Video Article

High Throughput SiRNA Screening for Chloropicrin and Hydrogen Fluoride-Induced Cornea Epithelial Cell Injury

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Abstract

Toxicant-induced ocular injury is a true ocular emergency because chemicals have the potential to rapidly inflict significant tissue damage. Treatments for toxicant-induced corneal injury are generally supportive as no specific therapeutics exist to treat these injuries. In the efforts to develop treatments and therapeutics to care for exposure, it can be important to understand the molecular and cellular mechanisms of these injuries. We propose that utilization of high throughput small inhibitory RNA (siRNA) screening can be an important tool that could help to more rapidly elucidate the molecular mechanisms of chemical cornea epithelial injury. siRNA are double stranded RNA molecules that are 19-25 nucleotides long and utilize the post-transcriptional gene silencing pathway to degrade mRNA which have homology to the siRNA. The resulting reduction of expression of the specific gene can then be studied in toxicant exposed cells to ascertain the function of that gene in the cellular response to the toxicant. The development and validation of *in vitro* exposure models and methods for the high throughput screening (HTS) of hydrogen fluoride- (HF) and chloropicrin- (CP) induced ocular injury are presented in this article. Although we selected these two toxicants, our methods are applicable to the study of other toxicants with minor modifications to the toxicant exposure protocol. The SV40 large T antigen immortalized human corneal epithelial cell line SV40-HCEC was selected for study. Cell viability and IL-8 production were selected as endpoints in the screening protocol. Several challenges associated with the development of toxicant exposure and cell culture methods suitable for HTS studies are presented. The establishment of HTS models for these toxicants allows for further studies to better understand the mechanism of injury and to screen for potential therapeutics for chemical ocular injury.

Video Link

The video component of this article can be found at https://www.jove.com/video/57372/

Introduction

Toxicant-induced ocular injury is a true ocular emergency because chemicals have the potential to rapidly inflict significant tissue damage. Unfortunately, treatments for toxicant-induced corneal injury are only generally supportive as no specific therapeutics exist to treat these injuries. The current treatment strategy is non-specific and primarily includes topical therapeutic treatments such as lubricants, antibiotics, and cycloplegics followed by anti-inflammatories (*e.g.*, steroids) once the cornea has re-epithelialized[1,2]. Despite the best current therapeutic treatment options available, long-term prognosis is generally poor due to progressive corneal clouding and neovascularization[2,3].

Animal models have traditionally been used to investigate chemical toxicity and understand mechanisms of injury. However, animal studies are time consuming and expensive. There are also efforts to reduce animal testing. For example, REACH legislation (EC 1907/2006) in the European Union has provisions intended to reduce animal testing. The provisions include a requirement that companies share data in order to avoid animal testing and obtaining approval from the European Chemicals Agency prior to performing proposed tests on animals. Under the provisions of REACH, animal testing should be a last resort. There is also the European Cosmetics Regulation (EC 1223/2009) that phased out the testing of cosmetics in animals. When animal studies are conducted, they are guided by the principles of 3Rs (Refinement, Reduction, and Replacement), which provide a framework for performing more humane animal research, reducing the number of animals used, and using non-animal alternatives where possible. For these reasons, the field of toxicology has sought to adopt *in vitro* assays that can provide insight into molecular mechanisms of toxicity and can be done in higher throughput[4]. This is a functional toxicology approach where toxicants are defined by their function rather than solely by their chemistry. Taken a step further, functional toxicogenomics seek to understand the role(s) that specific genes play in the effects of toxicants[5]. With the application of siRNA technology, screens to investigate gene function in the molecular and cellular responses to toxicants can be done at high throughput. siRNA are double stranded RNA molecules that are 19-25 nucleotides long that take advantage of the post transcriptional gene silencing pathway present in all mammalian cells[6]. These are synthetically made and designed to target a specific gene. When introduced into a cell, the siRNA is processed and one strand, the guide strand, is loaded into the RNA-induced silencing complex (RISC). The siRNA directs the RISC to a complementary region in an mRNA molecule, and the RISC degrades the mRNA. This results in the reduction of expression of the specific gene. The resulting reduction of expression of the specific gene can then be studied in toxicant exposed cells to ascertain the function of that gene in the cellular response to the toxicant. Such an approach has been used to further understand the mechanisms of ricin susceptibility and the AHR-dependent induction of CYP1A1[7,8].

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The Chemical Terrorism Risk Assessment (CTRA) list and the toxic industrial chemicals (TIC) listings have itemized select chemicals based on their toxicity and potential to be released during a terrorist, warfare, or industrial accident event[9]. We are applying an siRNA high throughput screening (HTS) toxicogenomic approach to the study of CTRA list toxicants, which have been identified to be at high risk of use in a terrorist incident. Traditional toxicology seeks to understand the adverse effects that chemicals have on living organisms; however, we have a further desire to understand the mechanisms of injury for the purpose of informing the development of therapeutics and therapeutic approaches, and possibly, to discover molecules which can be targeted for therapeutic development. This effort in some ways may be considered analogous to the use of high throughput siRNA screening and cell based assays in the drug discovery process[10]. A major difference would be that drug discovery typically seeks a singular target for therapeutic discovery whereas in our approach it is somewhat unlikely that there would be a singular target with high therapeutic value for the treatment of toxicant exposure. We anticipate that any effective treatment paradigm for toxicant exposure would require a multi-faceted approach to achieve high therapeutic value, and toxicogenomic data may vitally inform an effective treatment paradigm.

Benchtop automation brings high throughput methodology to laboratories outside the pharmaceutical or biotech industries. The *in vitro* studies at our institute have historically been traditional assays which are low throughput[11,12,13]. In the past few years, our laboratory has transitioned to the use of benchtop robotics to perform high throughput siRNA screening. Herein, we present the refinement of ocular cell models and the development of *in vitro* exposure methods for hydrogen fluoride (HF) and chloropicrin (CP) suitable for high throughput siRNA screening. Our goal is to identify molecules that regulate cellular injury in response to these toxicants. The targets of the siRNA library we selected include G protein-coupled receptors, protein kinases, proteases, phosphatases, ion channels, and other potentially druggable targets. HF and CP were selected for study by cross-referencing CTRA list agents with the ToxNet reports of industrial accidents to find those that present the greatest risk of ocular injury via vapor exposure[9,14]. CP (chemical formula Cl_3CNO_2, CAS number 76-06-2) was originally used as a tear gas in WWI[15]. It is currently used as an agricultural fumigant and functions as a nematicide, fungicide, and insecticide[16]. Hydrogen Fluoride (HF) is used in processes including alkylation in oil refineries and electrochemical fluorination of organic compounds[17]. HF (chemical formula HF, CAS number 62778-11-4) is a gas but in its aqueous form is hydrofluoric acid (HFA, CAS number 7664-39-3). Therefore, we elected to use HFA in our in cell exposure models. The SV40 large T antigen immortalized human corneal epithelial cell line SV40-HCEC was selected for study. Cell viability and the inflammatory marker IL-8 were selected as endpoints because targets that are involved in cellular injury should be reflected in the cell death and the inflammatory response. Specifically, if a target were to play a protective role in toxicant exposure, cell death and/or inflammatory cytokine production should increase when the target expression is inhibited by siRNA. The opposite would be true for targets that play a negative role. Also, chronic inflammation appears to play a role in cornea pathology after exposure, and intervention in cell death pathways may improve clinical outcome[2,18].

