Published in final edited form as:

Methods Enzymol. 2016 ; 568: 163–185. doi:10.1016/bs.mie.2015.09.029.

High Throughput Screening for Drugs that Modulate Intermediate Filament Proteins

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Abstract

Intermediate filament (IF) proteins have unique and complex cell and tissue distribution. Importantly, IF gene mutations cause or predispose to more than 80 human tissue-specific diseases (IF-pathies), with the most severe disease phenotypes being due to mutations at conserved residues that result in a disrupted IF network. A critical need for the entire IF-pathy field is the identification of drugs that can ameliorate or cure these diseases, particularly since all current therapies target the IF-pathy complication, such as diabetes or cardiovascular disease, rather than the mutant IF protein or gene. We describe a high throughput approach to identify drugs that can normalize disrupted IF proteins. This approach utilizes transduction of lentivirus that expresses green-fluorescent-protein-tagged keratin 18 (K18) R90C in A549 cells. The readout is drug ‘hits’ that convert the dot-like keratin filament distribution, due to the R90C mutation, to a wildtype-like filamentous array. A similar strategy can be used to screen thousands of compounds and can be utilized for practically any IF protein with a filament-disrupting mutation, and could therefore potentially target many IF-pathies. ‘Hits’ of interest require validation in cell culture then using in vivo experimental models. Approaches to study the mechanism of mutant-IF normalization by potential drugs of interest are also described. The ultimate goal of this drug screening approach is to identify effective and safe compounds that can potentially be tested for clinical efficacy in patients.

1. Overview of Intermediate Filaments and Their Associated Diseases

Intermediate filament (IF) proteins make up one of the three major components of the cytoskeleton, with the other two major groups being microfilaments (i.e., actins) and microtubules (i.e., tubulins) (Ku et al., 1999). IF proteins, as contrasted with actins and tubulins, have several distinct properties that include being the largest in terms of its family members [e.g., the keratin subgroup of IFs alone are encoded by 54 genes (Schweizer et al., 2006), relative insolubility, diverse structures, preferential expression in higher eukaryotes (e.g., they are not found in yeast), and extensive disease association (Fuchs and Weber, 1994; Omary et al., 2004). Another distinctive feature of IF proteins is their tissue and cell type selective expression. For example, keratins are the IFs of epithelial cells, desmin is found in muscle, neurofilaments in neuronal cells, glial fibrillary acidic protein (GFAP) in glial cells, and vimentin in mesenchymal cells. All these examples are cytoplasmic IF, as contrasted with lamins which reside in the inner aspect of the nuclear membrane of nucleated cells (Fuchs and Weber, 1994; Osmanagic-Myers et al., 2015; Schreiber and Kennedy, 2013).

In terms of human disease, IF mutations cause or predispose to >80 IF-associated human tissue-specific diseases (IF-pathies) (Omary, 2009; Worman and Schirmer, 2015) that can affect practically every organ in body depending on the distribution of the IF (Fuchs and Weber, 1994; Omary et al., 2004; Szevenenyi et al., 2008). The first IF mutation found to be directly linked to any human disease involved keratin 14 (K14) (Bonifas et al., 1991; Coulombe et al., 1991), which then led to multiple discoveries collectively showing that a broad range of human Mendelian-inherited diseases are caused by mutations in IF genes. Most of the known IF mutations are highly penetrant autosomal-dominant, though some of the IF gene mutations predispose to, rather than cause, disease per se (Omary et al., 2004; Usachov et al., 2015). For example, K14 mutations cause the blistering skin disease epidermolysis bullosa simplex (EBS); GFAP mutations cause Alexander disease (Brenner et al., 2001) (Brenner 2001); and K8 or K18 mutations predispose to the progression of several acute or chronic liver diseases (Ku et al., 2001; Strnad et al., 2010; Usachov et al., 2015). Most disease-causing mutations found in IFs occur in the more conserved central portion of the protein, which is a coiled-coil α -helical stretch of 310–350 amino acids termed the ‘rod’ domain (Figure 1). Mutations in ultra-conserved regions at the beginning or end of the rod domain result in disruption of the IF network from extended filaments into dots and short filaments (Figure 1), and generally lead to a more severe form of an IF-pathy (Coulombe et al., 2009; Lane and McLean, 2004).

