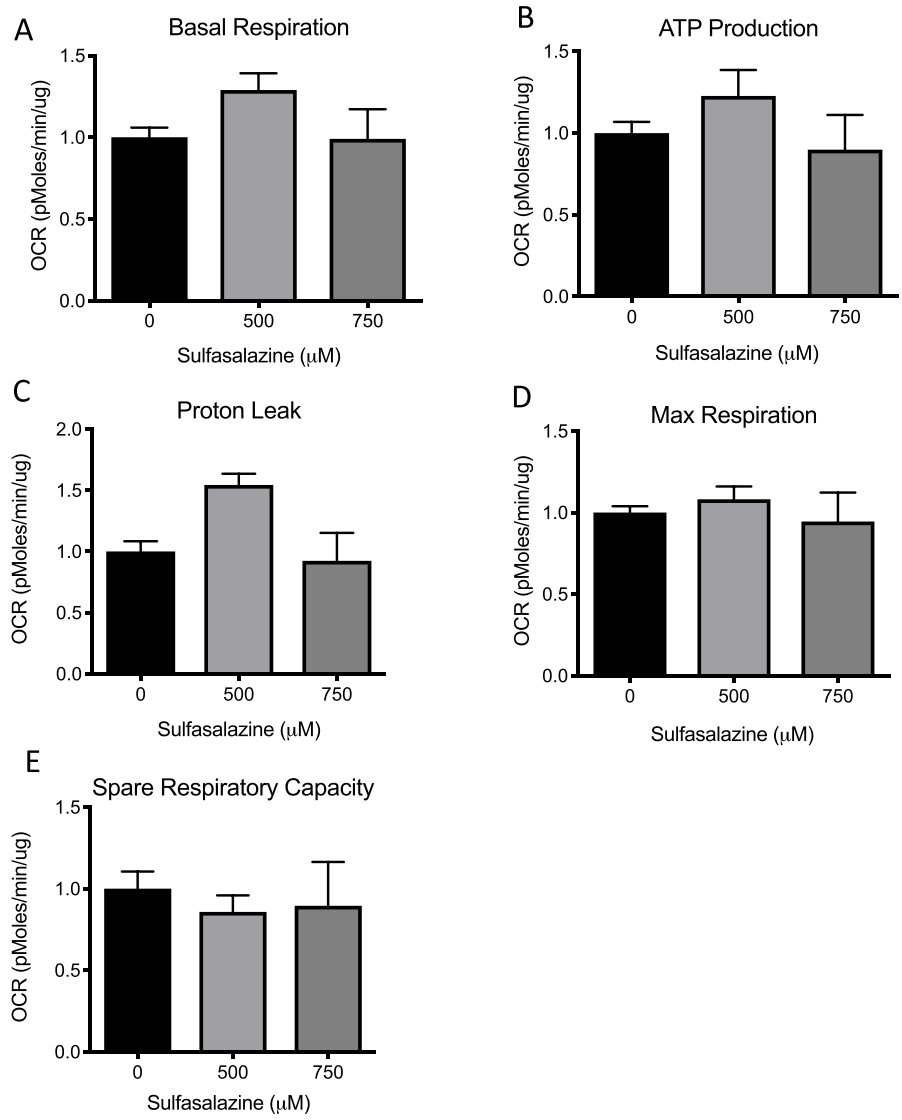

Fig. 3. Sulfasalazine does not alter placental mitochondrial respiration. Cellular mitochondrial respiration was assessed using the seahorse flux analyser following primary cytotrophoblast treatment with sulfasalazine (0–750 μM). (A–E) Sulfasalazine did not alter mitochondrial basal respiration, ATP production, proton leak, maximal respiration or spare respiration capacity. Data are mean ± SEM of 4 independent experiments in triplicate.

both involved in the placental regulation of sFlt-1 [10]. Thus, we next measured protein expression of these molecules after treating primary cytotrophoblasts with sulfasalazine. Sulfasalazine reduced phosphorylated ERK/12 protein expression by over 80% and also reduced total ERK1/2 protein (Fig. 2A–C). Additionally, both phosphorylated STAT-3 and activated STAT-3 protein expression were reduced by sulfasalazine, however total STAT-3 expression was not significantly altered (Fig. 2D – G) This data demonstrates that sulfasalazine, as well as reducing EGFR, also markedly affects the downstream signalling pathways.