Protocol

1. Cell Culture Maintenance

1. Grow the cell line SV40-HCEC at 37 °C, 5% CO_2, and 90% humidity in DMEM F-12 with 15% fetal bovine serum (FBS), 1% L-glutamine, 10 µg/L epidermal growth factor (EGF), and 5 mg/L insulin.
2. Passage the cell line every 3 to 4 days (depending on seeding density) to ensure that confluency never exceeds 80% during culture maintenance.
3. Detach the cells from flasks using a detachment solution (14 mL solution for every 150 cm^2 flask) and incubation at 37 °C for no more than 8 min.
4. Neutralize the detachment solution with an equal volume of cell culture medium and pellet the cells in 50 mL conical tubes by centrifugation for 7 min at 160 x g.
5. Re-suspend the cell pellet in medium (20 mL for every 150 cm^2 flask).
6. Count the cells with an automated handheld cell counter and seed in new flasks at a density that will allow them to grow for 3 to 4 days without exceeding 80% confluency.

2. Plate Cells for Experimentation

1. Check flasks of SV40-HCECs by phase contrast light microscopy to ensure that confluency is less than 80% and to assess general cell health.
2. Detach, pellet, resuspend, and count SV40-HCECs from flasks as described in steps 1.3 to 1.6 of cell culture maintenance. Use SV40-HCEC medium containing 0.5 µg/mL hydrocortisone (HCORT medium) to resuspend cells.
3. In a disposable medium bottle, prepare a suspension of SV40-HCECs at 17,857 cells/mL in pre-warmed HCORT medium.
    1. Prepare a sufficient volume of cell suspension for the number of plates to be seeded.
    2. Seed a minimum of 14 x 96-well plates with cells for every siRNA library plate to be studied.
4. Swirl the bottle to evenly suspend the cells, pour a sufficient volume of the cell suspension into a reservoir on the orbital shaker nest of an automated liquid handler, and store the bottle containing the cell suspension on a 37 °C warming plate during the cell plating process.
5. Use the automated liquid handler to add cells to plates at a density of 1000 cells per well (5000 cells/cm^2) in 70 µL of medium per well of a 96-well plate. Use a 50 µL/s pipetting speed for all steps.
    1. Run the orbital shaker at 100 rpm constantly during the seeding process.
    2. Mix the cell suspension three times (140 µL mix volume) using the automated liquid handler.
    3. Aspirate 140 µL of the cell suspension and dispense 70 µL into each well of two cell culture plates.
    4. Repeat steps 2.5.3 and 2.5.4 until all plates have been seeded with cells.
    5. Refill the cell suspension reservoir as needed during the seeding process.
6. After the plates have been seeded, remove them from the automated liquid handler and incubate them for 30 min at room temperature before transferring them to a cell culture incubator.

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7. Evaluate the cell density of every well for each plate the following morning using an automated imaging system which is housed in a cell culture incubator and exclude any plates from the study which have interior wells that are not between 15% and 22% confluent.

3. Transfect Cells with siRNA

1. Acquire siRNA library plates from the vendor pre-configured to contain 80 siRNA targets per plate (see **Figure 4**), with columns 1 and 12 left empty.
2. Reconstitute the siRNA library plate with siRNA buffer to a final concentration of 2 pmol/µL for each well of siRNA according to the manufacturer's instructions[19].
3. Transfect the cells 24 h after seeding with 4 pmol/well of siRNA and 0.3 µL/well of transfection reagent. Perform all steps according to the transfection reagent manufacturer's protocol (see **Figure 4** for plate layout)[20].
    1. Perform all the transfections using an automated liquid handler, and use a 25 µL/s pipetting speed for all steps. Use pre-configured tip boxes to address only the wells that will be transfected.
    2. Use the top half of the library plate to transfect a set of six replicate plates referred to as the "Top Plate Set" (six replicates per siRNA, n=6).
    3. Use the bottom half of the library plate to transfect a different set of six replicate plates referred to as the "Bottom Plate Set" (six replicates per siRNA, n=6).
    4. Use the term "Full Plate Set" to describe the 12 cell plates that have been transfected with library siRNA.
    5. Transfect wells B2-B6 of all plates in the Full Plate Set with negative pool siRNA after all top and bottom plate sets have been transfected with library siRNA.
    6. Also transfect wells B2-B6 of another two plates, that do not receive library siRNA and will serve as unexposed controls (one for the top plate set and one for the bottom plate set), with negative pool siRNA.
4. Incubate all cell plates in a cell culture incubator for 4 hours after all transfection mixes have been added and mix by gentle tapping every hour.
5. Use an automated liquid handler to wash all wells of the plates twice with pre-warmed HCORT medium diluted 1:5 in PBS. Use a 50 µL/s pipetting speed for all steps.
    1. Aspirate 100 µL from each well and discard that into a waste reservoir.
    2. Add to each well 100 µL/well pre-warmed HCORT medium diluted 1:5 in PBS which is contained in a different reservoir at a sufficient volume of for the number of plates.
    3. Repeat steps 3.5.1 and 3.5.2 for each plate.
        1. Empty the waste reservoir as needed.
        2. Refill the reservoir with HCORT medium diluted 1:5 in PBS as needed.
    4. Aspirate 100 µL from each well and discard that into the waste reservoir.
6. Refeed all wells of the plates with 100 µL/well pre-warmed HCORT medium, which is contained in a different reservoir at a sufficient volume of medium for the number of plates.
    1. Refill the reservoir with medium as needed.
7. Use wells C2-C6 of all plates as non-transfected controls.
8. Keep wells B7-B11 and C7-C11 of all plates non-transfected to be used as drug and vehicle controls for toxicant exposure.

4. Refeed the Cells the Following Day

1. Use an automated liquid handler to refeed all wells of the plates. Use a 50 µL/s pipetting speed for all steps.
    1. Aspirate 100 µL from each well and discard that into a waste reservoir.
    2. Refeed each well with 100 µL/well pre-warmed HCORT medium that is contained in a different reservoir and has a sufficient volume of medium for the number of plates to be refed.
    3. Repeat steps 4.1.1 and 4.1.2 as needed to refeed all cell plates.
        1. Empty the waste reservoir as needed.
        2. Refill the reservoir with medium as needed.

5. Positive Control Addition

1. Two days after transfection and two hours prior to exposure, prepare a 62.5 µM solution of the positive control cardamonin in HCORT medium to be used for HFA exposures and add to row B of an 8-row dilution reservoir.
    1. For CP exposure, prepare and use a 25 µM solution of SKF 86002.
    2. Prepare a volume of positive control sufficient for the number of plates to be tested.
2. Prepare a 0.625% solution of DMSO in HCORT medium (vehicle control) and add to row C of the same reservoir.
    1. For CP exposure, prepare and use a 0.5% solution of DMSO.
    2. Prepare a volume of vehicle control sufficient for the number of plates to be tested.
3. Use the automated liquid handler to add positive and vehicle controls to wells B7-B11 and C7-C11 of each cell plate and use a 50 µL/s pipetting speed for all steps. Use pre-configured tip boxes to address only the wells that will receive the positive and vehicle controls.