2. Current Targeted Therapeutic Approaches for IF-pathies

Mutations in most IF genes, with a few exceptions (e.g., α -internexin and a few of the keratins), have been linked to a human disease. The most pressing current obstacle in the IF field is that there isn’t a single direct cure or even partial therapy for any of the human IF-pathies. As such, the only current management of such diseases relates to life style remedies such as prevention of skin trauma in the case of EBS (Gonzalez, 2013), or to treating end organ damage such as diabetes or cardiac complications as is the case for some of the lamin disorders (Lu et al., 2011). However, several genetic (e.g., gene therapy or allele-specific

silencing) and pharmacologic approaches have been attempted in experimental systems (Table 1). For example, the natural product sulforaphane, which activates Nrf2-dependent transcription and upregulates K16 and K17, ameliorated skin blistering in K14-null mice (Kerns et al., 2010; Kerns et al., 2007). Similarly, forced overexpression of Nrf2 in astrocytes of R236H GFAP mutant mice improved the pathologic features of the GFAP mutation and restored mouse body weights to wild-type levels (LaPash Daniels et al., 2012). The antibiotic doxycycline was also used in K5-null mice and extended the survival of neonatal mice from less than 1 to nearly 8 hours, possibly via down-regulation of inflammatory cytokines (Lu et al., 2007).

Other siRNA-related approaches specifically knockdown mutant allele expression and clear mutant protein aggregation (Allen et al., 2013; Hickerson et al., 2015; Leslie Pedrioli et al., 2012). Furthermore, chaperone protein inducers and gene replacement therapy offer alternative approaches (Gentil et al., 2013). In addition, kinase signaling pathway inhibitors, such as JNK inhibitor SP600125 and ERK1/2 inhibitor selumetinib, benefited mice with cardiomyopathy due to *LMNA* mutation (Muchir et al., 2012; Wu et al., 2010). In these latter cases, specific kinases that are known to be activated in the context of lamin mutations were selectively and effectively targeted (Muchir and Worman, 2016). Lastly, Withaferin A, which inhibits NF- κ B and may manifest some of its biologic effect by binding directly to the soluble fraction of vimentin, has anti-angiogenic effects (Mohan and Bargagna-Mohan, 2016) that could impact some of the IF-pathies but this remains to be formally tested.

3. Unbiased Drug Screening to Target IF Mutations

Given that IF mutations, particularly those that result in more severe phenotypes, generally destabilize and disrupt the IF organization and network distribution, one potential general approach is to screen for compounds that stabilize IFs and potentially serve as direct or indirect chemical chaperones. Such compounds might function similar to taxol which stabilizes microtubules (Rohena and Mooberry, 2014). Other potential positive consequences of an unbiased screen is to identify compounds that target signaling pathways that are aberrantly activated or deactivated as a consequence of the mutation, or to even define novel pathways that are modulated by IF proteins that had not been previously appreciated. However, there are some potential drawbacks for an unbiased screen to keep in mind when planning experiments. For example, the screen is only as good as the assay that is being utilized for screening, so the design of the assay is critical, and some potentially powerful assays (e.g., in vitro filament assembly to identify potential compounds that bind directly to IF proteins) may not be readily suitable for high throughput screening. In addition, compounds of potential interest might have multiple functions or might have undesirable side effects, and it might also be difficult to define their function.

Utilization of such an approach was successfully carried out by screening a kinase inhibitor library in a 384-well plate (Kwan et al., 2015). This approach identified the pan-kinase inhibitor PKC412 as a compound that reverts disrupted K18 R90C filaments into a wildtype-like extended filament network (Kwan et al., 2015). Assessment of the mechanism of action of PKC412 on filament organization showed that it promotes dephosphorylation of non-muscle myosin heavy chain-IIA (NMHC-IIA), thereby enhancing NMHC-IIA association

with K8/K18 which in turn was shown to stabilize the keratin filament network (Kwan et al., 2015). The following section highlights details of the approach used by Kwan et al that can be implemented to screen for compounds that target any IF protein, and can also be used for other non-IF proteins that have a fluorescently tractable cellular organization.

