Validation of a High Throughput Gap-Junctional Intercellular Communication (GJIC) Assay Using Cigarette Smoke Condensates

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Abstract

Cigarette smoke condensate (CSC) is known to inhibit cell communication in a dose-dependent manner. We developed and evaluated a new high throughput GJIC assay for reproducible dose response, accuracy, and precision. An automated fluorescent microscope (ArrayScan®, Cellomics), the rat liver epithelial cell line WB-F344, and CSC from the 2R4F Kentucky Reference Cigarette and two single-tobacco cigarettes (i.e., Bright and Burley) were utilized in the evaluation. **Experimental:** Cells were stained with the fluorescent dye calcein. Stained cells were pipetted to unstained cells of the same type and incubated for 3 h with the test substance or solvent control (0.5% DMSO). After establishing the connexin channels, the transfer of the fluorescent dye to neighboring cells within 3 h was determined and expressed as "% stained cells" as a measure of gap-junctional intercellular communication. Phorbol-12-myristate-13-acetate (TPA), a well-known inhibitor of GJIC, was used as a control. **Results:** The assay showed a reproducible dose response. The repeatability and reproducibility for the 2R4F were 3.7% and 6.9%, respectively; the minimal detectable difference was 5.7 µg/ml. The EC_{50} value for TPA was 0.34 ng/ml, which is in the range reported in the literature for other GJIC assay designs. The EC_{50} values for CSC from the three cigarette types were 44 µg/ml for Bright, 49 µg/ml for the 2R4F, which is a mixture of Bright and Burley tobacco, and 56 µg/ml for Burley, which had a shallower slope than the other two cigarette types. **Conclusion:** This cell-based assay can determine the GJIC-inhibitory activity of CSC, and meets ISO criteria for precision and accuracy. The assay can also discriminate between different CSCs, as reflected by the EC_{50} values for the three cigarette types. In conclusion, this screening assay is an adequate tool to determine GJIC, which has been proposed to play a role in tumor promotion.

Introduction

Chemically induced inhibition of gap-junctional intercellular communication (GJIC) has been proposed to play a role in tumor promotion (Trosko and Chang, 1985). This end point has been considered as a possible predictor of tumor promotion and non-genotoxic carcinogens (Rivedal and Witz, 2005). Because tumor promoters can induce cell proliferation and down-regulation of intercellular communication, inhibition of GJIC by tumor-promoting compounds is considered to be a critical step in the removal of a cell from growth control.
Li et al. (2003) introduced the automated fluorescence microscope to measure the effect of single substances on GJIC. Ziambaras et al. (1998) introduced the manual technique of parachuting fluorescently labeled cells onto unlabeled cells to measure GJIC. We transferred Li's automated technique to WB-F344 cells (the most commonly used cells in GJIC assays) and combined it with Ziambaras' manual technique to develop a high-throughput GJIC assay that can determine the influence of complex mixtures, such as cigarette smoke, with high repeatability and reproducibility.

Objective

Develop a high throughput screening assay to detect GJIC inhibition activity of cigarette smoke condensate (CSC) by combining two techniques: an automated fluorescence microscope (Li et al., 2003) and parachuting of fluorescently labeled cells onto unlabeled cells (Ziambaras et al., 1998).

Materials and Methods

Test Substances
Cigarette smoke condensate (CSC) from a standard reference cigarette and 2 specially designed single-tobacco experimental cigarettes:
- Reference Cigarette 2R4F (a blend of Bright and Burley tobaccos)
- Bright cigarette
- Burley cigarette

Generation of CSC
- Cigarettes were smoked on a 20-port Borgwaldt smoking machine.
- Total particulate matter was collected on a Cambridge filter and dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 25 mg/ml DMSO.

Cell Culture
- Rat liver epithelial cells WB-F344 purchased from Health Science Research Resources Bank, Osaka, Japan (cat. no. ICRB 0193; http://www.jhsf.or.jp).
- Cells maintained in F-12K nutrient mixture (Kaighn's Modification) with 5% FCS and gentamicin (50 µg/ml) at 37 °C and 5% CO_2.