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1. Remove 10 µL of medium from wells B7-B11 and C7-C11 of each cell plate and discard it into rows G and H of the 8-row dilution reservoir.
2. Transfer 10 µL of the positive and vehicle controls to those wells and mix 3 times.

4. Return these cell plates to the incubator.
5. Repeat steps 5.3 to 5.4 until all cell plates have received positive and vehicle controls.

6. HF Exposure of Cultured Cells

CAUTION: HFA is corrosive and acutely toxic.

1. Perform all chemical exposure operations in a chemical fume hood wearing double nitrile gloves, a laboratory coat, disposable polyethylene sleeve protectors and safety glasses.
   1. Acquire HFA as a 48% solution.
   2. Dilute HFA to 1% with ultrapure water and store in 5 mL aliquots in 10 mL thick wall polyethylene vials to improve safety.
   3. Decontaminate pipette tips and reservoirs that have come into contact with HFA and any leftover liquid HFA with 2.5% calcium gluconate prior to disposal in the hazardous waste stream.

2. For each siRNA library plate under investigation, prepare a 0.0036% HFA medium solution by adding 288 µL of 1% HFA to 80 mL of pre-warmed HCORT medium in a 150 mL bottle.
   1. Swirl the bottle and incubate the dilute HFA in a 37 °C incubator in the chemical fume hood for 10 min.

3. Mix the HFA medium solution again by swirling the bottle and add the HFA medium solution to a reagent reservoir on a plate warmer.
4. Perform the exposure with two technicians working in tandem.
   1. Have the technician on the right aspirate 100 µL from each of the interior wells of a cell plate using a 12 channel pipettor and then pass the plate to the technician on the left.
   2. Have the technician on the left add 100 µL per well of the HFA medium solution.
   3. Repeat steps 6.4.1 and 6.4.2 for all cell plates that are to be exposed to toxicant.
   4. Also repeat steps 6.4.1 and 6.4.2 for unexposed control plates, utilizing fresh HCORT medium instead of the HFA medium solution.

5. Place the plates in the chemical fume hood incubator for 20 min and then return them to the cell culture incubator.
6. Repeat the positive control addition step (steps 5.1 to 5.5) as previously shown once all plates have been exposed and returned to the cell culture incubator.

7. CP Exposure of Cultured Cells

CAUTION: CP is acutely toxic and an irritant.

1. Perform all chemical exposure operations in a chemical fume hood wearing double nitrile gloves, a laboratory coat, disposable polyethylene sleeve protectors and safety glasses.
   1. Acquire CP.
   2. Dilute CP to 5% in DMSO and store in 10 mL aliquots in 10 mL scintillation vials to improve safety.
   3. Decontaminate pipette tips and reservoirs that have come into contact with CP and any leftover liquid CP with 2.5% sodium bisulfite prior to disposal in the hazardous waste stream.

2. Prepare a sufficient volume of pre-warmed HCORT medium containing 1x Pen/Strep and pre-warmed PBS for the number of plates to be exposed.
3. For each Top Plate Set or Bottom Plate set, add 8.04 µL of 5% CP in DMSO to a 50 mL aliquot of pre-warmed HCORT medium and mix well for a final CP concentration of 0.0008%. Cap the tube tightly and incubate the solution in a 37 °C incubator in the chemical fume hood for 1 h.
   1. Time the addition of CP to the 50 mL aliquots of medium so that each exposed Top Plate Set and Bottom Plate Set receives toxicant exactly 1 h after CP was added to the 50 mL aliquot of medium.

4. Remove a Top Plate Set and 1 unexposed control plate from the cell culture incubator and place them in the chemical fume hood near the end of the incubation period.
5. Mix the CP solution by inverting the tube and then decant it to a reagent reservoir at the end of the 1 h incubation period.
6. Perform the exposure with two technicians working in tandem.
   1. Have the technician on the right aspirate 100 µL from each of the interior wells of a cell plate using a 12 channel pipettor and then pass the plate to the technician on the left.
   2. Have the technician on the left add 100 µL per well of the CP medium solution.
   3. Repeat steps 7.6.1 and 7.6.2 for all cell plates of the Top Plate Set to be exposed to toxicant.
   4. Also repeat steps 7.6.1 and 7.6.2 for unexposed control plates, utilizing fresh HCORT medium instead of the CP medium solution.

7. Place the plates in a 37 °C incubator in the chemical fume hood for 10 min.
8. Add a sufficient volume of PBS and HCORT medium containing 1x Pen/Strep to reagent reservoirs near the end of the 10 min incubation period.
9. Remove the CP solution from cell plates using a 12 channel pipettor and replace it with 100 µL per well of PBS at the end of the 10 min incubation period.
10. Immediately remove the PBS from cell plates and replace it with 100 µL per well of HCORT medium containing 1x Pen/Strep.
11. Also repeat steps 7.9 and 7.10 for the unexposed control plates.
12. Return the cell plates to a standard cell culture incubator.

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13. Repeat steps 7.3 to 7.12 for the Bottom Plate Set.
14. Repeat the Positive control addition step (steps 5.1 to 5.5) as previously described once all plates have been exposed and returned to the cell culture incubator.

8. Sample Collection and Cell Viability Assay

1. Twenty four hours after exposure, prepare a solution of 0.5 mg/mL MTT substrate in PBS containing 10 g/L glucose and warm to 37 °C[21]. Prepare 10 mL of substrate for each plate to be assayed.
2. For the number of plates to be assayed, add a sufficient volume of MTT substrate solution to a reservoir on an automated liquid handler.
3. Use the automated liquid handler to collect sample and add MTT substrate using a 50 µL/s pipetting speed for all steps. Preconfigure tip boxes to address only those wells to be assayed.
    1. Aspirate 95 µL of medium from the inner 60 wells of the cell plate, and deposit 42.5 µL into each of two 384-well storage plates.
    2. Immediately add 100 µL per well of the MTT substrate solution to the cell plates, and incubate them at 37 °C in a cell culture incubator for 1.5 h.
    3. Refill the reservoir with MTT substrate solution as needed.
4. Incubate the cell plates at 37 °C in a cell culture incubator for 1 h.
5. Seal the 384-well storage plates and store them at -80 °C for subsequent analysis.
6. Repeat steps 8.3 to 8.5 for all top and bottom plate sets and the associated unexposed controls.
    1. Reuse tips between replicates if desired, but wash them when adding MTT substrate solution and when switching between plate sets.
7. After the 1 h incubation, add a sufficient volume of DMSO to a reservoir on an automated liquid handler.
8. Use the automated liquid handler to add DMSO and use a 50 µL/s pipetting speed for all steps.
    1. Aspirate 100 µL from each of the inner wells of the cell plates and discard the MTT substrate solution into a waste reservoir.
        1. Empty the waste reservoir as needed.
    2. Overlay each well with 100 µL DMSO.
        1. Refill the DMSO reservoir as needed.
9. Shake the plates on a plate shaker for 3 min.
10. Measure absorbance at 570 and 690 nm using a plate spectrophotometer.
11. Subtract the background and calculate the % cell viability by dividing the exposed absorbance values by the unexposed controls. Use the average unexposed negative pool siRNA control to calculate % cell viability for all targets.