4. High Throughput Steps to Identify Drugs that Target Intermediate Filaments

4.1. Methods for drug screening

The initial critical elements for drug screening include: (i) developing an assay with a clear high throughput-friendly readout that is biologically meaningful and useful; (ii) deciding on the libraries to test. The different options are covered in Section 5 but, in general, using an FDA-approved library as an initial step is practical (because it allows the potential repurposing of already approved and likely relatively safe drugs). In addition, the size of such libraries is generally manageable (500–2000 compounds); (iii) Linking with an institutional core or a private company to carry out the screening. The cost is also a factor that needs to be considered.

For our purposes, we used A549 cells (human lung carcinoma cell line) because in preliminary experiments we noted that they are readily transducible by GFP-K18 lentiviruses (GFP is fused with the N-terminus of K18), and they provide a clearly discernable dot pattern for the K18 R90C mutant or a well-defined filamentous pattern for K18 WT. We opted to use a simple epithelial cell line (i.e., A549 cells) that expresses keratins though we also tested mouse NIH-3T3 fibroblast cells that express vimentin but not keratins, and CHO (Chinese hamster ovary) cells that are similar to NIH-3T3 cells in terms of their IF expression profile. The general approach we utilized is summarized in Figure 2 and details of the apparatus setup are summarized in Figure 3.

For selection of the cell system to be used for screening, is also possible to use stable cell lines. One potential concern is the durability (and level) of expression of the IF protein of interest in the ‘stable’ cell lines on hand. Also, in the case of keratin stable overexpression, another potential limitation to keep in mind is the induction of expression of compensatory keratins (or other IFs). For example, even in our system, we observed that the percentage of cells with dots decreased gradually from ~ 80% cells with dots on the second day after transduction to ~50% on the fourth day (due to WT keratin overexpression). Another alternative system is to derive mutant IF-expressing cells from cells of animal tissue or from human primary cells, as described in the latter case by immortalizing keratinocytes from patients (with EBS due to K5 or K14 mutation) using simian virus 40 T antigen or papillomavirus 16 (Morley et al., 2003).

4.1.1. Materials

- A549 cells
- GFP-tagged K18-WT lentivirus(positive control)
- GFP-tagged K18-R90C lentivirus (negative control)

- Tissue culture 6- well plates
- Perkin Elmer View Plate-384 Plates
- Phosphate buffered saline (PBS)
- Complete medium
- 0.25% trypsin with EDTA
- 4% paraformaldehyde (PFA)
- 0.1% Triton-X
- 1:5000 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen H3570)

4.1.2. Methods

1. The day before transduction, trypsinize and count the A549 cells. Plate 0.6×10^6 cells per well (6-well plate) in 3–5ml of complete growth medium. Cell density should be 50–80% confluent on the day of transduction.
2. Transduce cells with GFP-WT or GFP-R90C K18 lentivirus. Spin the plate at 2,500rpm for 30min at 30 °C (6-well plates are used in allow to allow their centrifugation in order to enhance the transduction efficiency), then change the medium after 6 hours or next the day by simple aspiration. Incubate the cells at 37 °C in a 5% CO₂ incubator for 48 hours.
3. Trypsinize and count the cells, adjust to 400,000 cells/ml.
4. Seed the cells into the 384 plates using the automated Multidrop and add compounds from the drug library by using the pintool (Figure 3). Incubate the plates at 37°C in a 5% CO₂ incubator for 24 or 48 hours (depending on the duration that a biologic effect is anticipated).
5. Aspirate the medium from the plate and add 4% PFA with Multidrop, incubate for 15 minutes (22 °C). It is possible to also use other fixatives such as methanol. However, methanol can interfere with the GFP signal (Straight, 2007; Wang et al., 1996), though we have not found to be the case for GFP-K18. We opted to use PFA mainly because it works well for our purposes and for convenience (i.e., no need to maintain the methanol at –20 °C).
6. Wash the cells with PBS (3 times) using a Biotek plate washer.
7. Add 20ul 0.1% Triton-X using the MultiDrop, incubate for 5min (22 °C), then wash with PBS (3 times).
8. Add 40ul DAPI, incubate for 15 minutes (22 °C) then wash with PBS (3 times). We stain the nucleus with DAPI for two reasons. First, it helps determine the cell density in each well, so we can calculate the percentage of cells with dots by the software to generate the heatmap. Second, it provides useful information regarding cell integrity, by defining the morphology of the nucleus.
9. Add 20ul PBS, seal the plates and store at 4°C until imaging.

