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Small molecule antagonism of oxysterol-induced Epstein–Barr virus induced gene 2 (EBI2) activation

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ABSTRACT

The Epstein–Barr virus induced gene 2 (EBI2) was recently identified as the first oxysterol-activated 7TM receptor. EBI2 is essential for B cell trafficking within lymphoid tissues and thus the humoral immune response in general. Here we characterize the antagonism of the non-peptide molecule GSK682753A, which blocks oxysterol-induced G-protein activation, β-arrestin recruitment and B-cell chemotaxis. We furthermore demonstrate that activation triggers pertussis toxin-sensitive MAP kinase phosphorylation, which is also inhibited by GSK682753A. Thus, EBI2 signalling in B cells mediates key phenotypic functions via signalling pathways amenable to manipulation providing additional therapeutic options for inhibiting EBI2 activity.

1. Introduction

The Epstein–Barr virus induced gene 2 (EBI2 also known as GPR183) is a G protein-coupled seven-transmembrane (7TM) receptor that is predominantly expressed in B and T cells [1,2]. It regulates the trafficking of B cells within lymphoid tissues and is highly important for the generation of humoral immune responses [3,4]. EBI2 remained orphan for years; however, two independent studies recently showed that this receptor is activated by oxysterols, most potently by 7α,25-dihydroxycholesterol (7α,25-OHC) [5,6]. Binding of oxysterols to EBI2 induce Gαi activation, β-arrestin recruitment and ultimately migration of EBI2-expressing B and T cells. Thus, EBI2 functions as a chemo-attractant receptor. Interestingly, the main oxysterol generating cells within the lymphoid tissue were recently shown to be of stromal origin and these are required for efficient T cell-dependent plasma cell responses [7]. Moreover, we [8,9] and others [10] identified residues critical for oxysterol binding to EBI2 showing that the main anchor points are found in TM-II, -III, -VI and ECL2 of which several are located in the minor binding pocket [11].

The expression of EBI2 has been found to be dysregulated in several types of B cell malignancies and is thus reduced in e.g. diffuse large B-cell lymphomas [12] and chronic lymphocytic leukemia [13] and increased in post-transplantation lymphoproliferative disorders (PTLDs) [14]. EBI2 is also highly expressed in EBV-transformed lymphoblastoid B cells which phenotypically resemble PTLDs [15]. We recently showed that increased expression of EBI2 potentiates antibody-induced proliferation in B cells [16]. Thus, in malignancies where EBI2 expression is increased, this receptor may contribute to pathogenesis possibly by potentiating B cell proliferation. In such cases, blocking EBI2 activity could serve as a target for pharmacotherapy. Furthermore, this could also be envisioned to apply in autoantibody-mediated diseases such as lupus and rheumatoid arthritis. Finally, the up-regulation of EBI2 upon EBV infection may function to position B cells in specific lymphoid zones in order to increase overall viral survival. Blocking EBI2 activity may therefore serve as a novel route to treat EBV infection as no EBV-specific drugs are currently available. Of note, EBI2 is expressed both in the latent and lytic infection stages as opposed to e.g. the EBV-encoded 7TM receptor BILF1 or other EBV genes [1,17]. The desire to develop tool compounds for modulating EBI2 activity is exemplified well by an ongoing uHTS screen at the Sanford-Burnham Center for Chemical Genomics where a range of compounds able to antagonize 7α,25-OHC-mediated β-arrestin recruitment has been identified in a primary screen (PubChem BioAssay ID: 651636).

Abbreviations: 7α,25-OHC, 7α,25-dihydroxycholesterol; 7TM, seven-transmembrane; EBI2, Epstein–Barr virus induced gene 2; PTLD, post transplantation lymphoproliferative disorder; OE, over-expression; ptx, pertussis toxin..

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Simultaneous to the deorphanization of EBI2, we provided a characterization of a non-peptide inverse agonist (coined GSK682753A) that suppressed the apparent constitutive activity of the receptor [16]. Here we investigate the antagonistic properties of this compound and find that it potently suppresses 7α,25-OHC-mediated Gαi activation, β-arrestin recruitment and chemotaxis of primary B cells *ex vivo*. Furthermore, for the first time we demonstrate that 7α,25-OHC-induced activation of EBI2 triggers pertussis toxin (ptx)-sensitive MAP kinase phosphorylation which also is suppressed by GSK682753A.

2. Materials and methods

2.1. Ligands

7α,25-OHC was purchased from Avanti Polar Lipids and GSK682753A was synthesized in-house at GlaxoSmithKline. Both were dissolved in DMSO; final DMSO concentration was 0.1%.

2.2. Transfection and cell culture

CHO cells were grown in RPMI1640 containing 10% FBS, 2 mM glutamine, 180 u/mL penicillin and 45 μg/mL streptomycin (PenStrep) at 5% CO_2 and 37 °C. Stably transfected CHO FLP-In cells were grown in the same medium also containing 600 μg/mL Hygromycin. The CHO-K1 EA-arrestin cell line was grown in F-12 HAM medium containing 10% FBS, PenStrep and 250 μg/mL Hygromycin.

2.3. Receptor constructs

All constructs contained an N-terminal M1-FLAG tag to facilitate immunostaining. This does not affect the ligand-induced activity of EBI2. Mutations were generated using the Quick Change protocol.