9. Measure IL-8 Concentration in Cell Culture Supernatants

1. Measure the concentration of IL-8 in the cell culture supernatants using a no wash bead-based assay according to the manufacturer's instructions[22]. Create an assay plate layout to accommodate samples and standard curve.
2. Remove the 384-well storage plates from the -80 °C freezer, thaw at room temperature, and briefly centrifuge the plates to collect the sample in the bottom of the wells.
3. For the number of assay plates to be run, make a sufficient volume of anti-IL-8 acceptor beads and biotinylated anti-IL-8 antibody in assay buffer included in the kit. Use the ratio of 50 µL of anti-IL8 acceptor beads and 50 µL of biotinylated anti-IL8 antibody to 9.9 mL of assay buffer.
    1. Using a multichannel pipettor, add bead/antibody mixture to a black 384-well storage plate.
        1. Add acceptor bead/antibody to the wells designated for use for samples and standard curve according to the assay plate layout.
        2. Add a sufficient volume per well for the number of assay plates to be run.
4. Reconstitute IL-8 standard to 1000 pg/mL using cell culture medium containing 354 µM NaCl. Make a sufficient volume for the number of assay plates to be run.
5. Make eight twofold serial dilutions of the standard curve.
6. Add the standard curve in triplicate to the appropriate wells of a black 384-well storage plate according to the assay plate layout. Add a sufficient volume per well for the number of assay plates to be run.
    1. Similarly, add cell culture medium containing 354 µM NaCl with no IL-8 for background measurement.
7. Use an automated liquid handler to perform the assay. Use pre-configured tip boxes to address only the wells designated by the assay plate layout. Use a 50 µL/s pipetting speed for all steps.
    1. Transfer 8 µL of the acceptor bead-antibody mixture to the wells of the white 384-well shallow well assay plates.
        1. Add only to the wells designated for samples and standard curve according to the assay plate layout.
    2. Transfer 2 µL of the standard curve to the appropriate wells of the shallow well assay plates according to the assay plate layout.
    3. Prepare a 3.54 M NaCl solution and add a sufficient volume for the number of plates to be assayed to a shallow reservoir on the automated liquid handler.
    4. Adjust the salt concentration of the samples to match that of the standard curve by transferring 4.5 µL of the NaCl solution to the samples in the black 384-well storage plates.
    5. Mix the samples three times using a mixing volume of 30 µL, and transfer 2 µL of the samples to the white shallow well assay plates according to the assay plate layout.
        1. Change tips in between sample plates.

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6. Incubate for 1 h in the dark at room temperature.
7. For the number of assay plates to be run, make a sufficient volume of acceptor beads in assay buffer. Use the ratio of 200 µL of secondary acceptor beads to 12.3 mL of assay buffer.
8. Using a multichannel pipettor, add secondary beads to a black 384-well storage plate.
    1. Add secondary beads to the wells designated for use for samples and standard curve according to the assay plate layout.
    2. Add a sufficient volume per well for the number of assay plates to be run.
9. Using the automated liquid handler, transfer 10 µL of the secondary beads to the wells of the white 384-well shallow well assay plates.
    1. Add only to the wells that received samples and standard curve according to the assay plate layout and wash the tips between each plate.
10. Incubate for another hour in the dark at room temperature.

8. Seal the assay plates with clear plate seals and scan using a plate reader compatible with the assay. Use 0.2 mm distance between plate and detector, 180 ms excitation time, and 550 ms measurement time for the scan.
9. Import the raw data from the IL-8 assays into a spreadsheet.
10. Use automated curve fitting for the standard curve of the IL-8 assays, and then convert the raw data to pg/mL for each sample.

Representative Results

Exposure Method Development

We refined and evaluated the suitability of the human corneal epithelial cell line SV40-HCEC for use in HTS studies. SV40-HCEC were immortalized using the SV-40 large T antigen and were a gift from Dhanajay Pal[23]. There were too many variables explored in exposure methodology development to present concisely herein, and so, only some samples of findings we believe may be more broadly applicable to multiple toxicants are presented.

Toxicant Exposure by Spike or Medium Exchange

We initially sought to expose cells by spiking 5 µL of a working concentration of toxicant diluted in water, saline, or medium into 95 µL of medium already present into each well of cells in a 96-well plate to achieve the final desired exposure concentration. SV40-HCEC were exposed using this spike expose/refeed methodology to 1 X LD_{50} of HFA for 24 h (1 X LD_{50} = 0.02% HFA by this method when the HFA spike was diluted in medium), and wells were refed with fresh medium 10 min later. After 24 h, the cells were overlaid with MTT substrate as described above for the cell viability assay. Images were captured by photomicroscopy prior to solubilization of the formazan crystals with DMSO. Close examination of wells showed that a large degree of cell death was localized in one spot, and this is in spite of the fact that the plate was shaken for 15 s immediately after the spike was added to the entire plate (**Figure 1a**). This phenomenon was observed when the spike was added to the bottom of the well or when added at the meniscus. A higher magnification image of this area using phase contrast shows that cells are still present in the area unstained by MTT (**Figure 1b**). We also tested a medium exchange expose/refeed methodology where medium was removed from the wells, replaced with cell culture medium containing the 1 X LD_{50} of HFA (1 X LD_{50} = 0.035% HFA by this method) and wells were refed with fresh medium 10 min later. After 24 h, the cells were overlaid with MTT substrate as described above. This method achieved an apparently more even cell death response of cells within each well (**Figure 1c and 1d**). Unexposed controls are provided for reference and show no localized areas of cell death (**Figure 1e and 1f**). For CP exposures, the spike method did not result in a large degree of cell death localized to one spot within each well; however, the cell death response was more consistent from one iteration to the next using the medium exchange exposure method (data not shown).