10. Image the plates using the ImageXpress Micro system.

For the analysis of the drug effect on keratin filament organization, it is possible that a software program may need to be designed depending on the aim of the screening (e.g., in our case our readout was disappearance of dots which is an easier 'read' for a camera that appearance of filaments). The IXM uses MetaXpress Software Custom Modules software to allow image analysis and quantification. This software includes algorithms that the user can select to achieve the greatest discrimination of phenotypes. In our case, the two phenotypes are "puncta" or "dots" versus "filaments". We used the algorithms to achieve the greatest discrimination between negative control and positive control phenotypes. The algorithms offer discrimination for features that include size, shape and intensity distribution.

The imager will typically acquire five pictures/well (see Figure 4 A–C for examples of captured pictures taken from the screened wells), and the software will compare the profile of the pictures with the images from the negative control wells (which are treated with DMSO) and positive control wells (which are K18-WT transduced cells treated with DMSO, Figure 4 C). The 'heatmap' (Figure 4C) can provide general information about which wells and their corresponding compounds might decrease the numbers of dots/well/cell, but visual inspection is also essential not only for quality control but also to be sure that what appears to be a positive well is not simply because of cell death that effectively provides loss of dots (i.e., a pseudo-positive hit). It is possible that drugs may increase the presence of dots (e.g., by increasing the phosphorylation of the IF). We have not encountered this, except in few rare cases and, if one wishes to pursue such compounds then one potential follow-up test would be to assess their effect on normal keratins (or other IFs).

4.2. Validation of initial drug screening results

The next important step is to validate the findings of the high throughput screening by immunofluorescence staining (Figure 2) using commercial sources of the positive-hit compounds. Another aspect that may be tested at this stage is whether the test compounds can also correct the disorganized filaments from another mutation keratin (i.g., K14 R125C) or another mutation IF (GFAP, lamin, etc). Different doses of the compound should be tried based on the dose used in drug screening and IC50 (half maximal inhibitory concentration), and different time points of exposure to the drug should be checked. After the compounds are added to the cells, the change in cell proliferation and morphology should be monitored. Furthermore, animal models can also be used to validate the test compound, but the dose and duration and treatment plan would need to be optimized, depending on what is known about the compound(s) of interest.

4.2.1. Cell system materials—The materials here are identical to that is shown in Section 4.1.1 except that chamber slides are utilized to visualize the GFP fluorescence and to quantify the extent of filament normalization.

4.2.2. Cell system methods

1. Steps 1 and 2 are identical to what is described in Section 4.1.2.

2. After the 48 hours of transduction with the lentivirus constructs, trypsinize and count the cells and seed the cells into 4-well chamber slides (400,000 cells/ml; 1 ml per well).
3. Add the compound next day. Incubate slides at 37°C in a 5% CO₂ incubator for 24 or 48 hours.
4. Aspirate the medium from the slides and add 4%PFA, incubate at room temperature for 15min then proceed as described in Section 4.1.2 (steps 6–8).
5. Image the slides by fluorescent microscopy. It is possible to also use immune staining instead of GFP-tagged proteins but there are some advantage and disadvantages for such an approach. GFP-tagged proteins offer convenience and save the steps of having to immunostain. The GFP tag also allows following the efficiency of transduction. However, some compounds may have auto-fluorescence that can mimic GFP or other fluorescent signals, and this should be tested for any potential positive hits. There is also the potential of losing the GFP signal, depending on the fixation method as mentioned in Section 4.1.2.
6. Count the numbers of transfected cells with dots, dots and filaments, or mainly filaments; then calculate the percentage of cells with the three types of dots and /or filaments. This is done in triplicates to assess reproducibility and whether there is a significant increase in the number of cells with filaments rather than dots.