3.3. Sulfasalazine does not inhibit mitochondrial respiration

In addition to EGFR signalling, we have reported the mitochondria positively regulates placental sFlt-1 secretion. Thus, we wondered whether sulfasalazine may be acting via both of these pathways to reduce placental sFlt-1 secretion. We assessed mitochondrial respiration of primary cytotrophoblast cells using the Seahorse Flux analyser following treatment with sulfasalazine and found that sulfasalazine did not alter basal respiration, ATP production, proton leak or maximal respiration (Fig. 3A–D). This suggests that sulfasalazine does not inhibit placental mitochondrial respiration.

4. Discussion

Sulfasalazine is a novel potential therapeutic for preeclampsia, reducing the secretion of a key anti-angiogenic molecule sFlt-1 [5]. Here, we demonstrate that sulfasalazine inhibits EGFR and key downstream signalling molecules coincident with reducing placental secretion of sFlt-1. This suggests that reduced EGFR signalling is a mechanism by which sulfasalazine may exert its effects on reducing sFlt-1 secretion.

Over the last two decades, there has been an avid search to identify molecules that could reduce the anti-angiogenic factors of preeclampsia and mitigate endothelial dysfunction as a means to treat preeclampsia. Unfortunately, poor understanding of the pathogenesis of the disease has made targeted therapeutics difficult to design. Recently, we published work demonstrating EGFR signalling and mitochondrial function as two parallel pathways that regulate sFlt 1 secretion from placenta. We further showed that indeed, molecules that we have been testing as potential therapeutics for preeclampsia reduce either EGFR expression (esomeprazole and statins) or inhibit mitochondrial respiration (metformin) [10]. Our current findings further suggest EGFR signalling to be upstream of sFlt-1 and to be a highly druggable target by which potential preeclampsia therapeutics have effect.

Sulfasalazine is an anti-inflammatory medication that we have also shown holds promise as a potential therapeutic for preeclampsia. There are numerous reports to suggest sulfasalazine exerts anti-inflammatory properties via targeting both the HO-1 and NFkB signalling pathways [15–17]. In our recent publication however, we were unable to show that HO-1 or NFkB pathways are targeted by sulfasalazine to reduce sFlt-1 expression and secretion in the placenta [5]. In the current study, we provide the first evidence to demonstrate that sulfasalazine reduces EGFR mRNA and protein expression and down-stream signalling molecules ERK1/2 and STAT3 in placental tissue.

Sulfasalazine has been previously suggested to reduce EGFR signalling in malignant cells, with sulfasalazine abolishing EGFR phosphorylation in lung adenocarcinoma cells [18]. Additionally, other anti-inflammatory drugs, including Licofelene and sulindac, have also been shown to inhibit EGFR and downstream signalling activation and expression [19,20]. This effect has been reported in a number of cancer cell types, including colon cancer cells, adenocarcinoma and pancreatic carcinoma [19–21]. Given we have demonstrated EGFR signalling regulates sFlt-1 secretion, it is plausible that other anti-inflammatory molecules, along with sulfasalazine, may also inhibit placental sFlt-1 secretion. Further interrogation of these molecules, the effect of perturbing signalling and other upstream pathways that regulate sFlt-1 may provide further evidence to the role of EFGR signalling and identify new therapeutic leads able to treat or prevent preeclampsia.

When sulfasalazine is administered orally approximately one third is absorbed by the small intestine and detected within serum 1–2 h following ingestion [22]. Importantly, at an oral dose of 4 g, serum concentrations are equivalent to the doses used here and in our initial report investigating the potential of sulfasalazine to treat preeclampsia [22]. Additionally, given the concentration of sulfasalazine reaching the placenta has not been previously reported, our team are currently undertaking a pharmacokinetics human study to determine this among women with preterm preeclampsia (Australia and New Zealand Clinical Trials Registry 12617000226303).

In conclusion, we have demonstrated that sulfasalazine inhibits EGFR and down-stream signalling pathway expression and suggest that it is this mechanism via which sulfasalazine quenches placental secretion of sFlt-1. Further investigation of other therapeutics able to modulate EGFR expression or signalling, that are safe in pregnancy, may yield new leads and further our understanding of the pathophysiology of this disease.

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Conflicts of interest

None declared.

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