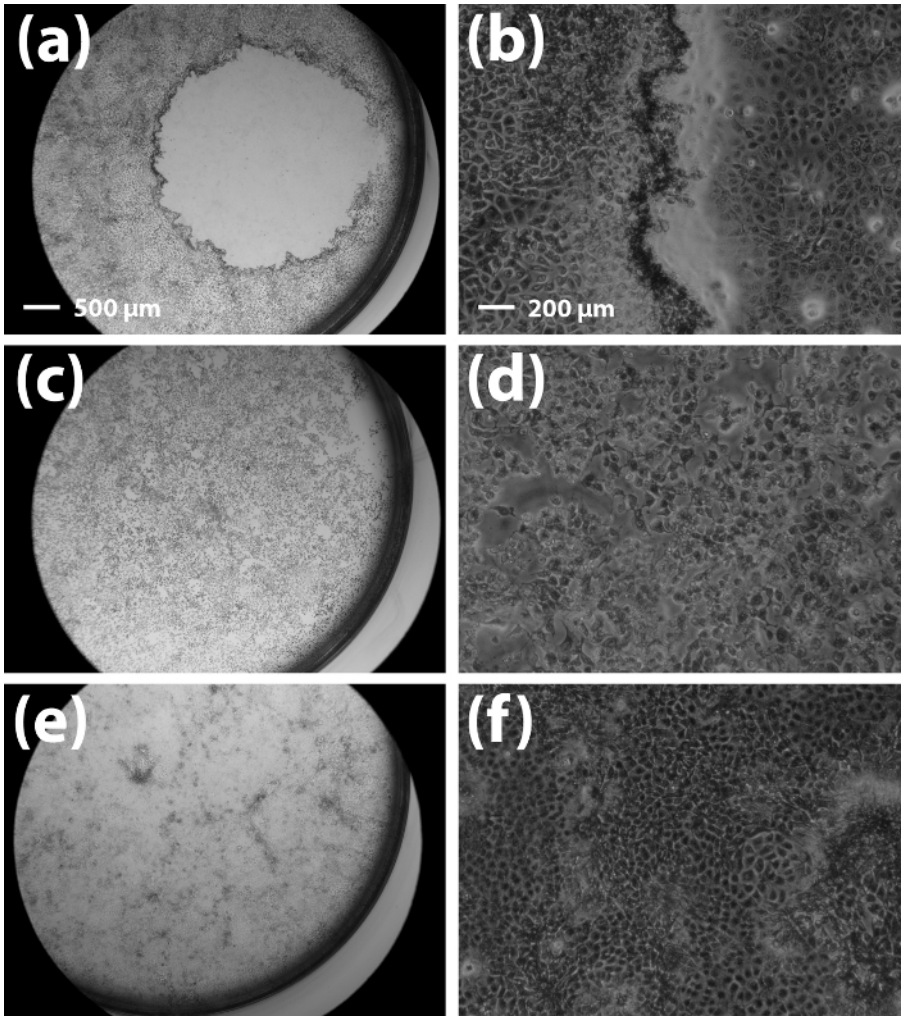

Figure 1: Cell death induced by spike and medium exchange exposure methods. All images were captured 1.5 h after addition of MTT substrate. Panel (a) is a bright field image of SV40-HCEC exposed to HFA spike expose/refeed methodology. Panel (b) is a higher magnification of the same well in panel (a) using phase contrast. Panel (c) is a bright field image of SV40-HCEC exposed by medium exchange expose/refeed methodology. Panel (d) is a higher magnification of the same well in panel (c) using phase contrast. Panels (e) and (f) are from an unexposed well, bright field and phase contrast higher magnification, respectively. Please click here to view a larger version of this figure.

Toxicant Stability in Medium

It was observed that once diluted in medium, the apparent cytotoxicity of HF and CP changes and then stabilizes. HFA was diluted in medium to 0.0036% and allowed to incubate at 37 °C for 1-60 min prior to exposing SV40-HCECs as described above. For HFA, the cytotoxicity increases within the first 5 min that HFA is diluted in medium and is then stable for at least an hour (**Figure 2a**). One way ANOVA followed by Dunnett's Multiple Comparison Test showed that % cell viability at all time points were significantly different from the 1 min time point. There were no significant differences between the 2.5 to 60 min time points. CP is organic and is first diluted to 5% in DMSO. It was then diluted in medium to 0.0008% and allowed to incubate at 37 °C for 5-120 min prior to exposing SV40-HCECs as described above. Once diluted in medium, the cytotoxicity of CP decreases over 60 min and then stabilizes for at least the next h (**Figure 2b**). One way ANOVA followed by Dunnett's Multiple Comparison Test showed that % cell viability at all time points were significantly different from the 5 min time point. There were no significant differences between the 60 to 120 min time points.

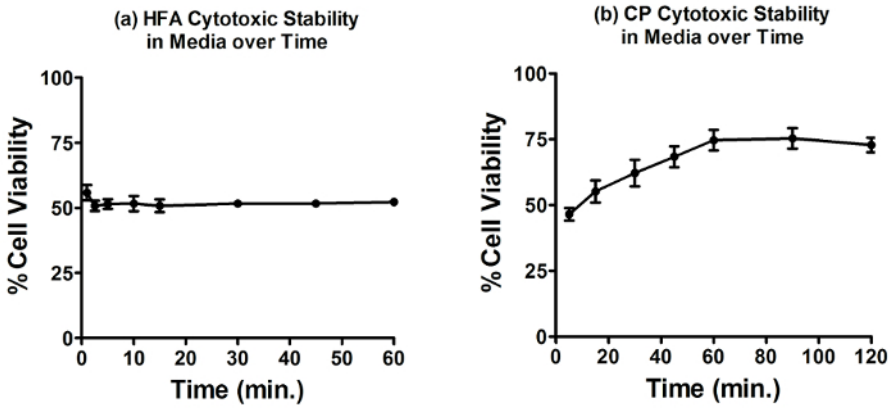

Figure 2: Toxicant loss of cytotoxicity in medium dilutions. The data are expressed as the average of the percent viability ± SD (n=6). Panel (a) shows the stability of 0.0036% HFA cytotoxicity when diluted in medium and incubated at room temperature for 1-60 min prior to exposing SV40-HCECs as described above. Panel (b) shows the stability of 0.0008% CP cytotoxicity when diluted in medium and allowed to incubate at 37 °C for 5-120 min prior to exposing SV40-HCECs as described above. Please click here to view a larger version of this figure.

Expose/Refeed or Expose Only

Another factor investigated in exposure methodology was whether to: A) add toxicant for 10 min, wash, refeed, remove plates from the chemical fume hood, and incubate 24 h in a standard cell culture incubator (expose/refeed method); or B) add toxicant, leave it on, remove plates from the chemical fume hood, and incubate 24 h in a standard cell culture incubator (expose only method). Safety is an important factor as it may not be permissible to remove plates of cells that contain diluted toxicant from the chemical fume hood because the toxicant will off-gas and expose personnel. A related consideration is the number of plates to be exposed at a given time since the more plates there are, there will be a greater volume off-gassing. In our HTS methods, we expose 48 x 96-well plates in a given study day. We placed exposed cell culture plates into a sealed bag and then used colorimetric gas detector tubes to assess CP and HF off-gassing from exposed plates. We found that exposing 48 plates to 1 x LD_{50} of HFA did not result in any detectable HF off-gassing (data not shown). For CP exposures, we found that there was enough CP off-gassing from 48 plates exposed to 1 x LD_{50} to present at least some safety concern (data not shown). Both expose/refeed and expose only methods were evaluated for HFA exposures. SV40-HCEC cells were exposed to HFA and viability was assessed by MTT assay 24 h after exposure. Dose response iterations were performed on different days. Compared to the expose/refeed method (**Figure 3a**), we found that the expose only method produced more consistent cell viability assay results (**Figure 3b**). Therefore, this method was selected as part of the final exposure methodology. For CP, any differences in the consistency of cell viability assay results between the two different exposure methodologies could not be evaluated since safety concerns precluded removing the CP exposed plates from the chemical fume hood for 24 h incubation in a standard cell culture incubator.

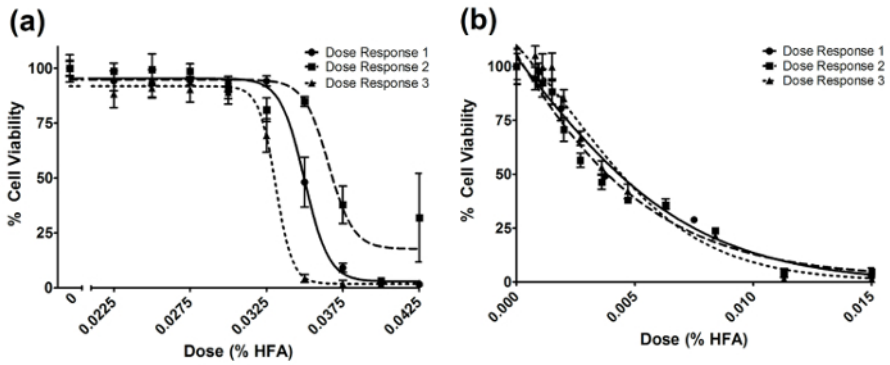

Figure 3: Exposure consistency with expose/refeed and expose only methods. The data are expressed as the average of the percent viability ± SD (n=6). (a) HFA was diluted in medium, incubated at room temperature for 10 min, and then used to expose cells by the expose/refeed method. (b) HFA was diluted in medium, incubated at room temperature for 10 min, and then used to expose SV40-HCEC by the expose only method. Please click here to view a larger version of this figure.