4.2.3. In vivo system materials

- K18 WT mice (that overexpress human K18-WT)
- K18 R90C mutant mice (that overexpress human K18-R90C)

The K18 WT mice (Abe and Oshima, 1990) and the K18 R90C mice (Ku et al., 1995) have been used extensively in numerous publications (Ku et al., 2007; Strnad et al., 2008) and are available upon request. The K18 R90C mice offer several advantages including being an in vivo model for keratin mutation induced liver injury and a model for mutation-induced keratin filament disruption (Ku et al., 2007). Similar mice that express other IF mutants would need to be utilized for any preclinical in vivo testing of drugs that target other IF-pathies.

4.2.4. In vivo system methods

1. Choose age and gender matched mice that express the mutant IF. In initial experiments, it is ideal to test both male and female mice since the effect may differ depending on the metabolism of the test drug. Mice are administered the test drug or carrier (DMSO, saline, other). Other caveats that go into consideration include the mode and frequency of delivery (oral, intraperitoneal, intravenous). This is why it is essential to carry out a detailed literature search to identify prior studies that may have tested the compound of interest in mice which could save a tremendous amount of experimental ground work.
2. Another important caveat is the potential expense to obtain sufficient compound for in vivo experiments. In general this is unlikely to be a significant issue unless the

compound of interest is unique or is a natural product that is available in limited amounts.

3. The in vivo readout will be correction of the abnormal filament organization which would then require subsequent testing to determine if the underlying IF-pathway phenotype is corrected or whether a predisposition to a disease phenotype can be ameliorated. In the case of the K18 R90C mice, the compound PKC412 normalized the disrupted keratin filaments in hepatocytes (similar to what was seen in cell culture, Figure 4 A–C) after 4 days of intraperitoneal administration and significantly protected from Fas ligand mediated liver injury (Kwan et al., 2015).

4.3. Assessment of the Mechanism of Action for Compounds of Interest

This is an important component that follows the identification of any potential compound of interest. The following questions need to be addressed, which represents that general approach used by Kwan and colleagues (Kwan et al., 2015) in defining the mechanism of action of PKC412 in normalizing mutant K18 filaments:

- a. Does the drug upregulate a compensatory keratin or other IF, or does it decrease the levels of the mutant IF: this should be one of the first questions to answer and can be addressed easily by assessing the levels of the mutant IF. In addition, the potential overexpression of a compensatory IF can be examined by carrying out high salt extraction (Snider and Omary, 2016) which is a simple and convenient method to assess whether new proteins now partition with the relatively insoluble IF fraction. Furthermore, a proteomic approach can be utilized to identify any new proteins if simple testing for potential IFs is unrevealing.
- b. Is the compound of interest a known enzyme inhibitor or activator, or does it have a known function: compounds with known functions can help narrow down the hunt for the mechanism of action of the compound of interest. For example, PKC412, which normalizes mutant K18 R90C filaments, is a known pan-kinase inhibitor which helped narrow down its likely mechanism of action to changes in phosphorylation in K18 or its binding partner K8, or in the phosphorylation of an associated protein (Kwan et al., 2015).
- c. Does the drug upregulate a keratin stabilizing protein or promote the binding of such protein to the mutant IF: This may relate to questions a and b (above). For example, in the case of PKC412, this compound leads to dephosphorylation of NMHC-II which in turn promotes its binding to K8 and helps stabilize the keratin filaments (Kwan et al., 2015) via an unknown mechanism. As mentioned above, a proteomic approach can help identify the presence of such a putative protein. In addition, expression profiling may be used to help identify up or down regulated proteins that may shed light on unique altered pathways.
- d. Does the drug work on more than one mutant IF: this is also relevant to know since a potential compound that affects more than one IF protein is likely to work via a conserved mechanism.

- e. Does the drug bind directly to the IF protein: an analogy here would be a taxol-like effect, and this is presently a missing holy grail for IF-pathies and the IF field in general since no such compounds are presently known. Direct binding can be tested using several modalities that include: (i) examining the effect of putative drug on in vitro filament assembly of purified IFs (protocols for IF in vitro assembly can be found in (Herrmann et al., 2004)), and (ii) testing the binding of a radiolabeled or derivatized form of the compound (if available) to the mutant and WT IF.