Reagents
- DMSO as solvent control (0.5%), Hoechst 33342 fluorescent dye, and phorbol-12-myristate-13-acetate (TPA) as positive control purchased from Sigma-Aldrich (Taufkirchen Germany).
- Hank's Balanced Salt Solution (HBSS) with Ca^{2+} + Mg^{2+}, calcein AM, and propidium iodide purchased from Invitrogen (Karlsruhe, Germany).
- Dye-loading buffer contained 5 µM calcein AM in HBSS; nucleus staining solution consisted of 10 µg/ml Hoechst 33342 in HBSS.

Automated Fluorescence Microscope (ArrayScan VTI, Cellomics, USA)
This system automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. Images were acquired for Hoechst 33342 and calcein using a 20x objective lens. Nine fields were acquired in each well. The key parameters for identifying the cells were nucleus area, nucleus perimeter, and fluorescence intensity of each cell.

GJIC Assay
Acceptor cells (20,000 per well) were seeded in 96-well plates and cultivated for 18 to 24 h.

Donor cells (1 million per flask) were seeded in T25 flasks, cultivated for 18 to 24 h, and stained with fluorescent dye calcein AM. Cells were washed with HBSS, dye-loading buffer was added, and cells were incubated at 37 °C for 20 min. Loading buffer was removed and cells were washed, then detached with EDTA-trypsin solution, centrifuged, counted, and resuspended in culture medium.

Stained donor cells were added to the acceptor cells at 4,000/well. Culture medium was aspirated before adding donor cells.

Test substances were applied and 96-well plates were centrifuged at 300 x *g* for 5 min, then incubated for 3 h at 37 °C and 5% CO_2. The medium was aspirated, the nucleus staining solution was added, and the cells were incubated for an additional 10 min at 37 °C. The transfer of calcein to neighboring cells was determined and expressed as "% stained cells" as a measure of GJIC.

References
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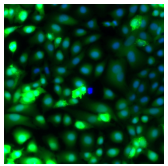
Results

Inhibition of GJIC

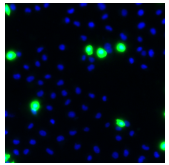
Solvent Control



(0.5% DMSO)

- no inhibition

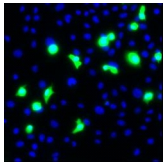
Positive Control



(1 ng/ml TPA)

- complete inhibition

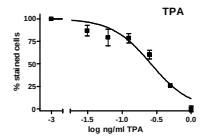
Cigarette Smoke Condensate: 2R4F



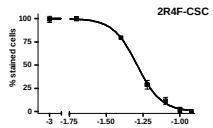
(0.12 mg/ml TPM)

- complete inhibition

Dose Response



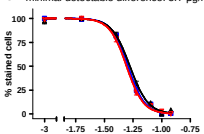
mean ± SEM, n = 36 (12/plate, 3 plates)



mean ± SEM, n = 36 (12/plate, 3 plates)

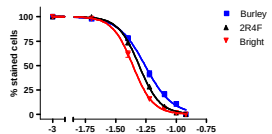
Repeatability and Reproducibility: 2R4F

- within-day variability: 3.7%
- between-day variability: 6.9%
- minimal detectable difference: 5.7 µg/ml



mean ± SEM, n = 12/plate

Discrimination of Cigarette Types



mean ± SEM, n = 36 (12/plate, 3 plates)

EC_{50} Values

Test Substance	EC_{50} Value
TPA (positive control)	0.26 (± 0.04) ng/ml
Bright (CSC)	44 (± 0.9) µg/ml
2R4F (CSC)	49 (± 0.9) µg/ml
Burley (CSC)	56 (± 2) µg/ml

Comparison	p Value
2R4F vs Bright	<0.0001
2R4F vs Burley	0.0008
Bright vs Burley	<0.0001

Remarks: mean ± SEM, n = 36 (12 EC_{50}/plate, 3 plates)

Summary and Conclusion

- The assay showed a dose dependency and very good reproducibility within 3 hours exposure of the cells for the tested substances.
- The EC_{50} value for TPA is in the range reported in the literature for other GJIC assay designs.
- The precision (repeatability and reproducibility) is better than precision standards in the bio-analytical industry for cell-based assays, which is in average 25%.
- The assay can discriminate between CSC from Bright and Burley, and 2R4F, which is a mixture of Bright and Burley tobacco.

This screening assay is an adequate tool to determine GJIC activity, which has been proposed to play a role in tumor promotion, in cells exposed to cigarette smoke condensate.

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