Cell Model Refinement

Medium Constituents

We adhered to the culture conditions established by the originating investigators for the passage and maintenance of these cell lines; however, we modified them for cells in experimentation. The cell culture medium prescribed by the originating investigators for the SV40-HCEC cells does not include hydrocortisone, but we utilized a low level of hydrocortisone (0.5 µg/mL) during exposure studies to suppress the occasional spike in cytokine production seen in naive SV40-HCEC (data not shown) which adversely affected data analysis from one iteration to the next during the refinement studies.

Phenotypic Stability

During our cell model refinement studies, we found that the SV40-HCEC begin to have decreased cytokine production in response to toxicant exposure starting around passage number 60 (data not shown). For this reason, we maintained cryostocks of low passage cells, executed all studies using cells under passage number 60, and monitored cytokine production during the screening process.

Plate Layout

An important source of experimental variability in high throughput studies is edge effects of multi-well plates. We found that the cytokine responses of cells in edge wells were not consistent with, or equivalent to, interior wells in both HFA and CP studies (data not shown). Median polish of the data, a statistical exercise used to minimize the edge effects on a data set, did not sufficiently resolve the issue (data not shown). Therefore, no cell plate edge wells were used for experimentation or controls (**Figure 4**).

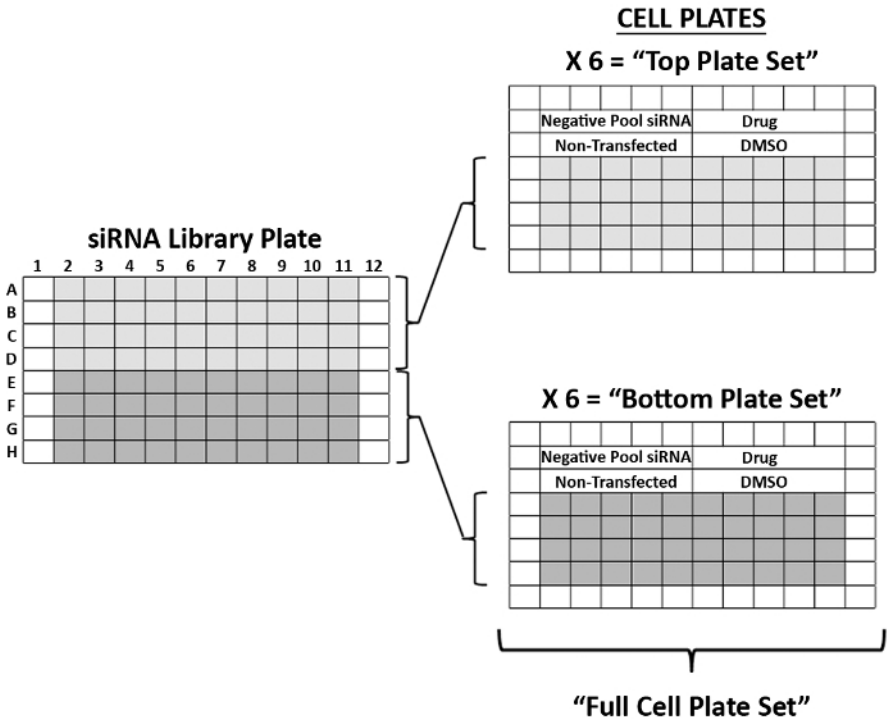

Figure 4: Library and cell plate layouts. The grey shaded areas in the Library Plate Layout represent wells that contain siRNA. The white wells are empty. The 40 targets from rows A-D of the Library Plate are used in the "Top Plate Set", and the 40 targets from rows E-H of the Library Plate are used in the "Bottom Plate Set". Wells B2-B6 are used for negative pool siRNA controls. Wells C2-C6 are non-transfected. Wells B7-B11 are used for either cardamonin or SKF 86002 positive control drugs as described, and wells C7-C11 are used for DMSO vehicle control. Please click here to view a larger version of this figure.

In Vitro Model Validation for HTS

We used Z' factor analysis to determine suitability of SV40-HCEC for use in HTS studies as this statistical test is used to determine assay quality for HTS[24]. For HF Z' factor analysis, cells were seeded at 1.25 x 10^3 cells/well, transfected with negative pool siRNA (as described above) 24 h after seeding, and exposed 48 h after transfection to 0.0036% HFA (1 x LD_{50}), n=3. For CP Z' factor analysis, cells were seeded and transfected as described above, and exposed 48 h after transfection to 0.0008% CP (1 x LD_{50}), n=5. A greater n was used for the CP studies owing to the greater variability in this exposure model. Z' factor was calculated for IL-8 expression data from unexposed *vs.* exposed cells and negative pool siRNA transfected unexposed *vs.* exposed cells for CP and HFA exposures. We also analyzed non-transfected cells to determine if there was an effect on assay quality due to siRNA transfection. Many iterations of the validation studies were performed while refining exposure and culture conditions in an effort to maximize the Z' factor results. SV40-HCEC passed validation and most iterations had Z' factor values greater than 0.50 for HF exposures (**Table 1**) and CP exposures (**Table 2**). Each replicate in these tables was from cells plated on different days.

Exposure Groups	Z'-Factor Analysis		
	Rep. #1	Rep. #2	Rep. #3
HF-Exposed Negative Control vs. Unexposed Negative Control	0.68	0.5	0.56
HF-Exposed vs. Naïve	0.4	0.68	0.56

Table 1: SV40-HCEC HFA Exposure Z` Factor Analysis of IL-8

Exposure Groups	Z'-Factor Analysis		
	Rep. #1	Rep. #2	Rep. #3
CP-Exposed Negative Control vs. Unexposed Negative Control	0.6	0.18	0.46
CP-Exposed vs. Naïve	0.42	0.29	0.42

Table 2: SV40-HCEC CP Exposure Z' Factor Analysis of IL-8

Primary siRNA Screening Results of HFA Injured Cells

Transfection optimization studies were performed prior to screening, and siRNA targeting cyclophilin b was used for these studies. An *in situ* RNA detection assay was used to measure cyclophilin b mRNA levels, and a high content analyzer was used to quantify the results. We achieved near 90% knockdown of cyclophilin b and typically no more than 5-10% cell loss with 4 pmol of siRNA/well and 0.3 µL/well transfection reagent (data not shown). Higher amounts of siRNA actually resulted in poorer cyclophilin b knockdown (data not shown). Similar levels of knockdown were achieved with 1.2 pmol/well of siRNA and 0.09 µL/well transfection reagent, but we elected to use 4 pmol per well in order to address any targets that could require greater amounts of siRNA to achieve effective knockdown. Target knockdown kinetics were also evaluated, and it was observed that target knockdown was maximal by two days post-transfection (data not shown). Target knockdown consistency across the plate was also validated (data not shown). The anti-inflammatory drugs cardamonin and SKF 86002 are used as positive controls for the inhibition of cytokine production for HFA and CP exposure, respectively. These were selected by testing a variety of compounds with known anti-inflammatory properties in exposed cells. The dose utilized in screening studies was selected by dose response optimization studies.