5. Available Libraries and Vendors for Drug Screening

There is a wide range of commercial compound libraries that are available to purchase (Table 2). In addition, some universities may have drug screening cores that are available for use by their faculty investigators. In general terms, it is ideal to select libraries that include already approved drugs or drugs that are ongoing clinical trials because of the usually detailed available information on their pharmacodynamics and safety profile. Examples include the Food and Drug Administration (FDA) approved drugs that are part of the BioFocus NCC and Prestwick libraries (Table 2). In addition, some vendors including Enzo Life Sciences, ChemDiv and Chembridge, can provide focused libraries that can target specific pathways, such as kinases, or biologic processes such as autophagy. Other vendors (e.g., Maybridge) provide libraries such as the HitFinder Library whereby compounds are selected to represent the overall diversity of the screening collection using a clustering algorithm. Such design libraries may shorten the drug discovery process and save some materials. Another cost-saving measure is multi-plexing the screening such that up three or four compounds may be tested per well.

6. Pearls and Pitfalls

There are several caveats to keep in mind:

- a. The design of the assay used for drug screening is key predictor of success. What is described in this chapter is a method to screen for compounds that normalize major organizational alterations in IFs. The hypothesis is that normalization of such severe IF dysorganization will ameliorate or even cure the underlying IF-pathway that is caused by the mutant IF and its consequent filament alterations.
- b. One limitation of the approach we describe is that it is not designed for more subtle IF mutations that can still cause significant patient morbidity and can lead to many IF-pathies. Assays with alternate selective readouts would need to be tailored for such situations.
- c. One potential benefit of an unbiased IF-related drug screening approach is that it could illuminate novel signaling pathways or interacting proteins, as was the case for identifying NMHC-II binding to keratins (Kwan et al., 2015).
- d. Once a hit compound is identified and validated in cell culture, it is important to use a systematic approach to define its mechanism of action, initially in cell culture, and then determine whether it is likely to work in vivo. This is the aspect that requires the most effort, because of multiple potential mechanisms that may be

involved. Also, some drugs may work in cell culture but may not be effective in animal studies for a number of reasons (e.g., pharmacodynamics, tissue/cell penetration), or may also impart protection independent of an IF mutation.

- e. One potential concern is whether a given screen will result in too many potential compounds to pursue. However, this has not been an issue for us and we are presently working on only 2 additional compounds (aside from PKC412) after screening several libraries totaling more than 3000 compounds and ending up with nearly 15 potential compounds that were then easily narrowing down to two. For example, triaging generally includes: (i) eliminating compounds that are known to be toxic or to have chemically reactive functional groups; (ii) selecting compounds that appear to be most effective in normalizing the mutant filament disruption phenotype; and (iii) selecting compounds that have a single or at most a limited number of known functions.

Acknowledgments

Our work is supported by US National Institutes of Health (NIH) grants DK47918 and DK52951, and the Department of Veterans Affairs (M.B.O.); and NIH institutional grant DK34933 to the University of Michigan. We thank Dr. Martha Larsen, Nicholas Santoro and Steve Swaney and for their tremendous assistance with the high throughput drug screening. We also thank Dr. Paula Gedraitis from Molecular Devices for her excellent technical support.

List of abbreviations

DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EBS	epidermolysis bullosa simplex
FDA	Food and Drug Administration
GFAP	glial fibrillary acidic protein
GFP	Green fluorescent protein
IF	intermediate filament(s)
IF-pathies	intermediate filament associated diseases
K	keratin(s)
NMHC-IIA	non-muscle myosin heavy chain-IIA
PBS	phosphate buffered saline
PFA	paraformaldehyde

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Figure 1. Prototype IF protein domains and consequences of IF mutation on filament organization

The schematic shows the three IF protein domains: a central α -helical coiled-coil relatively conserved 'rod' domain (310–350 amino acids), that is flanked by N- and C-terminal non- α -helical 'head' and 'tail' domains (of variable length depending on the IF protein) which, in turn, provide the exceptional structural diversity among IFs. The mutations responsible for the most severe IF-pathway phenotypes are typically located at ultra-conserved helix-initiation and helix-termination motifs at the beginning and end of the rod domain that cause disruption of the filamentous organization into short filaments or dots. Examples of IF

mutations that cause the type of filament disruption that is schematically shown include lamin A/C N195K mutation (causes Emery-Dreifuss muscular dystrophy) (Ostlund et al., 2001), GFAP R79H or R236H mutation (both cause Alexander disease) (Mignot et al., 2007; Wang et al., 2011), K14 R125C mutation (causes EBS) (Bonifas et al., 1991; Coulombe et al., 1991), and K18 R90C (predisposes to liver injury) (Ku et al., 1995). N, nucleus.