2.4. Membrane preparation

Membrane preparations of CHO Flp-In cells stably expressing M1-EBI2 wt or pcDNA5 were generated as previously described [9].

2.5. GTPγS binding assay

Measurement of GTPγS binding to CHO Flp-In M1-EBI2 wt or pcDNA5 membranes upon 7α,25-OHC agonism or GSK682753A antagonism was performed as described previously [9]. Briefly, 10 μg of membrane preparation was incubated 30 min in the presence of ligands at various concentrations and [^{35}S] GTPγS at 1 nM. Wheat-germ agglutinin-coupled SPA beads were subsequently added (2.8 mg/mL) followed by 30 min incubation. After centrifugation (1500 rpm, 5 min) the amount of GTPγS binding was measured using a TopCounter. Unspecific binding was determined by adding unlabeled GTPγS at 40 μM.

2.6. β-Arrestin recruitment

Recruitment of β-arrestin was measured using the PathHunter β-arrestin assay (DiscoveRx). cDNA encoding M1-EBI2 wt was fused to the PK1-tag and the small fragment of β-galactosidase and cloned into pcDNA3.1 + . Assays were performed in a CHO-K1 EA-arrestin cell line stably expressing β-arrestin coupled to the β-gal large fragment. Cells were seeded out at 20,000 /well in 96-well plates and transfected the following day with 50 ng DNA using FuGENE6 reagent (0.15 μL/well). 48 h after transfection, cells were stimulated with varying concentrations of 7α,25-OHC and/or GSK682753A for 90 min. β-Arrestin recruitment was detected as β-gal activity 60 min after addition of chemiluminescent substrate.

2.7. Chemotaxis

B cells were isolated from wt and EBI2-overexpressing C57BL/6 mice as previously described [16]. Chemotaxis was measured using 96-well ChemoTx plates with 5 μm pores. Various concentrations of 7α,25-OHC and/or GSK682753A were applied to the lower chemotaxis chambers and 200,000 B cells subsequently added to the filter. The plates were incubated for 5 h at 37 °C and the number of cells migrated into the lower chambers detected using the CellTiterGlo dye and a TopCounter.

2.8. MAP kinase phosphorylation

CHO cells stably expressing EBI2 or pcDNA5 were seeded out in 12-well plates. The cells were serum starved overnight and incubated with GSK682753A and/or 7α,25-OHC at varying concentrations (for EBI2 and pcDNA) for 10 min. Subsequently, the cells were washed twice, lysed in lysis buffer (100 mM Tris, 4% SDS, 20% glycerol) and centrifuged for 5 min at 1500 rpm. 15–20 μg protein was loaded on Bis–Tris 10% NuPAGE gels and run for 1.5 h at 140 V followed by blotting onto PVDF membranes for 1.5 h at 30 V. The membrane was blocked in TBST (1 × TBS with 0.1% Tween20) containing 5% BSA followed by incubation with rabbit anti-phopho ERK or anti-phospho p38 IgG antibody (1:1000). Following washing, the membrane was incubated in blocking buffer containing goat anti-rabbit-IgG HRP-conjugated antibody (1:10,000) and developed using Super-Signal West Pico substrate (Pierce). The amount of phosphorylation was measured using a FluorChem H2A camera. The membrane was subsequently stripped using Pierce stripping buffer (Pierce) and the procedure was then repeated with rabbit anti-ERK or anti-p38 IgG antibody to detect total ERK or p38 levels.

3. Results

3.1. GSK682753A antagonizes 7α,25-OHC-induced EBI2 activation

GSK682753A is a piperidine-based non-peptide molecule identified in a compound library screen as an EBI2 inverse agonist [16] (Fig. 1A). To examine the putative antagonistic properties of GSK682753A we initially sought to determine whether GSK682753A was able to block oxysterol-induced activation of EBI2 at the level of G protein activation. In agreement with previous studies [5,6], 7α,25-OHC, the most potent EBI2-activating oxysterol, induced GTPyS binding to membranes from CHO cells stably expressing EBI2 wt (but not to pcDNA5 controls) when present (Fig. 1B; [7α,25-OHC] = 1 nM, [GSK682753A] = 0). However, presence of GSK682753A dose-dependently blocked EBI2 activity with an IC_{50} of 0.2 μM demonstrating that GSK682753A indeed functions as an antagonist (Fig. 1B). To determine whether this antagonism is competitive or non-competitive, we investigated the effect of three different GSK682753A concentrations on 7α,25-OHC dose–response curves (Fig. 2A). As the presence of GSK682753A resulted in a right-ward shift in 7α,25-OHC potency curve with only limited effect on the efficacy, this suggest that GSK682753A functions as a competitive antagonist. This is further corroborated by Schild plot analysis showing a linear relationship between the concentration of GSK682753A and the log (dr-1) value (Fig. 2B; slope: 1.4). The K_d, as estimated from the *x*-axis interception, is 64 nM.

To characterize the antagonism of GSK682753A further downstream and in a G protein-independent pathway, we measured β-arrestin recruitment in transiently transfected CHO cells. 7α,25-OHC induced β-arrestin recruitment with an EC_{50} value of 0.2 μM in accordance with earlier studies [5,6] (Fig. 3A). GSK682753A also inhibited β-arrestin recruitment but with a slightly higher potency than in the GTPyS binding assay (IC_{50} value of 40 nM; [7α,25-OHC] = 1 μM) (Fig. 3B). We have previously shown that the Phe at position