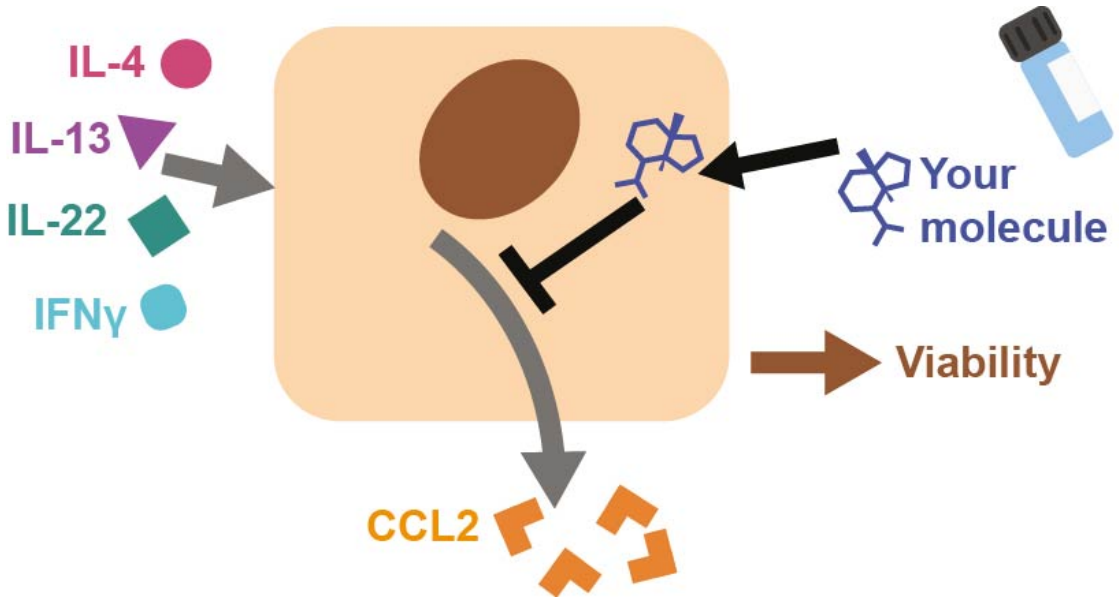
Keratinocyte CCL2 release

Inhibition of CCL2 release from IL-4, IL-13, IL-22 and IFNg-stimulated primary human keratinocytes
LEO Pharma Open Innovation assay number 1280



Disease relevance

The inflammatory skin disease atopic dermatitis (AD) is characterized by the T-cell cytokines including IL-4, IL-13, IL-22 and IFN-γ. In the present assay, keratinocytes are stimulated with a mixture of these cytokines, and the release of CCL2 (also called monocyte chemoattractant protein 1 (MCP-1)) is measured in the culture supernatant by proximity homogenous time-resolved fluorescence (HTRF). The purpose of the assay is to measure if test compounds are able to inhibit the levels of CCL2 released by the keratinocytes. Compound which inhibit keratinocyte CCL2 secretion may be expected to have efficacy in atopic dermatitis.
A known steroid, Betamethasone, inhibits CCL2 release in this assay with an EC_{50} of approximately 15 nM, and an Emax (plateau of the fitted curve) of approximately 60%. A high Emax shows that the compound is able to inhibit a large proportion of the secreted CCL2. A low EC_{50} value indicates that the compound is potent and is able to perform the inhibition at low concentrations.

Key assay parameters

Parameter	Description
Human primary keratinocytes (HEKa)	**Cells are frozen in passage 2. Cells are used in passage 3-6**
IL-4, IL-13, IL-22 and IFN-γ	**Atopic dermatitis-like stimulation: Recombinant human interleukin (IL) 4, 13 and 22 at 10 ng/mL and Interferon-γ at 1 ng/mL**
Effect	**Inhibition of AD stimuli induced CCL2 release from HEKa cells**
Positive control (~100% effect)	**AD-like stimulation + Terfenadine (CAS: 50679-08-8) at 10 microM**
Negative control (~0% effect)	**AD-like stimulation and 0.1% DMSO**
Reference compound	**Betamethasone (CAS: 378-44-9)**
Incubation time	**48h**
Capacity, run-time and requirements	**The assay takes 3 days to perform.**

Method description

Cell culture:
HEKa are human epidermal keratinocytes isolated from adult skin. The cells have been cryopreserved at the end of the primary culture stage in a medium containing 10% DMSO. Sterile cell culture work applies.