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Figure 2. Flow chart of the cell transduction and validation drug screening setup

A549 cells (or any cell culture system that is used for the screening assay) are seeded into culture plates. After allowing cells to adhere for one day, cells are transduced with GFP-WT K18 or GFP-R90C mutation K18, together with WT K8 (which result in normal control filament organization or in disrupted filaments, respectively, as exemplified in the schematic in Figure 1 or in the immunofluorescence staining shown in Figure 4). It is important to co-transfect with both a type I and type II keratin to allow filament formation. Fresh media is then added on Day 3 followed on Day 4 by transfer (after trypsinization) to the 384 plates for drug screening (see also Figure 3) or to chamber slides for specific drug validation. For validation, the test compounds are added in to the chamber slide followed by 1 or 2 days of drug treatment then cell fixation and visualization by microscopy.

A. Thermo Labsystems multidrop plate dispensers

B. Sciclone ALH3000 with a V&P drug dispenser

C. Bio-Tek ELx405 plate washer/aspirator

D. ImageXpress Micro Cellular Imaging and Analysis System

Figure 3. The drug screening high throughput apparatus

The cells are seeded into 384-well plates using the multidrop plate dispenser (A) followed by addition of the test compounds using the drug dispenser (B). After 1 or 2 days of incubation in the presence of the drugs, cells are fixed, permeabilized and mounted with DAPI using an automated washer/aspirator system (C). Images are then acquired using an ImageXpress system (D) that is coupled to a computer to allow data analysis.

Figure 4. Examples of drug screening phenotypes and a typical heat map as visualized by ImageExpress

(A) Negative control cells which were transduced with GFP-K18 R90C lentivirus and treated with DMSO for 48 hours (arrows highlight the dots). (B) Positive control cells which were transduced with GFP-K18 WT lentivirus and treated with DMSO for 48 hours. (C) GFP-K18 R90C lentivirus transduced cells treated with a compound that results in normalization of the disrupted keratins. (D) The first two columns (far left) of the 384-well plat represent GFP-mutant IF transduced cells treated with DMSO for 48 hours as a negative control (primarily red or non-green color), while the two columns on the far right represent GFP-WT IF transduced cells treated with DMSO as a positive control. The middle columns represent GFP-mutant IF transduced cells with different drug library compounds after 48 hours. Green indicates few dots/well while red indicates mainly dots/well. Several wells in the middle columns are bright green, indicating the added compound may have corrected the IF mutant phenotype or is too toxic in those wells (i.e., dead cells will also have few dots and represent false positives).