The high throughput siRNA screening strategy we utilized is industry-standard and involves three major steps: 1) primary screening, 2) library deconvolution, and 3) target validation. In primary screening, the phenotype of each target is evaluated utilizing a mixture of different siRNA sequences as a single reagent to target each gene. Targets are then down-selected to library deconvolution. In this step, each of the different siRNA sequences that are pooled to target each gene in primary screening are tested separately. Targets undergo another down-selection into target validation in which the target phenotype is confirmed with drugs, phenotype restoration, and/or siRNA from another vendor. For our primary screen, we elected to perform a focused sub-genomic screen rather than a genome wide screen for reasons of economy. Microarray data from HF and CP injured mouse corneas and Ingenuity Pathway Analysis (IPA) were used to focus our target library for the primary screen (manuscripts in preparation). Briefly, mouse corneas were exposed to toxicant vapor using a vapor cup similar to previously described methods[25]. Exposed cornea buttons and controls were harvested at various time points post exposure. RNA was isolated, and the quality and amount of RNA monitored. All microarray experiments were performed according to the manufacturer's protocol[26]. Raw signal intensities were normalized and analyzed by principal component analysis (PCA) to determine the significant sources of variability in the data. Genes were rank ordered by statistical significance. The data were mined and compared to gene lists of preconfigured siRNA libraries. From these gene lists, we selected 3120 targets for screening. The siRNA library was screened in SV40-HCEC cells (see **Figure 5** for a flow chart of the HTS protocol for the primary screen). Endpoint assessments included IL-8 levels in cell culture medium by no-wash bead-based assay and cell viability by MTT assay. The cells were exposed to 0.0036%HFA, as previously described, which is approximately 1 x LD_{50}. Cell viability was assessed by MTT assay 24 h after exposure. The effect of each siRNA pool on cell viability was analyzed for statistical significance relative to the exposed negative pool average for each plate using strictly standardized mean difference (SSMD)[27]. A dual-flashlight plot of the SSMD and cell viability fold-change is shown in **Figure 6**. The hit selection thresholds chosen for targets that exacerbated cell death or improved cell viability were SSMD ≤ -1.28 and SSMD ≥ 1.28, respectively.

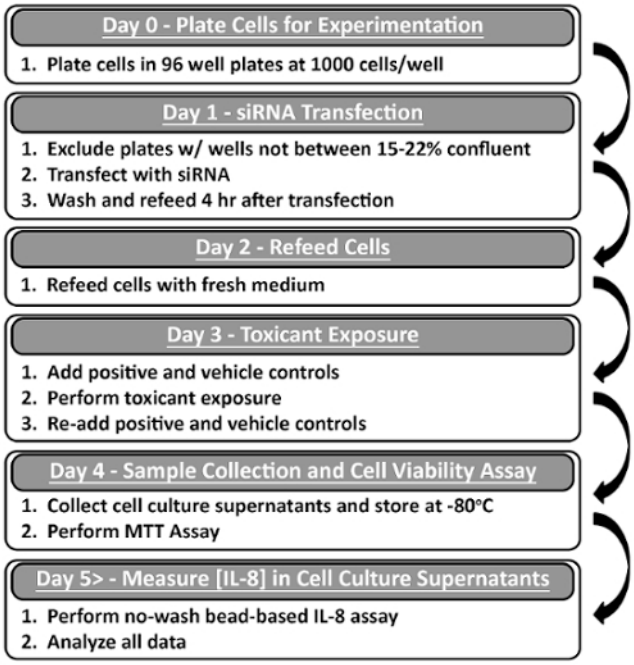

Figure 5: Primary Screen HTS protocol Flow Chart. This flow chart shows the essential steps that are performed on each day for the HTS protocol. Please click here to view a larger version of this figure.

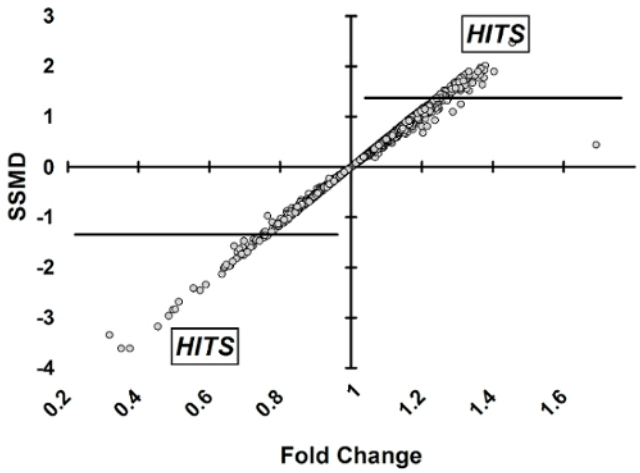

Figure 6: Dual-Flashlight Plot of the siRNA Effect on Cell Viability of HFA-Exposed SV40-HCEC Cells. Results shown for each target are the average of six replicates (n=6). Cell viability data are expressed as a fold-change relative to the exposed negative pool control siRNA, and statistical significance was analyzed by SSMD. Targets which had a SSMD ≤ -1.28 or ≥ 1.28 were selected as hits. Please click here to view a larger version of this figure.

The effect of each siRNA pool on HFA-induced IL-8 production was analyzed for statistical significance relative to the exposed negative pool average for each plate. IL-8 in cell culture supernatant was assessed assay 24 h after exposure. A dual-flashlight plot of the SSMD and IL-8 fold-change is shown in **Figure 7**. The hit selection thresholds chosen for targets that decreased IL-8 production were a 40% decrease and SSMD ≤ -1.0. The hit selection thresholds chosen for targets that increased IL-8 production were a five-fold increase and SSMD > 1.28.

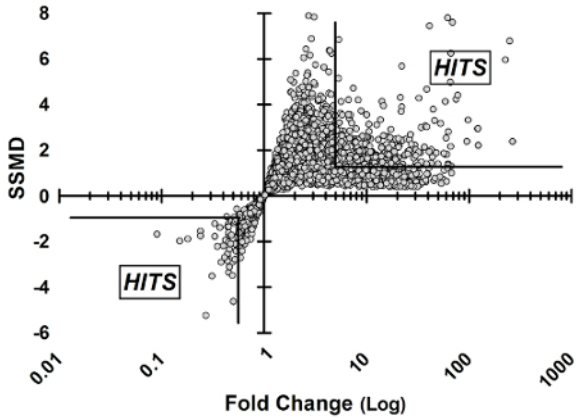

Figure 7: Dual-Flashlight Plot of the siRNA effect on IL-8 Production by HFA-Exposed SV40-HCEC Cells. Results shown are the average of six replicates (n=6). IL-8 expression level data are expressed as a fold-change relative to the exposed negative pool siRNA, and statistical significance was analyzed by SSMD. Hits were defined as targets which had a 40% decrease in IL-8 levels and SSMD ≤ -1.0, or a five-fold increase in IL-8 levels and SSMD > 1.28. Please click here to view a larger version of this figure.