Initiating Cultures from Cryopreserved HEKa Cells:

- Thaw a vial of the frozen HEKa stock in a 37°C water/bead bath.

- Transfer the cell suspension into 30 ml T75 cell culture flask.
- Take 15 ml out and dispense over into 2xT75 (15 ml each).
- Set up for assay 5-6 days later or pass it on.

Maintenance of Stock Cultures

- Change the culture medium to freshly supplemented medium, 24 to 36 hours after establishing a secondary culture from cryopreserved cells. For subsequent subcultures, change the medium 48-72 hours after establishing the subculture.
- Only passage up to 3-5 in the assay.

Subculture of HEKa

- View and confirm 80% confluence.
- Remove all of the culture medium from the flask.
- Add 3 ml of TrypLE Express solution to the flask. Rock the flask to ensure that the entire surface is covered.
- Immediately remove all 3 ml of TrypLE Express solution from the flask.
- Add 1½ ml (T75) (3 ml T175) of fresh TrypLE Express solution to the flask.
- View the culture under a microscope. Incubate the flask at room temperature until the cells have become completely round, approximately 8-10 minutes.
- Tap the flask very gently to dislodge cells from the surface of the flask.
- Add 3-4 ml (T75) (7 ml T175) of Trypsin Neutralizer solution to the flask and transfer the detached cells to a sterile conical tube.
- Centrifuge the cells at 170 x g for 7-10 minutes. Observe the cell pellet.
- Remove the supernatant from the tube, being careful not to dislodge the cell pellet.
- Resuspend the cell pellet in 15 ml medium with HKGS supplemented(cat no S-001-K) but without hydrocortisone (when use for assay). Pipette the cells up and down with a 10 ml pipette to ensure a homogeneous cell suspension.
- Determine the concentration of cells in the suspension.
- Dilute the cells in supplemented medium and seed new culture in a T175 flask. Incubate the cultures in a 37°C, 5% CO2/95% air, humidified cell culture incubator.

Assay Day 0:

- Trypsinize the cells as described above.
- Resuspend pellet in 10 ml assay medium (EpiLife Medium with HKGS Kit but without hydrocortisone)
- Filter the cells though a 37µm strainer.
- Count cells using a cell counter.
- Seed HEKa cells in 384-well view plates 3500 c/w/40 µl.
- Place the plates with lids on in a humidity chamber with lid (24.5x24.5cm box added H2O in the bottom).
- Incubate plates over night at 37°C, 5% CO_2/95% air

Assay Day 1:

- Prepare compound-containing source plate, i.e. titrations in DMSO to be transferred to the assay plate.
- Add 80 nL compounds and controls from the source plate to the assay plate (with cells).
- Add 40 µl stimulation (recombinant human interleukin (IL) 4, 13, 22 at 10 ng/mL and Interferon-gamma at 1 ng /mL) in medium with supplement, but without hydrocortisone.
- Incubate plates at 37°C, 5% CO2/95% air for 2 days.

Assay Day 3:

- Remove plates from incubation and equilibrate to room temperature for 30 minutes.
- Transfer 8 µl supernatant to white proxi 384-well plates for CCL2 detection.
- Add 8 µl media to the detection plate.
- Add 4 µl CCL2 HTRF detection reagent (to the white proxi 384w).
- Seal and incubate for 4 hours and read in a plate reader.

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Data analysis and calculations

CCL2 concentration in the supernatants is measured using homogeneous time-resolved fluorescence resonance (TR-FRET). The assay is quantified by measuring fluorescence at 665 nm (proportional to CCL2 concentration) and 620 nm (control). A ratio 665/620*1000 is calculated.