Table 1

Experimental therapeutic approaches for IF-pathies

Interventions	Test systems	Comments	Reference
Genetic approaches			
K16–14 hybrid transgene	<ul style="list-style-type: none"> K14-deficient mice 	Expressing a hybrid K16–14 transgene in the epidermis of mice null for K14 restored a wild-type phenotype to newborn epidermis, of note, K18 expression was not protective	Hutton 1998
Ectopic expression of desmin	<ul style="list-style-type: none"> K5^{+/-} mice crossed with Desmin^{tg/+} transgenic mice 	Stable expression of desmin rescued K5 null mice, which served as a model for severe EBS	Kirfel 2001
siRNA-(K9 R163Q)	<ul style="list-style-type: none"> AD293 cells CD1 female mice 	A allele-specific siRNA for mutant K9, which can lead to epidermolytic palmoplantar keratoderma, inhibits mutant allele expression in vitro and in mice	Leslie 2012
siRNA-(K12 R135T)	<ul style="list-style-type: none"> AD293 cells 	A potent allele-specific siRNA reduce the expression of mutant K12 which can cause Meesmann epithelial corneal dystrophy	Allen 2013
siRNA-(K6 N171K)	<ul style="list-style-type: none"> 293FT cells Swiss wobster mice 	Specific siRNA for mutant K6, which causes pachyonychia congenital, inhibits mutant but not WT K6 expression	Hickerson 2015
Pharmacologic approaches			
Sulforaphane	<ul style="list-style-type: none"> K14-deficient mice 	Sulforaphane ameliorates skin blistering by inducing K16/K17 in epidermis via Nrf2-dependent and independent pathways	Kerns 2007 Kerns 2010
Doxycycline	<ul style="list-style-type: none"> K5^{-/-} mice 	Doxycycline extended survival of neonatal K5 ^{-/-} mice from < 1 to up to 8 hours via suppression of inflammatory cytokines	Lu 2007
PKC412	<ul style="list-style-type: none"> A549 cells K18 R90C mice 	PKC412 normalizes K18 Arg90Cys mutation-induced filament disruption and disorganization by enhancing keratin association with NMHC-IIA in a myosin dephosphorylation regulated manner	Kwan 2015
Celastrol	<ul style="list-style-type: none"> SW13^{-IF} cells transfected with mutant NF-L 	Celastrol, an inducer of chaperone proteins, induced HSPA1 in motor neurons and prevented formation of NF inclusions and mitochondrial shortening induced by the expression of NF-L Q333P mutation	Gentil 2013
Farnesyltransferase inhibitors (FTIs)	<ul style="list-style-type: none"> Lmna^{HG/HG} mice 	ATB-100 improves disease phenotypes in mice with Hutchinson-Gilford progeria syndrome, and R115777 prevents the onset and progression of existing cardiovascular disease	Yang 2006 Capell 2008
SP600125	<ul style="list-style-type: none"> LmnaH222P/H222P mice 	The JNK pathway inhibitor, SP600125 prevents cardiomyopathy caused by <i>LMNA</i> mutation	Wu 2010
Selumetinib	<ul style="list-style-type: none"> LmnaH222P/H222P mice 	The ERK1/2 pathway inhibitor, selumetinib preserves cardiac function and improves survival in mice with lamin A/C gene mutation	Muchir 2012

Table 2

Available Libraries and Vendors for Drug Screening

Vendors	Library names	Library size	Comments	Websites
Evotec (NIH Molecular Libraries Small Molecule Repository)	BioFocus NCC	446	NIH collection of FDA-approved drugs that have a history of use in human clinical trials	http://nihsmr.evotec.com/evotec/
Prestwick Chemical	Prestwick	1280	100% approved drugs (FDA, EMA and other agencies), high chemical and pharmacological diversity, known bioavailability and safety in humans	http://www.prestwickchemical.com/index.html
Sigma-Aldrich	LOPAC (Library of Pharmacologically Active Compounds)	1280	A collection of molecules that span a broad range of cell signaling and neuroscience areas	https://www.sigmaaldrich.com/united-states.html
MicroSource	Spectrum	2400	Drugs that reached clinical trial stages; known biologically active compounds; natural products	http://www.msdiscovery.com/
Boston and Kansas University	Chemical Methodology Library Development (CMLD)	3000	Boston and Kansas University Collection	http://cmd.bu.edu/ https://hts.ku.edu/resources/compounds
Enzo Life Sciences	Mutiple	4000	Toxicity(487), drug repurposing(786), pathway targeting(215), chemical genomics(801), receptor de-orphaning(1134), natural products(502)	http://www.enzolifesciences.com
ChemDiv	Mutiple	1.5×10^6	Targeted Diversity Libraries Focused Libraries Design Libraries	http://www.chemdiv.com/
Chembridge	Mutiple	1×10^6	Diversity Libraries (Pre-selected diversity libraries) Targeted and Focused Libraries (Kinase, GPCR, Ion Channel CNS & Nuclear Receptor Libraries)	http://www.chembridge.com/index.php
Maybridge	Multiple	53,000	Hitfinder(Representatives of the overall diversity of the screening collection) HitDiscover	http://www.mavbridge.com/