Discussion

Herein we describe our methods and results on the development of a high throughput cornea epithelial cell screening model for the study of HF and CP injuries. We also present the results from the primary siRNA screen for HF injury. There were many challenges to the development of HTS models for the study of TIC injuries. Methods that we could find in the literature related to the study of HF, HFA or CP in cell culture models were of little help. Most *in vitro* studies on the fluoride ion involve oral cells and utilized sodium fluoride and not HF or HFA (for some examples, see[28,29]). Some methodology on the *in vitro* exposure of bronchial epithelial cells to CP has been published by Pesonen *et al.*[30]. Ultimately, we found no specific methods in the literature related to the HTS study of HF, HFA or CP, and the vast majority of challenges and variables related to the study of these toxicants in HTS required development. Particular attention was paid to achieving the high degree of exposure consistency across time and numerous screening plates necessary for a successful HTS study.

One type of challenge encountered was related to the application of toxicant to cell culture systems. Some toxicants are either reactive with or unstable in aqueous solutions. The toxicants we selected for study are not regarded as having these instability issues. An agricultural study investigating photohydrolysis of CP showed that a 0.001 M solution of CP in water had a half-life of 31.1 h when exposed to an 1100 lux xenon light source (sunlight intensity of an overcast day) for 10 days, 12 h per day[31]. There was no measurable hydrolysis over the same 10 day period when the solution was stored in the dark. The proton and fluoride ion of HFA are clearly stable in water. There are, however, additional stability considerations with regard to toxicant dilutions made in cell culture medium. We found that there was an initial change of cytotoxicity to some degree when the toxicants were first diluted in cell culture medium that then stabilizes after a brief period of time. We believe that this is due to the toxicant binding or complexing with constituents in the medium. Cell medium contains calcium, magnesium, protein, *etc.* that are known to interact with the fluoride ion[32,33]. CP is known to have oxidative reactions with biological thiols, but it may also bind DNA, proteins, and other nucleophiles[34,35]. Once these reactions and interactions have completed or reached equilibrium, the amount of available toxicant stabilizes and thus the apparent cytotoxicity stabilizes. We initially explored the use of saline and water dilutions of toxicants for working stocks used for exposures to avoid toxicant interactions with medium constituents in working dilutions. The use of saline would also preserve the acid component of HFA injury for exposures performed by medium exchange. We found that the cell death responses to toxicants in water and saline dilutions were more variable than the method that used cell culture medium dilutions with medium exchange (data not shown). Regarding the spike exposure method with HFA, the variability may be due to inconsistency in the spike dispersing in the well leading to cellular targets competing with medium constituents for the available fluoride ion. The reasons for the variability in HFA exposures by medium exchange with HFA saline dilutions are entirely unclear. In any event, exposure methods using saline dilutions for HFA were abandoned and not explored for CP. The toxicity of our HFA dilutions in medium probably closely mimics studies using sodium fluoride because the cell medium buffers the free proton in our HFA dilutions.

Other challenges we encountered were related to refinement of the cell culture models for use in HTS. We found it necessary to modify the cell culture medium constituents for cells in experimentation, specifically hydrocortisone and antibiotics. We utilized a low level of hydrocortisone (0.5 µg/mL) during exposure studies to suppress the occasional spike in cytokine production seen in naive SV40-HCEC. This low amount of hydrocortisone did not adversely affect the responses of the cells to toxicant and was below levels typically used to suppress cytokine production by cultured cells in response to stimulant[36,37]. The exact reasons why naive SV40-HCEC cells occasionally produce excess cytokines are not known, but it is possible that the cells are variably sensitive to the minor stressors that occur during cell passaging and plating. Many labs routinely include antibiotics in cell culture maintenance medium, but some antibiotics, tetracyclines in particular, have been shown to exhibit anti-inflammatory effects on cultured cells including corneal epithelial cells[38]. In general, our laboratory avoids the use of antibiotics whenever possible since these can potentially confound studies. We also investigated the phenotypic drift over multiple passages of our cells in culture. It is common for immortalized cells to undergo genetic and/or phenotypic drift over multiple passages, if not maintained in the exponential phase, if allowed to reach confluency, or if exposed to certain environmental stressors[39,40,41]. Therefore, it is critical during a high throughput screen that cell lines are properly maintained and that the screen is completed using cell passage numbers that maintain the known phenotypic response. Multi-well plate edge effects is the phenomenon that cells grown in the outer perimeter wells of multi-well plates often do not respond the same or with the same consistency as cells grown in interior wells[42]. There are statistical exercises, such as median polish, that can be utilized to minimize edge effects on a data set[43]. However, there is no statistical exercise that can eliminate edge effects on a particular target or control if it is always tested in an edge well, and varying the plate layout between replicates to move targets or controls off of edge wells is not logistically

practical or cost effective. It is our opinion that the decision to include the use of edge wells in cell-based HTS assays should be made cautiously. We found it necessary to eliminate the use of cells grown in edge wells for controls and targets.

Our HTS cell model and exposure methodology for *in vitro* HF injury was used to successfully complete the primary siRNA screen of 3120 targets. SSMD was used for hit selection because it was proposed specifically to measure the magnitude of difference between a control and an siRNA[27]. Our results for cell viability show that there was a linear relationship in the dual-flashlight plot between SSMD and fold-change for virtually all targets. For this reason, we used the single criterion of SSMD for hit selection for this endpoint. The IL-8 data differed in that some targets had weak but consistent effects while others showed strong but inconsistent effects. Therefore, we used both fold-change and SSMD for hit selection criteria for this endpoint. The SSMD values used for hit selection for both cell viability and IL-8 data were chosen because cutoffs between 1 and 1.645 (or -1 and -1.65) achieve low false discovery and non-discovery rates[44]. There were some targets that were hits in both the cell viability and IL-8 data. In these cases, preference for inclusion in the deconvolution library was given to those targets that exerted their effects in the same direction of improving overall health (improved viability and decreased IL-8) or exacerbating injury (increased cell death and increased IL-8). Overall, we selected a total of 250 targets between the cell viability and IL-8 endpoints to make up the deconvolution library for HF injury. The primary screen for *in vitro* CP injury is in progress.

In summary, we have developed exposure and culture conditions suitable for the HTS study of ocular injury by HF and CP. Several variables in exposure and culture conditions were refined and optimized, and the models were validated to be suitable for HTS studies by Z' factor analysis. We have further proven the utility of these models by completing the primary screen of an siRNA library in the HF model. There are many injuries and diseases that are of no or limited interest to the pharmaceutical or biotech industry, toxicant injury by CP and HF included, and the advent of benchtop robotics facilitates the HTS study of injuries and diseases in virtually any laboratory. High throughput genomic data sets can greatly contribute to the understanding of the roles that specific genes play in injury or disease which can help to more efficiently focus follow on *in vitro* or *in vivo* studies.

Disclosures

The authors have nothing to disclose.

DISCLAIMER: The views expressed in this article are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. This research was supported by an interagency agreement between NIH/NIAID and the USAMRICD, and in part by an appointment to the Postgraduate Research Participation Program at the U.S. Army Medical Research Institute of Chemical Defense administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC.

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