% Effect:
The capacity of the test compound to inhibit CCL2 release is normalized to the signal in the negative control wells with keratinocytes incubated with 0.1% DMSO (0%) and 10 µM Terfenadine (100%), which fully inhibits the CCL2 release.

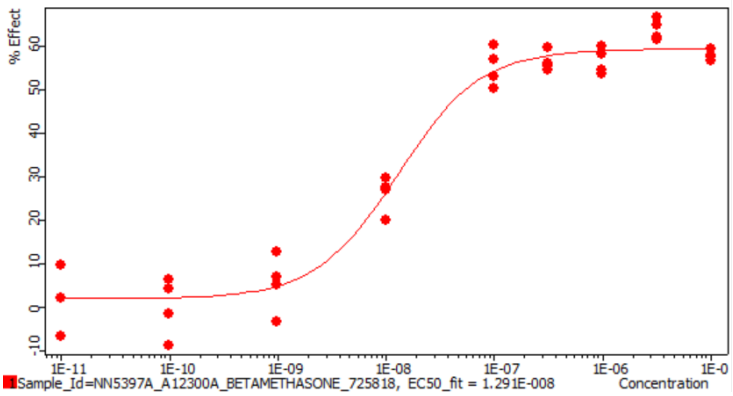

Figure: A typical concentration-response curve with the reference compound Betamethasone, EC_{50} = 13nM and Emax = 60% (partial inhibition).

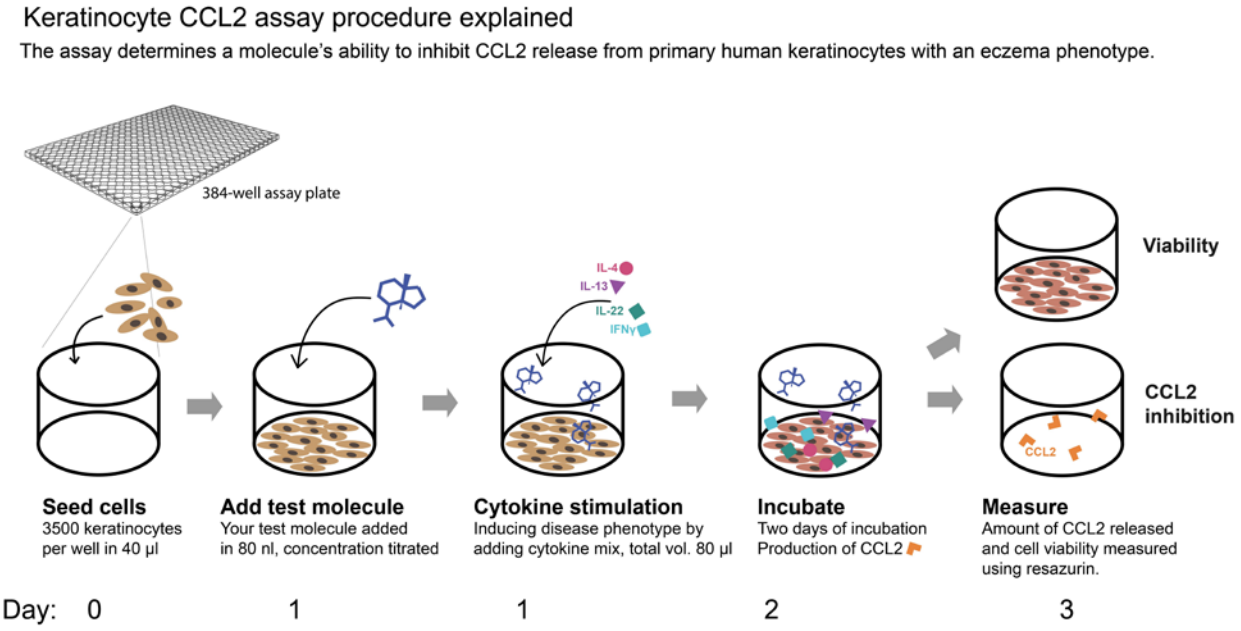

Figure: The picture illustrates the process of running the assay, from day 0 – seeding, up to day 3 – read out.